Ex vivo crystallin expression in a bioengineered anterior lamelarcornea

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INTRODUCTION: Crystallins are water-soluble structural proteins found in the eye lens and cornea that are involved in remodelling and protecting the cytoskeleton, apoptosis inhibition and resistance to stress and play a crucial role in maintaining the transparency of both the lens and cornea. Although their function is partially unknown, these proteins are able to reduce light scattering and improve transparency by driving the light pathway though the cornea and lens [1]. In the present work, we generated a biomimetic bioengineered anterior lamelar cornea by tissue engineering using natural biomaterials and evaluated the *ex vivo* synthesis of several major crystallins as a quality control step of these corneas intended for future clinical use.

METHODS: Primary cell cultures of human cornea epithelial and stromal cells were established from sclera-corneal tissue biopsies using explant and enzymatic digestion methods and grown in specific media [2]. A bioartificial model of the human anterior cornea was constructed by using fibrin-agarose biomaterials containing stromal keratocytes and epithelial cells on top. Epithelial stratification was induced by air-liquid culture technique. These artificial anterior lamelar corneas were histologically assessed to identify the presence of crystallins Cry-αA, Cry-αB, Cry-ζ and Cry-λ1 by immunohistochemistry using human native corneas as controls. Then, mRNA oligonucleotide microarrays were used to determine the expression of these crystallins in cell cultures of epithelial and stromal cells and bioengineered corneas.

RESULTS: Immunohistochemical expression of Cry-αA was very intense in control corneas and bioengineered corneas, although expression was higher in controls. However, the analysis of mRNA expression by microarray (Figure 1) showed higher expression in the artificial corneas as compared to the primary cell cultures. For Cry-αB, expression was higher in bioengineered corneas than control native corneas by both immunohistochemistry and microarray. Then, the analysis of Cry-ζ expression showed moderately positive expression in human control corneas and artificial corneas by immunohistochemistry and microarray, although the mRNA expression tended to decrease as compared to primary cell cultures. Finally, Cry-λ1 expression was positive in the epithelium of human native corneas, but not in the stroma, and mildly positive in bioengineered human corneas by immunohistochemistry. Microarray analysis, however, showed low expression in all samples.

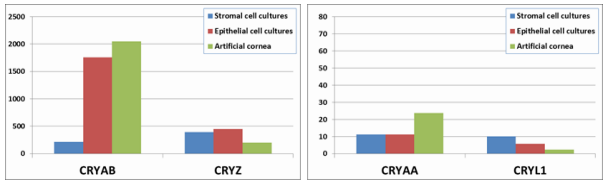

Figure 1. mRNA expression of the four crystallin genes analysed in this work as determined by microarray in stromal keratocytes (blue), epithelial cell cultures (red) and artificial anterior lamellar corneas (green).

DISCUSSION & CONCLUSIONS: These findings suggest that this bioengineered cornea model is able to express relevant crystallin proteins ex vivo that would be responsible for the optical properties of these artificial tissues and support their use in clinical trials.

ACKNOWLEDGEMENTS: This study was supported by the Spanish Plan Estatal de Investigación Científica, Desarrollo e Innovación Tecnológica (I+D+I) 2013-2016 from the Spanish Ministry of Economy and Competitiveness (Instituto de Salud Carlos III), grant FIS PI14/0955 (co-financed by FEDER funds, European Union).

Development of artificial elastic cartilage for tissue engineering applications

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INTRODUCTION: Elastic cartilage (EC) is a type of cartilage present in the outer ear, Eustachian tube and in the core of the epiglottis [1]. The structure and function of the EC can be affected by several conditions and traumas and there are few therapeutic alternatives for these patients. Tissue engineering offers the possibility to design and generate different kinds of tissues or organs, including the human cartilage, but very few works have focused on the development of human elastic cartilage substitutes.

In the present work, we generated a bioengineered model of human elastic cartilage using elastic chondrocytes and natural biomaterials, and we analysed the sequential maturation and differentiation levels of cells and ECM at different development times.

METHODS: Elastic chondrocytes were isolated from small biopsies of the human ear cartilage using collagenase digestion. Cells were expanded ex vivo using specific culture media. Then, bioengineered human elastic cartilage substitutes were constructed using nanostructured fibrin-agarose biomaterials with the cultured cells immersed within. Artificial tissue samples were harvested at weeks 1, 2, 3, 4 and 5 for histological and immunohistochemical analyses, and the sequential development of elastic cartilage patterns were determined by identification of acid proteoglycans, elastic fibres, collagens type I and II, and aggrecan. The chondral phenotype was evaluated by S-100 immunohistochemistry.

RESULTS: The results of the present work demonstrate that fibrin-agarose hydrogels are adequate hydrogels for the encapsulation of human elastic chondrocytes for tissue engineering purposes. Cells formed complex cells clusters resembling the native cartilage. Histological analyses revealed a progressive increase of the amount of cells inside the scaffold, showing positive expression of the chondrocyte marker S-100 in all bioengineered tissues. In addition, a progressive synthesis of proteoglycans and elastic fibres was observed, especially in cell-rich tissue areas. The most significant immunohistochemical results revealed a sequential synthesis of collagen type I, a weak reaction for collagen type II (Figure 1) and a positive reaction for aggrecan in some territorial areas.

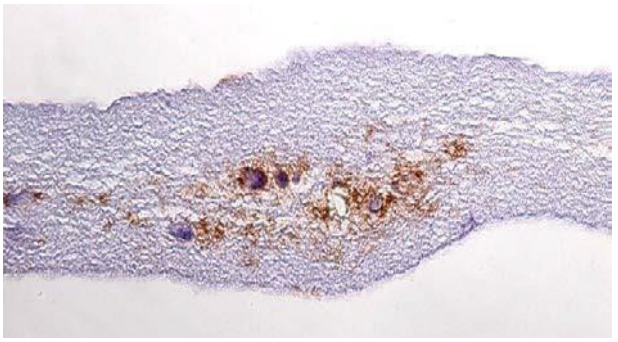

Fig. 1. Collagen II expression in a fibrin-agarose scaffold containing cultured elastic chondrocytes as determined by immunohistochemistry.

DISCUSSION & CONCLUSIONS: This study demonstrates that nanostructured fibrin-agarose biomaterials are appropriate scaffolds for elastic cartilage tissue engineering, allowing the synthesis of major ECM components. Therefore, these biomaterials could be used for the generation of biocompatible and biologically active human elastic cartilage tissue-like substitutes by tissue engineering. However, further molecular, biomechanical and in vivo studies will be needed.

ACKNOWLEDGEMENTS: This work was supported by grant SAS PI-0653-2013, Junta de Andalucia, Spain.

Reduction of thrombo-inflammation during ischemic reperfusion injury using endothelial surface modification with PEG-conjugated phospholipid in an allogeneic transplant model

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INTRODUCTION: Ischemic reperfusion injury (I/R injury) disrupts the endothelial cell integrity and involves a series of adverse events on the endothelial cell surface including activation of the complement and coagulation systems. This I/R-induced thromboinflammation is potentially deterrent for both graft and patient survival in clinical transplantation. Here, we hypothesized that ex-vivo coating of the endothelial surface with PEG-lipid will protect against I/R injury. In this study, we used en-bloc kidney transplant model in pigs to evaluate the effect of PEG-lipid coating on kidney graft.

METHODS: Kidneys were retrieved *en bloc* from young pigs weighting 30-35 kg. After 24 hours of static cold preservation, one kidney within the *en bloc* package was randomly selected for incubation (at least 45 minutes) with PEG-lipid solution without contamination of the contralateral kidney. Following implantation and reperfusion, sequential blood and tissue samples were obtained from *en bloc* kidney grafts. Blood sampled were analyzed for coagulation and complement factors as well as pro-inflammatory cytokines IL-1β, IL-6, MCP-1 and tissue factor. Tissue samples were histologically evaluated for I/R-injury. Coating of the renal tissue with PEG-lipid was analyzed with fluorescence microscopy. Data are presented as (median and range) and were statistically analyzed by Wilcoxon test.

RESULTS: Immunofluorescence microscopy confirmed deposition of PEG-lipid on the endothelial, interstitial and tubular structures of treated kidney without contamination of the contralateral control. PEG-lipid treated kidneys had less thromboinflammation compared to untreated control as evaluated by TAT (119 μg/ml (50 – 145) vs. 218 μg/ml (98.7 – 302), $p < 0.001$) C3a (122 ng/ml (103 – 166) vs. 218 ng/ml (178 – 302), $p < 0.01$) and C5b-9 (140 ng/ml (69.1 – 232) vs. 214 ng/ml (119 – 650) $p < 0.01$). Similarly, PEG treated kidney demonstrated reduced expression of IL-1β (91.0 pg/ml (66.8 – 140) vs. 191.5 pg/ml (77.3 – 330), $p < 0,01$), IL-6 (14.8 pg/ml (12.8 – 19.3) vs. 27.0 pg/ml (18.3 – 29.5), $p < 0,05$), MCP-1 (20.3 pg/ml (11.1 – 23.2) vs. 28.5 pg/ml (20.3 – 37.5), $p < 0,05$) and tissue factor (174 pg/ml (144 – 222) vs. 367 pg/ml (258 – 476), $p < 0,01$) in relation to control. At 6 hours post transplant, the total urine output was higher in PEG-lipid treated kidney compared to its control (55.7±13.4 ml vs. 35.2±17.2 ml, $p < 0,05$). Histological evaluation demonstrated reduced expression of complement markers such as C5aR, C3b, and C5b-9 in PEG-lipid treated kidney graft in comparison to control.

CONCLUSIONS: Short-term PEG-lipid incubation of kidney allografts after prolonged cold preservation reduced both tissue- and fluid-based thromboinflammation during reperfusion. This simple treatment modality might represent a novel and effective strategy to reduce I/R-induced tissue damage in clinical transplantation.

ACKNOWLEDGEMENTS: The authors thank Lillemor Funke and Susan Lindblom for technical assistance

A novel bioengineering application of cardiac pericytes for vascular replacement in paediatric patients with congenital heart defects

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INTRODUCTION: Neonates born with severe congenital heart defects (CHD) require the repair or replacement of vessels and valves shortly after birth to survive. Vascular prostheses currently used are unable to match the child growth and need to be replaced repeatedly, exposing young patients to risky operations. We are working on the generation of tissue-engineered vascular grafts (TEVG) made of CorMatrix patches cellularised with patients' cardiac pericytes (CPs). Our aim is to build living grafts able to grow and remodel over time allowing definitive correction of the defects.

METHODS: CPs were isolated as previously described [1] from surgical myocardial leftovers of neonates and infants receiving palliative surgery for correction of CHD. CPs seeding onto CorMatrix was optimised and cell viability tested with CalceinAM-EthDIII staining, in both grafts grown in static culture and inside a rotating bioreactor. Cell proliferation was assessed by EdU incorporation for 48hrs. Survival of cells after freezing/thawing of TEVG was tested. Production of extracellular matrix (ECM) was investigated by colorimetric measurement of the fibrillar collagen in cells lysates and supernatants, and by fluorescent immunostaining for collagen type I (COL) in cells, TEVG and decellularised cells monolayers. The vascular plasticity of CPs was tested culturing cells and CP-grafts with an inductive media for 2 weeks. Recruitment of endothelial cells (ECs) was tested by a Boyden chamber migration assay in which ECs were let migrate for 48hrs to the graft. All experiments have been done on N=2 to 6 cell lines.

RESULTS: CPs grow very well on CorMatrix, with >95% cells viable after 10 days of culture and 64±6% cells proliferating 48hrs post-seeding. The freezing/thawing of TEVG resulted in 70% viable cells 48hrs post-thawing. CPs grown on CorMatrix maintain the pericyte-phenotype, expressing NG2, Vimentin and PDGF-Rβ (as we showed in [1]). We detected fibrillar collagen in both cell lysates (5.5±0.8µg Coll/10^5 cells) and supernatants (0.1±0.02µg Coll/10^5 cells*hr). Moreover, when grown on plastic dishes, CPs produce and release their own ECM made of COL. Also, CPs repopulating the CorMatrix produce new COL matrix (**Fig. 1**). CPs, grown on both glass coverslips and CorMatrix, differentiate into smooth muscle cells (SMCs) expressing contractile filaments αSMA, Calponin (**Fig. 2**) and SM-MHC. Last, CPs do not differentiate into ECs, but when seeded on CorMatrix they attract ECs to the graft (400 vs 10 ECs/mm^2 in TEVG vs native matrix).

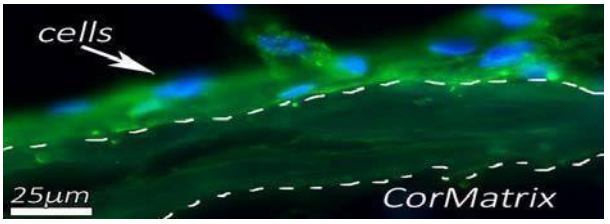

Fig. 1: Fluorescence image of TEVG: production of ECM. Collagen type I is shown in green fluorescence, nuclei by blue fluorescence of DAPI.

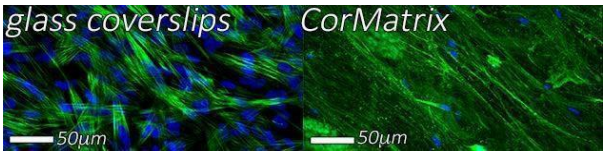

Fig. 2: Fluorescence image of CPs differentiated into SMCs. In green fluorescence, filaments of Calponin; nuclei are in blue (DAPI).

DISCUSSION & CONCLUSIONS: We demonstrate that CPs, cells so far under-investigated and that we can isolate in high number from surgical leftovers, are able to synthetize their own ECM promoting the growth and remodelling of the TEVG over time. The great differentiative capacity into SMCs promises the generation of contractile grafts resembling the physiologic function of an artery. In addition, the recruitment of ECs to the luminal side of the graft is crucial to help preventing thrombotic complications. For all these reasons, CP-TEVG might represent a new, promising and definitive solution for correction of CHD. Our current research is paving the way for a preclinical study in a large animal model of CHD.

ACKNOWLEDGEMENTS: British Heart Foundation PG/15/32/31398; the Sir Jules Thorn Award 2014

Temperature controlled dual hypoxic chamber design for in vitro ischemia experiments

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INTRODUCTION: Ischemia under body temperature is a well-described phenomenon with numerous scientific models available to simulate the conditions in vitro. Cold-ischemia however is a less investigated field, even though the beneficial effect of cooling is obvious. The aim of the present work is to study in vitro cold-ischemia and to design a device, which most precisely establishes the necessary conditions.

METHODS: Two aluminium boxes with neoprene gasket were modified to meet the requirements of in vitro ischemic experiments. Thermoelectric coolers (TEC) were used to set the temperature of the chambers. The dissipated heat was removed by a water cooling system. The power of the TEC modules was adjusted by DC-DC converters. Temperature is controlled with TSM125 thermostat. The chambers can be separated from the system by manual valves. Two plastic container with bubble stones were built in the gas flow to keep the humidity at 100% when body temperature is used. A micro pressure controller and a rotameter was built in to avoid the unequal gas flow in the chambers when humidifier is only used one side. Alphasense O2-A2 oxygen sensor with transmitter board was used with a custom designed display electronics to measure the oxygen level. Ischemia was performed by using glucose free cell culture media and keeping the cells in the hypoxic chamber. Seven hours of oxygen-glucose deprivation was performed on 4mm circular rat calvaria explant. XTT (tetrazolium salt) assay was performed after the ischemic period to monitor the cell survival.

RESULTS: The system is able to keep the samples at any temperature between 0-37°C. To achieve this voltage and the current of the TEC modules were set to 7.5V and 4.5A respectively. The power of the TEC elements is enough to reach the operation temperature within 10 minutes. With the nitrogen flow set to 1 litre/minute with 0.8bar pressure the gas consumption was 25 bars during the 7 hours of the experiment. The dual chamber formation allows to run parallel measurements comparing different temperature conditions. XTT assay results demonstrated that cells are unable to survive 7 hours of ischemia under body temperature (37°C), in contrary, most cells survive the same condition under 4°C.

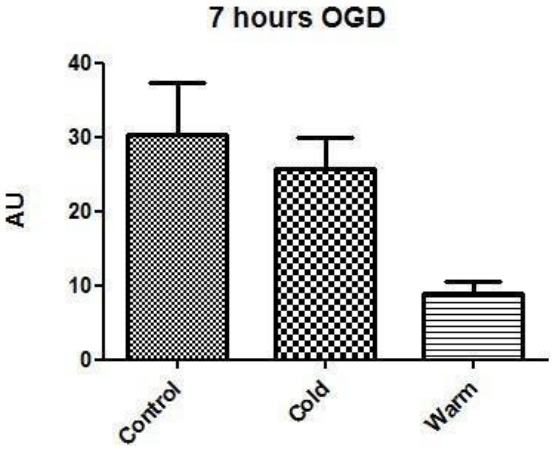

Fig. 1: Cell viability after 7 hours in vitro cold and warm ischemia

DISCUSSION & CONCLUSIONS: This work introduces a custom designed device, which is able to study in vitro ischemia at different temperature values. In addition, the dual chamber design allows parallel experiments at the same time with different settings. Cell culture experiments confirmed the known beneficial effect of cooling on cell survival in this model as well, as results further experiments can be designed to study cold ischemia on cellular, tissue and organ level.

ACKNOWLEDGEMENTS: The authors thank Lacerta Technologies for sponsoring this research.

Engineering functional bioartificial lymph nodes using polycaprolactone scaffolds

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INTRODUCTION: The quality of life of chronic lymphedema patients is dramatically reduced and is mainly the result of breast cancer therapy in developed countries [1]. Conservative treatment is still the gold standard. Surgical techniques so far offer moderate results with a high morbidity [2]. Thus we introduce a novel approach of functional bioartificial lymph nodes using PCL scaffolds.

METHODS: Human lymph nodes were harvested and processed into small fragments (1 mm) to fix them with fibrin glue in sterile cylindrical PCL scaffolds (5 mm diameter & height). For in vitro analysis the bioartificial lymph nodes were cultured in 48-well plates using cell culture media and an incubator for 4 weeks. The constructs were imaged after 1, 3, 7, 14 and 21 days using an inverted microscope for cell migration studies. In addition cell proliferation was evaluated by a fluorimetric dsDNA quantification kit. For the animal study we used 14 female immunodeficient nude mice (nu/nu, 4 weeks old). All bioartificial lymph nodes were implanted nearby the right femoral artery (sample > right groin, control > left groin). The first group (n = 7) were euthanized after 8 weeks, the second group (n = 7) after 16 weeks. Tissue samples were collected in formalin for further examinations (µCT, histology, IHC). Human LYVE1 antibodies were used to detect new lymph vessel formation.

RESULTS: Cell migration and proliferation was observed in vitro during different time points. Two cell types (round cells vs. adherent cells) were microscopically visualized. In vivo results showed a good integration of the constructs in the host tissue. After 8 weeks a significant vascularization of the bioartificial lymph nodes could be seen during explantation compared to the control site which was confirmed by µCT scans. Histological and immunohistochemical examinations revealed a regeneration of lymph node tissue and new lymph vessel formation outgoing from the bioartificial lymph nodes.

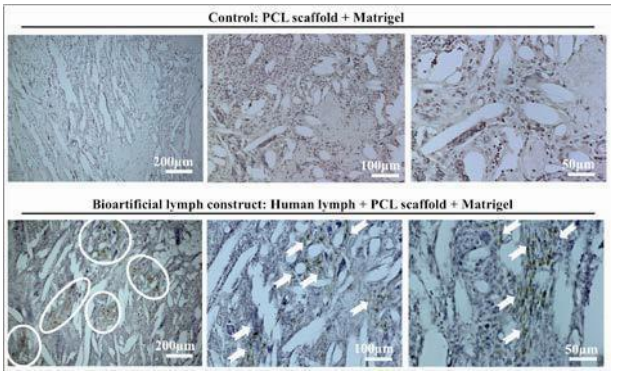

Fig. 1: Positive staining of lymph vessel formations in samples using human LYVE1 antibodies.

DISCUSSION & CONCLUSIONS: Adequate causative surgical treatment of chronic lymphedema with low morbidity is still lacking. Our new innovative approach shows an induction of lymph vessel formation in vivo which is an important precondition for surgical lymphedema treatment. Hence we engineered functional bioartificial lymph nodes. Further animal studies (lymphedema animal model) are necessary to show an improvement of lymphedema using bioartificial lymph nodes.

ACKNOWLEDGEMENTS: This study was funded by the German Society of Lymphology (Deutsche Gesellschaft für Lymphologie – DGL).

Interaction of muscle-derived stem cells and endothelial progenitor cells in co-culture: tissue engineering applications

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INTRODUCTION: Tissues are highly complex three dimensional structures made up of different cell types and their interactions. Co-culture systems that can mimic the interactions of the native tissues combined with 3D scaffolds are a favourable strategy for tissue engineering. This study aims to create a fragment of functional artificial tissue, based on co-culture of muscle-derived stem cells (Myo) and endothelial progenitor cells (EPCs) on a chemically modified, 3D elastomer surface. For this purpose, the bidirectional interactions between Myo and EPCs were investigated and interface between co-culture and poly(dimethylsiloxane) (PDMS) was analysed. PDMS is highly biocompatible and biostable material, with positive extracellular matrix deposition throughout the scaffold. Its suitability for microporous scaffold is also shown. Therefore, it is an attractive candidate for tissue engineering applications. Nevertheless, its fragility is a restrictive factor for surgical applications. Therefore, we aimed to create a chemically modified PDMS with improved physical properties.

METHODS: Growth and migration characteristics, colony forming rate, Vp1u uptake, differentiation capacity were investigated in both (Myo and EPCs) cell types. Direct co-culture system was used in this study. Matrigel 2D assay was applied to analyse tube forming capacity. To chemically modify PDMS elastomer, triacetoxy(vinyl)silane, vinyltrimethoxysilane and methacrylic/acrylic acid and other water-soluble monomers were used to form novel copolymers and terpolymers. The novel elastomer was microstructured using a 3D printing technique. The morphology of the cells grown on the scaffolds was evaluated by scanning electron microscopy.

RESULTS: To confirm the multipotency, Myo cells used in the study were induced to differentiate into three lineages: bone, adipose and muscle. Myogenic differentiation was confirmed by cell fusion index, adipogenic differentiation – by Oil Red staining, and osteogenesis – by Alizarin Red staining and increased alkaline phosphatase activity. EPCs, recognized as attaching spindle-shaped cells, were assayed by co-staining with 1,1'-dioctadecyl-3, 3,3',3'-tetetramethylindocarbocyanine perchlorate-labeled acetylated low-density lipoprotein and fluorescein isothiocyanate-conjugated *ulex europaeus* lectin, characteristics of endothelial lineage. Both types of cells were assessed according their capability to uptake Vp1u.

To assess the angiogenic ability of EPCs (control) and co-cultured cells, their capacity to form capillary-like networks *in vitro* was evaluated. The data obtained showed that Myo and EPCs in co-culture were tend to self-assemble and form organised structures with segregated cell-type partitioning. Moreover, incorporation of Myo cells into Matrigel increased the capacity of EPCs to form capillary-like networks.

Next, the interaction of co-cultured cells with chemically modified 3D elastomer surface is studied. For this purpose, four types of differentially modified PDMS samples were obtained. Initial biocompatibility studies showed promising results. The best biocompatibility demonstrated elastomer synthetized of 2-hydroxyethyl methacrylate (HEMA) and di-epoxy-terminated PDMS. However, its microfabrication using 3D printing should be optimised.

DISCUSSION & CONCLUSIONS: This work suggests that co-culture of Myo and EPCs is mutually beneficial to each other, suggesting the promising approach for tissue engineering applications. However, the conditions for PDMS chemical modification extensive cellular response studies should be carried out to find optimal 3D structuring parameters to form a scaffold mimicking natural cellular niches.

ACKNOWLEDGEMENTS: This work is supported by the grant from the Research Council of Lithuania, No. SEN-13/2015.

A novel protocol to characterise the mechanical properties of spinal cord tissue and benchmark candidate biomaterials for CNS tissue-engineering

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INTRODUCTION: A number of tissue-engineered approaches are currently in development to promote repair in the injured spinal cord. In addition, it is widely regarded that the mechanical properties of a tissue have the potential to profoundly affect cell behaviour [1]. As such, to promote optimal cell growth and host-tissue integration in the CNS, biomaterial candidates will need to be designed with the biomechanical properties of spinal cord tissue in mind. Nevertheless, given the viscoelastic nature of cord tissue, accurate mechanical characterisation has so far been difficult. Thus, the aim of this project was to develop a reliable testing protocol able to probe the mechanical properties of spinal cord tissue, and one which might ultimately be used to mechanically screen biomaterials for CNS tissue-engineering applications.

METHODS: Wistar rats (male) weighing between 380 – 480 g were culled and spinal cords displaced using a fluid-filled syringe. These were then immediately placed in AQIX RS-I (Liquid Life) tissue-preservation media ready for mechanical testing.

Cord sections were then cut to approximately 5 mm in length, and height measurements for each section taken using a contact angle machine. A mean height for each specific section was then calculated, and this used to generate a 0.5 % dynamic strain. Sections were then placed in a Dynamic Mechanical Analysis (DMA) system (Bose Electroforce 3200), where they were subjected to an ascending frequency sweep of 1 – 100 Hz. To test for destructive changes, a 1 Hz validation frequency was repeated after the full frequency sweep had been completed. Pre- and post-DMA sample volumes were also measured as a means of geometric validation. Sections were kept on ice until the point of geometric measurement, however, they were allowed to equilibrate to room temperature before mechanical testing began. All samples were maintained at 100 % humidity throughout.

RESULTS: DMA testing revealed that rat spinal cord compression modulus was in the range of 1 – 10 kPa. This was found to be a strain-rate dependent phenomenon, whereby tissue stiffness increased non-linearly with frequency. We also found that DMA testing caused a small (< 1 kPa) change in the 1 Hz validation frequency.

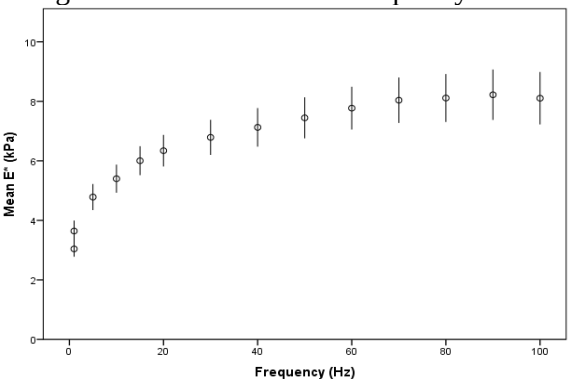

Fig. 1: Compound modulus values (E) of rat spinal cord using an ascending sweep of 1 – 100 Hz. Error bars depict ± 1 SEM (n = 4).*

DISCUSSION & CONCLUSIONS: Values obtained for rat spinal cord compression modulus were in accordance with previously published literature [2]. Yet, in contrast to other mechanical characterisation techniques available, our protocol was able to elucidate the range of modulus values seen in spinal cord tissue over its viscoelastic range. If future tissue-engineered therapies are to be successful, it is likely that careful consideration must be given to the biomechanical properties of native spinal cord tissue. Our novel testing protocol offers one such way in which promising new biomaterial candidates could be easily tested and benchmarked against endogenous cord tissue, and we anticipate that it may add a useful new addition to the CNS tissue-engineers toolbox.

ACKNOWLEDGEMENTS: This research was kindly funded by contributors to the UCL MBPhD Programme.

Lateral cartilage integration in an *in vitro* defect model utilizing surgical adhesives

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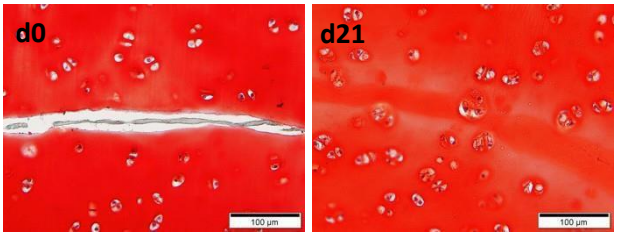
INTRODUCTION: Traumatic articular cartilage injuries often do not heal exhibiting incomplete integration of the fractured surfaces and are a major cause of early osteoarthritis. Enhancing integration of the opposing tissue surfaces at the defect site using surgical adhesives may lead to improved regeneration. In this study, two clinically approved adhesives, a combination of glutaraldehyde and albumin (BioGlue®) frequently used in thoracic aortic surgical repair [1], and a fibrin glue (TissuCol®) were investigated. Additionally, two long-term stable fibrin gels developed by our group [2] were assessed. For all adhesives, their suitability for immediate bonding and long-term lateral cartilage integration was investigated in a biomechanical in vitro model and tissue culture.

METHODS: Porcine cartilage blocks were harvested from the femoropatellar groove. Disc/ring composites were generated and treated at the defect interface with either BioGlue® (CryoLife, Guildford, UK), TissuCol® (Baxter, Unterschleißheim, Germany), or two different formulations of long-term stable fibrin (25 or 50 mg/ml fibrinogen [2]). Constructs receiving no glue treatment served as control. In a custom-made biomechanical push-out test system, adhesive strength was assessed as measure for cartilage integration either 2 hours after glue application or after culture of the constructs for up to 3 weeks in standard chondrocyte medium. The integration site was examined histologically and immunohistochemically for cytotoxic effects (MTT staining) and for major cartilage ECM components.

RESULTS: For control constructs without glue treatment, adhesive strength values were <5 kPa over the whole culture period, no tissue development was observed at the defect site. Using BioGlue®, immediate bonding led to a high adhesive strength of 157.7±32.5 kPa. However, already after one day in culture, in BioGlue®-treated constructs severe cytotoxic effects were observed. Application of TissuCol® led to immediate bonding values of only 13.5±3.9 kPa, but no cytotoxicity was detected over the course of the experiment. Upon *in vitro* cultivation, TissuCol®-treated cartilage constructs exhibited strongly increased adhesive strength of 28.8±16.1 kPa after 1 week and 88.7±51.9 kPa after 3 weeks. A slightly higher increase in adhesive strength was found for both long-term stable fibrin gels (not statistically different). These findings correlated well with cell invasion over time into all three fibrin gels and distinct synthesis of cartilage-specific ECM components such as glycosaminoglycans and collagen II within the gels (Fig. 1).

Fig. 1: TissuCol®-treated disc/ring cartilage constructs immediately after treatment (d0) and after 21 days of culture (d21). Distinct cell invasion and strong GAG deposition (safranin-O staining) was observed within the fibrin gel at the defect interface after in vitro culture.



DISCUSSION & CONCLUSIONS: Our results demonstrate that clinically available adhesives can lead to distinct cartilage bonding. BioGlue® did not appear suitable due to its cytotoxic effects. When using biocompatible fibrin, lateral cartilage integration strength increased strongly over time, however, immediate bonding was rather low. Thus, for clinical use, the development of new adhesive materials that balance the need for strong immediate bonding, adequate cell compatibility, and long-term integration would be beneficial.

ACKNOWLEDGEMENTS: Funding from the European Union is acknowledged, FP7/2007-2013, HydroZONES, grant agreement n. 309962.

Differentiation of adipose-derived mesenchymal stem cells to contractile smooth muscle cells for fibrin-based vascular tissue engineering

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INTRODUCTION: Autologous approaches for vascular tissue engineering are a promising alternative to scaffolds based on decellularized xenogeneic arteries that bear an unclear risk for immunological rejections. Vascular scaffolds can be generated from fibrin gels which are formed by thrombin-based fibrinogen polymerization by a specific moulding technique [1]. Fibrinogen can be easily isolated from peripheral blood and thus displays a high biocompatibility. However, scaffolds as such still have an insufficient stability. In natural arteries stability is conferred by the tunica media consisting of connective tissue fibers and smooth muscle cells (SMC) which, however, cannot be isolated directly from donor tissues. Therefore, we here demonstrate the differentiation of contractile SMC from adipose tissue-derived mesenchymal stem cells (ASC) as a suitable autologous tissue source, their characterization and their application for reseeding of fibrin scaffolds to form an artificial autologous tunica media.

METHODS: The stromal vascular fraction was isolated from adipose tissue of 7 different donors after collagenase digestion and homogenous ASC were obtained after cultivation for 2-3 passages. Tri lineage differentiation and surface marker expression confirmed their mesenchymal stem cell nature [2]. ASC were treated with transforming growth factor-beta1 (4ng/mL), bone morpho-genetic protein 4 (2.5ng/mL) and sphingosyl-phosphorylcholin (1µg/mL) for 4-12 days (SMC-diff). Contractile SMC marker expression was determined by Westernblot and IHC and functionality was assessed by gel contraction assays. Fibrin gels (10 mg/mL fibrinogen) and vascular scaffolds were seeded with SMC-diff or untreated ASC, cultivated under static or dynamic (25dyn/cm^2) conditions for 13 days and assessed for tensile stability.

RESULTS: SMC-diff showed a typical SMC phenotype after 4 days and a maximal expression of smooth muscle myosin heavy chain, smoothelin and alpha smooth muscle actin after 8 days in 6 out of 7 donors. Contraction of SMC-diff-seeded fibrin gels was 68.1% whereas gels seeded with untreated ASC did not contract. Fibrin gels seeded with SMC-diff (Fig. 1) had a 1.9-fold higher tensile stability (maximal force until rupture) than unseeded gels. In the reseeded fibrin scaffolds SMC-diff aligned under dynamic cultivation parallel to the flow and conferred a 1.7-fold higher tensile stability than under static conditions.

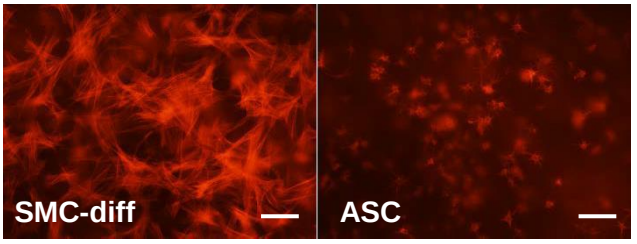

Fig. 1: Fibrin gels (10 mg/mL) seeded with SMC-diff or ASC were stained with for α-actin filaments with Phalloidin-TRITC after 72h. Scalebar 100 µm

DISCUSSION & CONCLUSIONS: It was shown that an autologous approach combining contractile SMC differentiated from ASC and fibrin gels exhibits a promising scaffold for the generation of the medial layer of the arterial wall.

ACKNOWLEDGEMENTS: This project was funded by Biofabrication for NIFE (supported by the german Lower Saxonian Ministry for Science and Culture and the VolkswagenStiftung).

Development of a collagen-based film for limbal stem cell transplantation

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INTRODUCTION: Limbal stem cell deficiency (LSCD) is a condition caused by a depletion of limbal stem cells (LSCs), which reside in the periphery of the cornea and replenish the transparent outer epithelial layer. A depletion of this stem cell population may occur due to external damage or dysfunctions of the limbal niche, leading to infiltration by the non-transparent, vascularised mucous membrane known as the conjunctiva, which can then cause blindness. LSCDs can be treated by *ex vivo* culturing of LSCs on a carrier before transplantation to the affected cornea. Currently, the gold standard carrier is amniotic membrane (AM), however this has several drawbacks relating to the need for harvest from donated tissue. The aim of this work is to address these shortcomings by developing a collagen-based film with tailored structural and biological properties via the use of crosslinking techniques and biomolecule coatings respectively.

METHODS: Collagen-based films consisting of 0.5wt% collagen/0.044wt% hyaluronic acid were dried at room temperature before assessing the effect of dehydrothermal (DHT), UV/riboflavin (UV/Ribo) and 1-Ethyl-3-3-dimethyl aminopropyl carbodiimide/N-hydroxysuccinimide (EDC/NHS) crosslinking on mechanical and degradation properties. The biochemical properties of films were also varied through the surface attachment of biomolecules native to the limbal niche, including fibronectin, vitronectin and type IV collagen. Cell studies utilised a human corneal epithelial cell line, hTCEpi, and primary human LSCs to assess proliferation (Click-iT EdU assay), gene (qPCR) and protein (immunofluorescence) expression. Human macrophages were used to assess *in vitro* immunogenicity by measuring expression of the inflammatory cytokine, TNFα(ELISA).

RESULTS & DISCUSSION: The fabrication process produced thin, transparent collagen-based films. Different crosslinking methods elicited a spectrum of mechanical properties (within the range of AM and native cornea[1]), with EDC/NHS producing the greatest stiffness and UV/Ribo producing the highest toughness. EDC/NHS crosslinking also significantly reduced enzymatic degradation. These findings were important to the surgical handling performance of the materials and their maintenance of structural integrity *in vivo*.

In addition to UV/Ribo crosslinked and fibronectin coated films inducing the highest rates of proliferation in hTCEpi cells, these fabrication techniques were also able to maintain expression of LSC markers. Furthermore, human LSCs were successfully expanded from limbal tissue explants on the collagen-based films and shown to maintain expression of the LSC marker, p63α (Fig. 1).

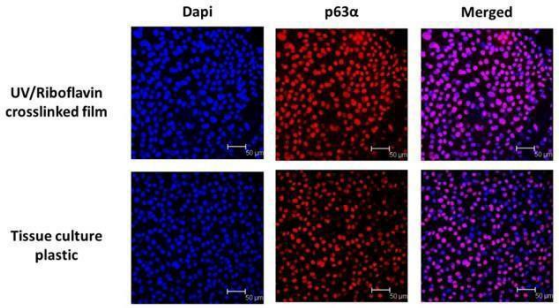

Fig. 1: Human LSCs expanded from limbal tissue explants showing nuclei (blue) and p63α expression (red) (scale bars represent 50 µm).

Finally, treatment of collagen-based films with different crosslinking techniques or fibronectin coating did not increase macrophage expression of TNFα, highlighting the potential for successful transplantation of these materials.

CONCLUSIONS: These findings can be used to inform the design of a collagen-based film that can successfully replace AM in the treatment of LSCD.

ACKNOWLEDGEMENTS: The authors would like to thank funding sources including the Irish Research Council (GOIPD/2013/269) and Enterprise Ireland (CF20144003).

Human mesoangioblasts and fibroblasts co-culture enhances repopulation of bioengineered oesophagi

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INTRODUCTION: Final repair of long-gap oesophageal atresia still remains a clinical challenge since primary anastomosis is not feasible and therapeutic options are associated with severe complications. In this scenario, tissue engineering is emerging as a clinical reality, with the aim to develop functional replacements through the combination of biomaterials and stem cells. Engineering the oesophageal muscle layer appears to be a key factor in fostering tissue regeneration *in vivo*, however it has not been successfully established yet. Aim of this study is to mature an oesophageal muscularis externa using decellularized matrices, natural templates that preserve tissue-specific extracellular information [1]. Mesoangioblasts were selected as best cellular candidates displaying a preferential commitment towards the smooth muscle fate [2] and combined with stromal cells using static and 3D dynamic culture.

METHODS: Decellularization of rat oesophagi was performed using our established detergent-enzymatic protocol [3]. Mesoangioblasts (hMABs) were isolated from human skeletal muscle biopsies; fibroblasts (mFBs) were obtained from mouse hindlimb muscles by enzymatic digestion. Different ratios of hMABs and mFBs were injected in the oesophageal muscle layer and cultured up to 11 days in static or dynamic culture using customised bioreactors.

RESULTS: Fibroblasts increased invasion of hMABs across the scaffold layers, favouring an overall homogeneous distribution. In particular, a combination of 85% hMABs and 15% mFBs enhanced cell engraftment without over-proliferation of stromal cells. hMABs proliferation and differentiation was not affected by the presence of fibroblasts, as detected with KI67 and SM22/Calponin stainings. The majority of proliferating and smooth muscle differentiating hMABs were observed within the muscle layer, suggesting a strong cell-matrix interaction.

Fibroblasts' positive effect on tissue re-colonization was observed also in the dynamic system, where the bioreactor provided a beneficial

3D culture setup, therefore significantly enhancing growth and differentiation of hMABs.

DISCUSSION & CONCLUSIONS: Effective cell engraftment is a pivotal requirement as well as the major challenge for successful development of bioengineered organs.
In this study, human mesoangioblasts have been identified as a successful cell source for oesophageal muscle engineering thanks to their high plasticity. A finely regulated co-culture with tissue-specific fibroblasts maximizes their migration capacity, leading to optimal matrix colonization with no fibrotic effects.
The crosstalk between mesoangioblasts, fibroblasts and oesophageal extracellular matrix could be a key factor in the development of functional engineered oesophagi.

DISCLOSURES: The authors have nothing to disclose.

A cell-responsive biomimetic extracellular matrix material for periodontal tissue regeneration

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INTRODUCTION: Periodontal diseases are often associated with irreversible bone loss. Though traditional therapies can be effective in preventing the progression of such diseases, the regeneration of the lost tissues is limited and further therapies are needed. Periodontal tissue engineering that combines supportive biomaterials and stem cells can potentially serve as a promising alternative to conventional periodontal treatments.

Cell adhesion is vital to survival, proliferation and the overall maintenance of tissue integrity. The extracellular matrix (ECM) not only provides anchorage, but also plays a role in determining cell fate and assisting with cellular migration. This project aims to evaluate the cell-responsiveness and regenerative capacity of a novel, injectable biomimetic hydrogel material designed to augment periodontal regeneration by controlled local delivery of target-specific biomolecules.

METHODS: A sterile protease sensitive poly-ethylene glycol (PEG) hydrogel functionalised with 2.5 mM of cRGD was synthesized as adapted from Lutolf et al[1]. Circular disks of the gel were created and are swollen in mesenchymal stem cell growth media (MSCGM™) for *in vitro* testing purposes. NUNC™ chamber slides were pre-coated with 2% agarose 24 hours prior to the experiment, onto which the swollen gels were placed. Mesenchymal stem cells (P<6) were seeded directly onto the circular hydrogel discs and allowed 2 hr for attachment prior to addition of 600µl of MSCGM per chamber. A control setup included a non-functionalised PEG gel and a chamber slide with a functionalised PEG gel placed directly onto the glass slide. Phalloidin conjugated to Alexa ® 488 and DAPI were used to fluorescently label the cytoskeleton and nucleus respectively. Confocal microscopy enabled visualization of these fluorescent stains.

RESULTS:

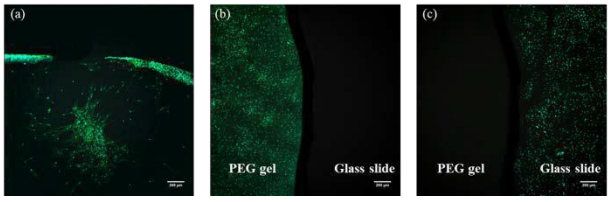

Fig. 1: (a) Attachment of MSCs onto the RGD functionalised gel placed on an agarose coated glass slide. (b and c) Two different focal planes across the same field of view along the curvature of a gel disk placed on a glass slide.

As seen from figure 1 (a), MSCs have successfully attached to the RGD functionalised PEG gel, however no cells appear to attach to the agarose. Interestingly, the cells here appear to cluster towards the periphery of the gel. Conversely, figure 1 (b) and (c) show successful attachment of cells onto the RGD functionalised gel as well as the glass slide respectively.

DISCUSSION & CONCLUSIONS: This preliminary experiment successfully assesses the feasibility of a chamber slide setup. We see that agarose prevents cell attachment to the glass slide directing cells to selectively attach to the RGD functionalised polymer gel instead. Additionally, this experiment was important to evaluate whether this set up would allow for successful confocal imaging.

The biophysical and biochemical properties of the chosen synthetic cell-responsive ECM material influence the homing and behaviour of stem/progenitor cells. The chemical composition, cross-linking density, and enzymatic degradation of the hydrogel defines the capacity of the material to influence cell homing, cell migration, and cell differentiation, and thus the regenerative potential of the cells within the hydrogel material. An ongoing study involves the assessment of cell viability and cell morphology within the gel with varying concentrations of linear and cyclic RGDs, prior to any further customization. Cell migration is to be determined using confocal microscopy and time lapse renders.

ACKNOWLEDGEMENTS: The authors would like to thank The Research Council of Norway (NFR) 143978/F20 for providing financial support to this project.

Stem cell isolation from bladder washes

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INTRODUCTION: Autologous human urothelial cells isolated from bladder washes have a high proliferative capacity *in vitro* and a strong potential for application in clinical regenerative medicine (1). As part of quality controls we analyzed telomere length and oncogene regions in cultured cells from bladder washes.

METHODS: Cells were harvested from bladder washes from healthy pediatric patients (n=10) undergoing urological interventions requiring catheterization or cystoscopy (non-urological interventions or diagnostic cystoscopy). Primary cultures were established in 10-cm^2 culture wells. After 2–3 weeks, at sub-confluence, cells were further expanded in 25-cm^2 flasks. Immunoassays were used to evaluate urothelial specific markers such as cytokeratins and uroplakins. The expression of human mesechymal stem cell markers was analyzed by flow cytometry. The expression of the senescence marker (CDKN2A/p16) was evaluated with RT-PCR. Relative telomere length of cultured cells was measured by real time quantitative PCR and FISH. Oncogene regions were studied witharray-CGH

RESULTS: Human urothelial cells isolated from bladder washes expressed typical urothelial markers such as cytokeratins and uroplakin IIIa. In 5/10 samples cells were proliferative also after 14 passages. In these cell cultures, telomeres increased in length after each passage. Cells that were proliferative after passage 8 did not expressed the senescence marker CDKN2A/p16, but in cultures with decreasing proliferative capacity CDKN2A/p16 was expressed at passage 8 and telomere length did not change over time. To rule out if genetic instability played a role in the progressive elongation of the chromosomes, we analyzed chromosomal regions containing known urothelial oncogenes and found that these regions presented no gene dose aberrations. Analysis of cells cultures with increased telomere length in culture showed that part of the population could be characterized as urothelial stem cells (Table 1)

Table 1. Characterization of cells with flow cytometry by using mesenchymal stem cell markers in 3 different cell populations (A, B and C) isolated from bladder washes. All samples were negative for CD14, CD31, CD34, CD45, CD80 and HLA class II.

	Mean % positive cells		
Marker	**A**	**B**	**C**
CD73	93	91	64
CD90	59	85	64
CD105	10	18	9
HLA class I	96	96	60

DISCUSSION & CONCLUSIONS: Cell harvesting from bladder washes has a potential for isolation of different cell populations including urothelial stem cells. The cells did not presented gene dose alterations during the time in culture according to the array CGH results, but an increase in telomere length. In ongoing studies we are investigating the possible mechanisms for the increased telomere length. In the future, stem cells could be selected for autologous cell expansion and re-implantation to the patient for tissue expansion in reconstructive regenerative medicine. However, pre-transplantation quality controls including: cell characterization in respect to cell type, proliferative capacity, genetic stability and programmed senescence are crucial to secure biosafety to the patient

ACKNOWLEDGEMENTS: To Swedish Society of Medical Research, Swedish Society of Medicine, HRH Crown Princess Lovisa's Memorial foundation and Promobilia Foundation.

In vitro engineering of colon epithelium

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INTRODUCTION: Inflammatory diseases of the human GI tract may result in loss of the mucosa. These conditions are currently treated with surgery to remove part of the intestine or with expensive medications that only dampen symptoms. An *in vitro* culture system has been developed in which colonic epithelial crypt cells are suspended in Matrigel and overlaid with medium containing a well-defined set of growth factors [1,2]. The resulting organoids have been shown to have regenerative potential [3]. However Matrigel is not a suitable substrate for clinical application. The main aim of this project is to find a well-defined, reproducible scaffold which supports the *in vitro* proliferation of colon epithelial cells, and can be used to deliver the cells in the form of a regenerative tissue graft.

METHODS: Gelatin and PET nanofibre and microfibre scaffolds were electrospun. Gelatin scaffolds were crosslinked with 25mM EDC and 10mM NHS in 90% ethanol for 24 hours at 4°C.

The colon cancer cell line, HT29 was grown on scaffolds for 7 days. Cell proliferation was assessed by measuring cell metabolism with the Presto Blue assay. Formation of tight junctions was investigated by Zo-1 immunostaining.

RESULTS: HT29 cells were equally viable on all the electrospun scaffolds, as demonstrated by Presto Blue results in figure 1. Immunostaining of the tight junction marker Zo-1 is shown in figure 2. The images indicate that the smaller nanofibres better support tight junction formation, as the 'chicken-wire' Zo-1 expression is more clearly seen. It also seems that gelatin microfibres do not support tight junction formation in HT29 cells.

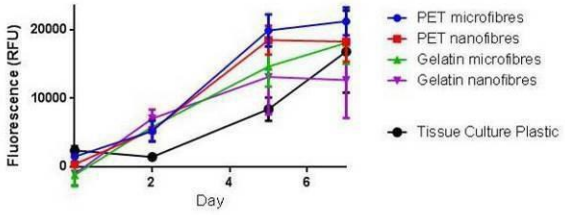

Figure 1: Presto Blue data showing HT29 cell proliferation over 7 days on electrospun scaffolds and tissue culture plastic. Data points are mean ± SD (n=3)

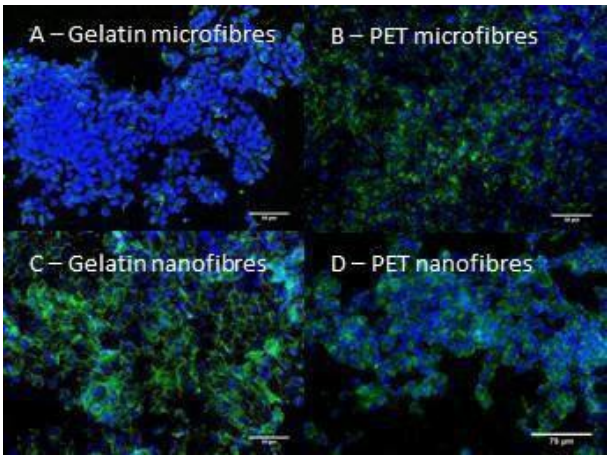

Figure 2: Immunostaining for Zo-1 in HT29 cells after 7 days culture on electrospun scaffolds. Zo-1 in shown in green, and Hoechst nuclear stain is shown in blue.

DISCUSSION & CONCLUSIONS: There are no published studies in which colon epithelial cells have been cultured on gelatin or PET electrospun scaffolds. Therefore this novel work shows that colon epithelial cell lines are viable and able to form tight junctions on these scaffolds. Further data will be obtained from more cell lines and different scaffold forms. The most appropriate scaffolds will then be chosen for use with primary colon cells.

Primary epithelial crypt cells have been isolated from human colon. Organoids are generated when crypt cells are cultured in Matrigel with media containing a well-defined combination of compounds and growth factors, including Wnt3a, R-Spondin, Noggin and EGF.

The next step in this project will be to seed primary colon crypt cells onto the chosen scaffolds. Scaffold effect on primary cell growth and differentiation will then be investigated.

ACKNOWLEDGEMENTS: This research is funded by the BBSRC doctoral training partnership (DTP).

Extensible gelatin scaffold for tissue engineered cartilage

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INTRODUCTION: Although promising results in the cartilage tissue engineering have been reported, problems such as inflammatory reactions to the implanted materials, poor cell-to-cell communications, and toxic degradation products [1]. One of the major concerns is poor integration of the tissue engineered cartilage with the host tissues. Indeed, gaps have been found between the implanted chondrocyte/scaffold construct and recipient tissue in several studies [2]. Consequently, the development of new biodegradable scaffolds for improving engineered tissue integration shall possess a critical role in cartilage tissue engineering.

METHODS: In this study, microfluidic technology was used to prepare an expandable gelatin scaffold with a honeycomb-like structure [3]. The physical properties of the highly organized scaffold were characterized. Primary chondrocytes were harvested from rabbits, expanded, and seeded into the expandable scaffolds. The *in vivo* performances of the chondrocyte/scaffold construct were verified in a rabbit model of osteochondral defects. Animals were sacrificed 6 months after surgery, and the regenerated cartilage tissues were retrieved and analyzed by using mechanical test and histology.

RESULTS: The untreated osteochondral cylindrical defects did not repair, and some fibrous tissues were noticed. The defects transplanted with tissue engineered chondrocyte/scaffold constructs showed cartilage regeneration with good integration with recipient tissue. Histological analysis revealed that implanted chondrocytes distributed uniformly, maintained functional phenotype and secreted components of extracellular matrix (ECM).

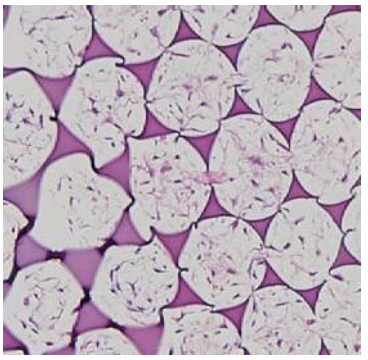

Fig. 1: In vitro chondrocyte culture in highly organized scaffold: H&E stain revealed the cells secrets abundant ECM and migrate via interpore. The scaffold is stacking by hexagonal structure.

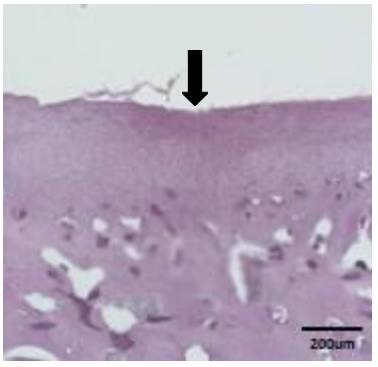

Fig. 2: Histological sections showed well integration between engineered cartilage and host tissues formation. Mild surface fibrillation was noted at the newly formation cartilage.

DISCUSSION & CONCLUSIONS: Due to the high swelling ratio and porosity, our expandable gelatin scaffold is able to fill any disfigurations and demonstrates locomotion similar to the pseudopodium of some microorganism. Animal study revealed that the expandable scaffold had high integration efficiency, and the chondrocytes within this scaffold possessed normal phenotype with adequate ECM production. In conclusion, the expandable scaffold improves chondrocyte/scaffold construct integration with the host tissue and benefits cartilagerepair.

ACKNOWLEDGEMENTS: This study was supported by Taipei Tzu Chi Hospital, The Buddhist Tzu Chi Medical Foundation, and the Ministry of Science and Technology, Taiwan.

DISCLOSURES: The authors confirm that there are no conflicts of interest.

histological analysis of in vitro co-culture and in vivo mice co-transplantation of stem cell-derived adipocyte and osteoblast

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INTRODUCTION: Many researchers have focused on the role of adipocytes in increasing efficient bone tissue engineering and osteogenic differentiation of stem cells. However, previous reports have not reached a definite consensus on whether adipocytes positively influence *in vitro* osteogenic differentiation and *in vivo* bone formation. We investigated the adipocyte influence on osteogenic differentiation from adipose-derived stromal cells (ADSCs) and bone formation through histological analysis *in vitro* and *in vivo*.

METHODS: ADSC cultured on porous membranes and differentiation : The ADSCs were seeded on inverted transwell cell and were induced adipocyte in adipogenic induction media (Gibco BRL) for 14 days. Subsequently, ADSCs were seeded inside the insert. The medium was refreshed with osteogenic induction media (Gibco BRL) for 14 days. After 30 total days of ADSC culture, fully osteogenic differentiated cells were identified using standard alizarin red staining.

Co-transplantation of osteoblasts and adipocytes differentiated from ADSCs : The osteoblasts were mixed with adipocytes in 1 ml of type I collagen hydrogel (Sigma) and injected into the subcutaneous dorsum of the mice mice (BALB/c-nu, 8-week-old females; SLC, Tokyo, Japan). 4 and 6 weeks after injection, the mice were sacrificed. Bone formation was measured by histological analysis and plain radiogram (soft x-ray; Softtex, Sofron Co., Tokyo, Japan).

RESULTS: ADSCs co-cultured with ADSCs on the top and bottom of transwell membrane were intensely stained compared with the top of ADSCs co-cultured with the bottom of adipocytes.

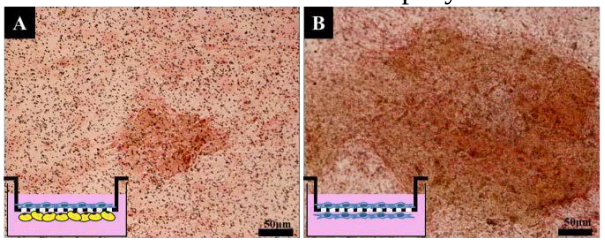

Fig. 1 : Influence of adipocytes on the osteogenic differentiation of ADSCs in a co-culture system.

The implants were evaluated by histological analysis and plain radiogram. From the histology of the implants, new bone and adipose formation occurred in both groups. However, The osteoclast density was significantly higher in the osteoblast-like cells-only transplantation group than in the co-transplantation group.

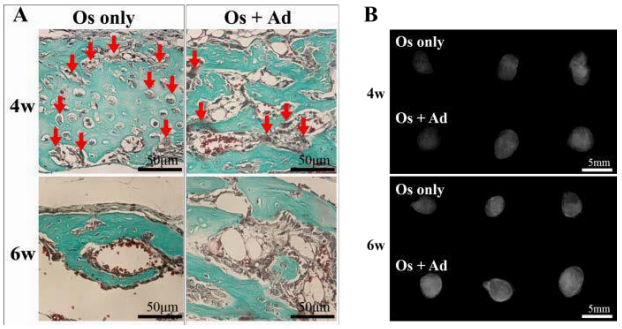

Fig. 2 : Influence of adipocytes of co-transplantation system.

DISCUSSION & CONCLUSIONS: This study demonstrates that adipocytes inhibit osteogenic differentiation of ADSCs *in vitro* but enhance the efficacy of osteogenesis *in vivo*. In this regard, we think that the secretion level of cytokines(PPAR-γ, leptin, adiponectin) from adipocytes will bring out change in *in-vitro* and *in-vivo* condition.

ACKNOWLEDGEMENTS: This research was supported by a grant (HI14C-2755-020014) of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), (PJ.00995602) from the Next-Generation BioGreen 21 Program of Rural Development Administration funded by the Korean government and (10047890) from the Industry Sources Development Project and funded by the Ministry of Trade, Industry and Energy.

Hydroxyapatite-silk fiber-silk fibroin tri-composite scaffolds for bone tissue engineering

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INTRODUCTION: Bone fracture, breakage and other bone related defects have always been a major concern in the medical field. This is mainly due to shortage of bones for transplantation as well as morbidity of donor site (autografting) [1]. Even after transplantation immune rejections (allografting) have been often observed from the recipient. The growing demand for a bioactive, biocompatible, biodegradable, load-bearing, osseointegrative tissue-engineering scaffold for *in vivo* bone tissue regeneration brought us with the current research idea [2]. In this study, we developed a highly porous and biocompatible silk based composite scaffold by incorporating osteoinductive hydroxyapatite (HA).

METHODS: Silk fibroin (*Antheraea Assama*, a non-mulberry Indian endemic variety) fibre reinforced and HA deposited scaffolds were characterized for their physical properties using FTIR, XRD and FESEM. *In vitro* compatibility of scaffolds was quantified with MG63 cells and human bone marrow stem cells (BMSCs) followed by expression profile assessment of Col I, BSP, OPN genes using RT-PCR and immunostaining.

RESULTS: The results show that the addition of HA and silk fiber led to significant increase in scaffold stiffness with nearly similar pore interconnectivity and scaffold porosity. Fiber reinforcement of scaffolds made them stiffer and rigid, hence preferred by bone cells. Comparatively reduced water uptake and elevated biodegradation time could further be justified by fiber reinforcement. *In vitro* proliferation studies suggested ~2.0 and 1.8 fold increase for MG63 and BMSCs respectively when cultured on tri composite scaffold (MSMF HA) and were significantly higher than other groups ($p \leq 0.05$). A similar result was obtained for ALP activity where maximum value was recorded for MSMF HA group. Additionally ~8 and 10 fold increment of BSP and col-I expression profile in human BMSCs on same group scaffolds was recorded.

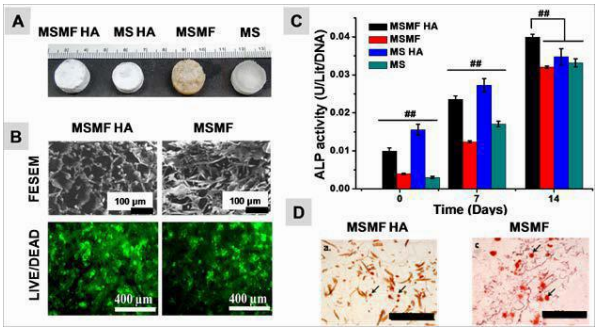

Fig. 1: A) Gross morphology of scaffolds, B) Live-dead staining of bone cells, C) ALP expression profile of MG63 cells cultured on HA silk scaffolds ($^{\#\#}p \leq 0.01$), D) Alizarin red staining of scaffold sections showing mineralization (Scale 400 µm).

DISCUSSION & CONCLUSIONS: The aim of this study was to develop a three dimensional scaffold by incorporating osteoinductive HA particles into a highly porous and extremely biocompatible silk-based scaffold. The different ALP activity profiles were related with the degree of crystallinity or surface roughness due to the HA deposed and variation in mechanical strength due to fiber reinforcement. It is known that incorporation of varying length of fiber led to enhanced ALP activity, expression of functional markers, improved mechanical strength and proliferation when cultured with hMSCs for osteogenic differentiation [3]. The major contribution of the HA was to guide the deposition of the newly formed bone mineral and help in their nucleation.

ACKNOWLEDGEMENTS: Department of Biotechnology, Indian Council of Medical Research, Department of Science and Technology, Government of India and IIT Guwahati are acknowledged for their generous funding.

Kidney regeneration through host stem cell recruitment supported by a novel anti-inflammatory scaffold

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INTRODUCTION: Polylactic acid-polyglycolic acid copolymer (PLGA) is a material with useful applications in biomedicine, however, the production of acidic by-products during *in vivo* degradation is a serious concern, because these by-products can produce inflammatory reactions. In this work, magnesium hydroxide, $Mg(OH)_2$, was incorporated into a PLGA scaffold to neutralize the acidic environment. The manufactured $Mg(OH)_2$-incorporated PLGA scaffold (PLGA/$Mg(OH)_2$) is expected reduction of inflammation and stimulation of host stem cell recruitment into the scaffold.

METHODS: A PLGA/$Mg(OH)_2$ scaffold was fabricated by a freeze drying method. For regenerativity analysis of the scaffold, the right kidney was exposed through an incision on the back, and was subjected to partial nephrectomy. Mice (ICR) were divided into 3 groups, 1) control, 2) PLGA and 3) PLGA/$Mg(OH)_2$. For histological, IHC and real-time PCR analysis, the implanted scaffold was harvested at 0, 1, 2, 4, 6, 8, and 12 weeks after operation.

RESULTS: PLGA/$Mg(OH)_2$ scaffold showed more swelling and faster degradation through all observed period than PLGA scaffold. At week 6, the scaffold was not observed with bare eyes.

In histological analysis, several pores appeared at week2. At week 4, PLGA/$Mg(OH)_2$ scaffold showed tight cell density and the premature glomerular structure, at week 6, mature glomeruli were showed, and at week 8, the number and size of glomeruli were increased. At week 12, the scaffold region was replaced by regenerated renal tissue. The glomerular number per unit area of the PLGA/$Mg(OH)_2$ was 14.67±1.53, and PLGA was 10.00±2.00 at week 12 (control, 9.33±1.15).

The acute inflammation was observed around the scaffold at week 1, and monocytes were predominant. From week 2, monocytes migrated into the degrading scaffold and differentiated into macrophages, and then joined together to form foreign body giant cells. The number of immune cell was significantly reduced in the PLGA/$Mg(OH)_2$ scaffold compared to the PLGA. In IHC analysis, the expression of T cell markers, pro-inflammatory factors and fibrosis-related factors was mainly found at the implanted PLGA scaffold. The expression levels of stem cell and renal differentiation markers in the PLGA/$Mg(OH)_2$ scaffold were higher than those in the PLGA.

DISCUSSION & CONCLUSIONS: The PLGA/$Mg(OH)_2$ scaffold produced low immunity/inflammation/fibrosis and high anti-inflammatory factor expression . The increased host cell recruitment was revealed by enhanced glomerular number and the expression of genes related to renal differentiation. Therefore, the incorporation of $Mg(OH)_2$ into the PLGA scaffold resulted in a reduced inflammatory response and enhanced renal reconstructivity as a promising scaffold for renal regeneration.

ACKNOWLEDGEMENTS: This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (2014R1A1A3049460); (NRF-2014M3A9D3033887); funded by the Korea government (MSIP) (NRF-2015R1C1A1A01053509); funded by the Ministry of Education (2015R1D1A3A03020378); and supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare (HI14C1642).

Isolation and immortalisation of tonsillar keratinocytes for three-dimensional tissue engineered tonsil mucosal models

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INTRODUCTION: The generation of tissue engineered epithelial models is often hampered by the limited proliferative capacity of primary epithelial cells. This study aimed to isolate primary keratinocytes from human tonsils removed during routine tonsillectomies by enzymatic digestion and then immortalise these cells using a Rho kinase inhibitor. Immortalised cells were used to create functional tissue-engineered tonsillar tissue that could be used to model tonsillitis.

METHODS: The efficiency of cell isolation using two different enzymes (trypsin and dispase) was compared. The growth of primary tonsil keratinocytes was measured and compared to cells cultured in the presence of the Rho kinase inhibitor Y27632. Immortalised keratinocytes were used to develop a tissue-engineered model of tonsil epithelium using primary tonsil fibroblasts and de-epithelialized dermis. These models were then incubated with *Streptococcus pyogenes* to model tonsillitis and the expression of pro-inflammatory cytokines measured by cytokine array and ELISA.

RESULTS: Enzymatic digestion of tonsillar tissue with trypsin resulted in the isolation of significantly more keratinocytes compared to dispase isolation. Keratinocytes cultured without the Rho kinase inhibitor Y27632 survived in culture for less than 10 population doublings whereas cells cultured in the presence of this inhibitor grew for over 30 population doublings without changing their phenotype. Tonsil keratinocytes and fibroblasts cultured in three dimensions produced a multi-layered differentiated epithelium that histologically resembled the surface epithelium of normal tonsils and responded to *S.pyogenes* by increasing expression of pro-inflammatory cytokines.

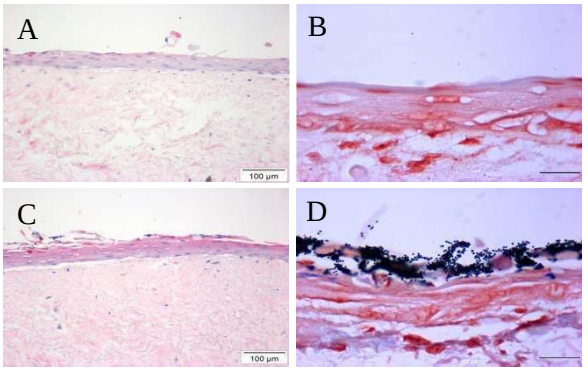

Fig. 1: H&E and Gram stained images of uninfected control (A-B) and S. pyogenes (C-D) infected tissue engineered tonsil mucosal models.

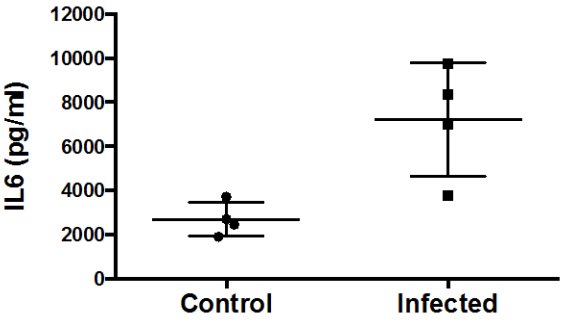

Fig. 2: IL-6 release from uninfected controls and S. pyogenes infected tissue engineered tonsil mucosal models.

DISCUSSION & CONCLUSIONS: Tonsil keratinocytes can be successfully isolated and cultured *in vitro*. Y27632 was able to markedly prolong the life-span of keratinocytes without any deleterious consequences to the cell phenotype making these cells useful for a number of applications which require longer term culture. A functional tissue engineered model of tonsil epithelium was generated which will provide a useful tool for studying cells in a more physiologically relevant way.

Generation of Nucleus pulposus cells from stem cells: first steps towards intervertebral disc regeneration

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**Authors equally contributed to this work*

INTRODUCTION: The nucleus pulposus (NP) is the central part of the intervertebral disc (IVD) and plays a key role in spine kinematic. At birth, the NP is populated by two cell types: the notochord cells (NTCs) known as the NP progenitors and the nucleopulpocytes (NPCytes) which are responsible for the synthesis of NP-specific extracellular matrix [1, 2]. The early decline of the NTCs followed by the progressive disappearance of the NPCytes initiates an irreversible degenerative cascade in the NP ultimately leading to the loss of IVD biomechanical properties. In this context, repopulating a damaged NP with regenerative cells seems to be a promising approach for long-term regeneration of IVD [3]. Our objectives in this study are (i) to generate notochord precursors from human induced pluripotent stem cells (hiPSC) and (ii) NPCyte-like cells from human adipose stromal cells (hASC).

METHODS: Both Nodal and Wnt signaling pathways are required for the formation of the notochord in the mouse embryo. Notochord cells arise from progenitors expressing FoxA2/Brachyury. Human iPSC differentiation was thus induced with activin A and CHIR99021 (inhibitor of GSK-3). In parallel, NPCyte-like cells were generated from hASC cultured in the presence of both Transforming Growth Factor-β1 (TGF-β1) and Growth Differentiation Factor 5 (GDF5).

RESULTS: Our results showed that 30% of differentiated hiPSC expressed both FoxA2 and Brachyury after 24 hours of treatment by activin A and CHIR99021, whereas activin A alone induced only 10% double positive cells. The differentiation of hASC towards NPCyte lineage is robust and highly reproducible (9 patients). In addition, the transplantation of these engineered NPCyte-like cells in *nude* mice subcutis demonstrated that they survived, maintained their specialized phenotype and secretory activity *in vivo*. Finally, we have analyzed the role of TGF-β1 and GDF5 canonical pathways during differentiation. Our results indicated that the acquisition of the NPCyte molecular identity was mainly governed by the Smad2/3 pathway while the acquisition of NPCyte morphology was controlled by the Smad1/5/8 pathway.

DISCUSSION & CONCLUSIONS: The combination of both engineered cell types may show a great potential for the restoration of the biological equilibrium of the NP and thus provides a long-term regenerative strategy for degenerated IVD.

ACKNOWLEDGEMENTS: This study was supported by INSERM and grants from the Fondation pour la Recherche Médicale (FRM pionner de la recherche "Bioingénierie"), the AO Foundation (S-12-14G), the Fondation de l'Avenir pour la Recherche Médicale Appliquée (FARMA ET3-683), the region Pays de la Loire (BIOREGOS II) and the Agence Nationale pour la Recherche (ANR générique 2014 REMEDIV). PC is the recipient of a doctoral grant from the AXA Research Fund.

Comparing the myocardial glycoprofile across non-regenerative and regenerative models: an inner look at the extracellular environment

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INTRODUCTION: Recent studies have begun to focus on the expression of glycans during the extracellular remodeling after ischemic injury [1]. Since a comparative analysis of the myocardial glycoprofile across different species is still missing, our aim was to investigate glycans, especially sialic acid residues expression, across regenerative and non-regenerative animal models. We hypothesize that a differential glycan expression across these models will enable us to tailor a regenerative treatment for the infarcted myocardial tissue.

METHODS: Lectin histochemistry and lectin microarray analyses were performed on heart ventricle samples from healthy Lewis and Fischer rats (n=3), Romanov sheep and *cmlc2* zebrafish. Hearts were removed and fixed in 4% PFA and cryopreserved for cryosectioning. Sections of 5 μm thickness were stained with H&E, Masson's trichrome and Wheat Germ Agglutinin (WGA) and *Sambucus nigra* (SNA-I) lectins. Membrane associated proteins were extracted from snap-frozen myocardial samples and fluorescently labelled. Labelled membrane extracts were incubated on a 48-lectin microarray for 1 h. Microarrays were imaged using a G2505 microarray scanner (Agilent). All statistical analyses were performed using Minitab Express™ (Minitab® Software).

RESULTS: Distinct patterns of glycan expression were found by lectin histochemistry analysis across sheep, rat and zebrafish myocardium (Fig. 1 A,B), as expected from different species. Sialic acids (Neu) and *N*-acetylglucosamine (GlcNAc) expression was assessed using WGA lectin. Moreover, specific α-(2,6)-linked Neu was detected across all the species by binding to SNA-I lectin, showing an extracellular localisation in correspondence of the capillaries structures. Hierarchical clustering analysis on two strains of healthy rat has shown two clusters having a 58% similarity, remarking the difference in binding intensity between the two strains of rat (Fig.1C).

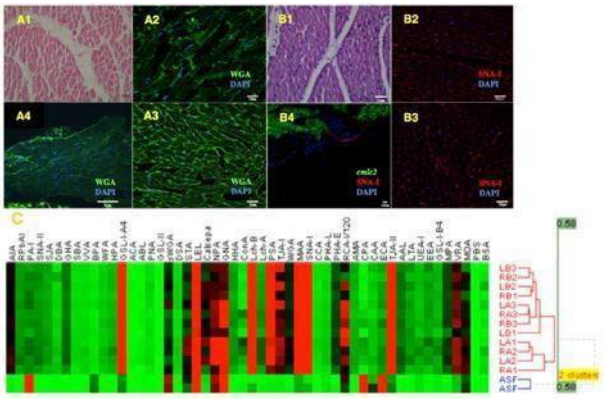

Fig. 1: Interspecies glycans expression in cardiac ventricle samples from non-regenerative and regenerative models. (A,B): H&E (A1) and WGA lectin staining on healthy sheep (A2), rat (A3) and zebrafish (A4); Masson's trichrome (B1) and SNA-I lectin staining on healthy sheep (B2), rat (B3) and zebrafish (B4). (C): Lectin microarray on healthy snap frozen myocardial left (L) and right (R) ventricles from protein extracts of Fischer (a) and Lewis (b) rats.

DISCUSSION & CONCLUSIONS: Neu and GlcNAc residues, which are involved in cardiac signalling and functionality [2], were consistently expressed across both regenerative and non-regenerative models. Moreover, a 58% intra-species similarity was detected in rat myocardial samples and current investigations are quantifying the analogy with zebrafish.

ACKNOWLEDGEMENTS: Seventh Framework Programme Grant Agreement no.: 317304; Centre for Microscopy & Imaging funded by NUI Galway and PRTLI, Cycles 4 and 5, National Development Plan 2007-2013; Science Foundation Ireland (SFI) and European Regional Development Fund under Grant Number 13/RC/2073

Regeneration of osteochondral defects by spatial modulation of endochondral ossification within engineered cartilage grafts

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INTRODUCTION: A tissue engineered implant is an attractive treatment for osteochondral (OC) defects as it has the potential to regenerate the joint surface. Treating OC defects involves restoring the damaged cartilage and subchondral bone. Current treatments such as mosaicplasty result in donor site morbidity [1]. One promising route is to recapitulate the process of endochondral ossification by which long bones are formed. Chondrogenically primed bone marrow derived stem/stromal cells (BMSCs) typically progress along an endochondral pathway, becoming hypertrophic and being replaced with bone. However, under certain conditions, specifically low oxygen or in the presence of joint-like mechanical loading BMSCs have been shown capable of differentiating into more phenotypically stable articular cartilage [2,3]. The objective of this study is to engineer a cartilaginous template (CT) using BMSCs and to explore the potential of such a developmentally inspired implant to regenerate lapine OC defects. It is hypothesised that this CT will undergo spatially defined differentiation *in vivo* in response to the unique environmental conditions within an OC defect, resulting in the development of newly regenerated hyaline cartilage region overlying a layer of endochondral bone.

METHODS: The CT was fabricated as follows; Rabbit BMSC were expanded until P2. Cylindrical hydrogel constructs were formed (4x4 mm) using a 1.5% RGD modified alginate, seeded with 20E6 cells/ml. The constructs were maintained in chondrogenically defined media supplemented with TGF-β3 for 4 weeks, and then analysed for chondrogenic markers. Defects (4x4 mm in diameter) were made in the medial femoral condyle of the hind leg of New Zealand white rabbits using a biopsy punch and a surgical burr. Two defects were made per rabbit (one in each femur), empty defects served as controls (n=6). Animals were sacrificed after 3 months.

RESULTS: There was no significant difference between experimental and control groups in terms of bone volume within the osseous regions of the OC defects. However, cartilage matrix deposition in the chondral region was enhanced in the CT group, specifically sGAG and collagen type II deposition. All CT treated defects stained positive for sGAG compared to limited staining in the empty controls. Additionally, collagen type II staining in CT groups demonstrated cellular alignment similar to that of native cartilage (Fig 1C) compared to an unorganised structure in the control (Fig. 1B). Mechanical testing results complement this demonstrating that repaired tissue was significantly stiffer in the CT group (Fig 1A).

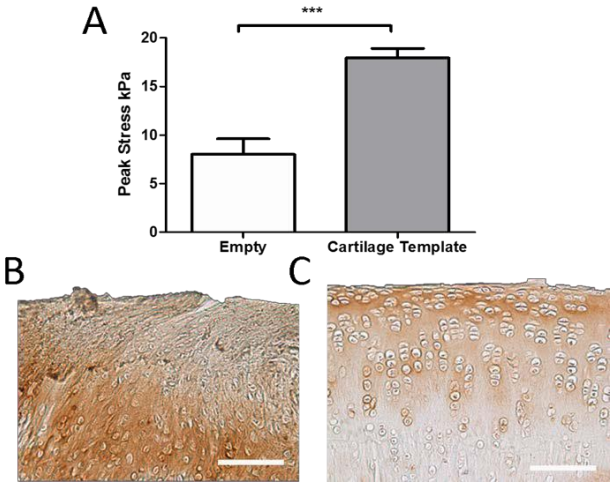

Fig. 1: (A) Indentation testing. Collagen type II staining for (B) empty and (C) CT treated defects. Scale bar- 100 μm, (***)p<0.001, n=6.

DISCUSSION & CONCLUSIONS: These findings demonstrate that local environmental conditions are controlling the fate of the CT *in vivo*. The pre-implanted CT had a homogeneous distribution of matrix components. This template prompted a more hyaline cartilage repair, pointing to the potential for developmentally inspired soft tissue templates, engineered using BMSCs, to regenerate OC defects.

ACKNOWLEDGEMENTS: Science Foundation Ireland (SFI).

Autologous bone marrow mononuclear cells in the treatment of type I ununited anconeal process in dogs

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INTRODUCTION: The ununited anconeal process (UAP) is a well-known lesion observed in young dogs after 6 months of age and the therapy for this lesion is well defined and supported. The present study aimed to describe the use of autologous bone marrow mononuclear cells (BMMNCs) in the treatment of UAP in large dog breed dogs.

METHODS: Four cases of monolateral type I UAP in four German Shepherd dogs (three females and one male) were selected for treatment. The inclusion criteria were age (between 7 and 9 months), presence of type I UAP, and absence of degenerative lesions in the elbow joint. The dogs were referred for monolateral lameness and pain during passive movement of the elbow with a small reduction in the normal range of motion. The radiographic examination performed while the dogs were under general anesthesia revealed mediolateral flexed views of the elbow, UAP without any signs of secondary degenerative joint disease (DJD), and few signs of elbow incongruity, in all dogs. In addition, radiographic examination of the contralateral joint showed the complete union of the ossification centre of the anconeal process. Computed tomography (CT) analysis of the elbows detected evident and irregular fragmentation of the anconeal processes of the olecranons, which were maintained in situ by fibrous tissue. The owners were reluctant to allow invasive surgical therapy, such as ulna osteotomy, and thus cellular therapy was proposed as an alternative. The autologous BMMNCs were obtained from the bone marrow collected from the iliac crest of each dog using a Jamshidi biopsy needle connected to a heparinised syringe (2500 IU/20 ml body mass). The mononuclear cell fraction was separated by gradient centrifugation using Ficoll separating solution (1.077). The cells were washed twice with sterile phosphate-buffered saline solution, stained with a nuclear dye, and counted in a Burker chamber, and then suspending in 1 ml of fibrin glue immediately before injection. BMMNCs were injected, under CT guidance, in the ossification centres of the anconeal processes affected by UAP.

RESULTS: The dogs exhibited no local or systemic adverse reactions after treatment. Radiographic and CT examinations performed at 21, 90, 180, and 270 days after treatment indicated the progressive fusion of the ossification centre, which was completed at 90 days, without any signs of secondary DJD at follow-up after 270 days. One of the treated dogs died at 6 months because of an unrelated complication. The ulna was taken from the treated elbow of this dog for microradiographic and histological study, which revealed the formation of new bone between the anconeal process and ulna.

DISCUSSION & CONCLUSIONS: In dogs affected by UAP, reattaching UAP with a unique screw in association with proximal ulnar osteotomy to allow the proximal ulna to move to a more favorable position relative to the humerus may provide the best means to achieve a good outcome. This surgery is only appropriate when an early diagnosis has been made and where the loose anconeal process has not changed shape. In chronic cases of UAP, the most appropriate treatment is usually removal of the anconeal process. In general, if UAP is not surgically treated, lameness rapidly progresses causing severe pain and degenerative joint disease. The results obtained in this study using a few cases suggest a possible alternative and innovative application of cell therapy to the treatment of type I UAP in dogs

Architecture of the corpus spongiosum urethra

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INTRODUCTION: Urethral reconstruction is performed in patients with urethral strictures or for correction of congenital disorders. As the corpus spongiosum (CS) is important in supporting the urethra, tissue engineering of the urethra should ideally be combined with reconstruction of a CS. For that purpose, detailed knowledge of the architecture of the CS is needed for scaffold designing. The objective of this study is to true size visualize and measure the architecture of the vascular lumen of the CS of the penis.

METHODS: The penises of two fresh male cadavers were dissected. One was frozen into ice in flacid (non-erectile) state, in the other one an artificial erection was induced by saline infusion before freezing into ice. At six different sites of the penis sections were taken using a cryomacrotome and stained by a modified Mallory-Cason procedure. Sections were obtained both in transverse and frontal planes. After digitalization, images were edited in Adobe Photoshop. A distinction was made between tissue and lumen. On transverse and frontal sections area measurements were performed using Image J software. Furthermore, a three dimensional reconstruction was made in Virtual Proteins software, and a physical 3D model was made using stereolithography.

RESULTS: In transverse sections the mean area percentage of the vascular lumen was 60% in the flacid CS, and 77% in the CS of the erect penis. In the frontal sections the area percentages of the vascular lumens of the flacid and erect penis were 53% and 74% respectively. Although some variation was observed, overall no significant difference was found between the section sites. These results implicate that with erection, there is a transverse increase of 129% and a longitudinal increase of 140% of the penis. The three dimensional reconstruction showed connections between the vascular spaces in all possible directions. In general, the structure of the frontal sections was different from the transverse sections. In the frontal plane incomplete septae were more abundant, creating more connections between the relatively larger vascular cavities than in the transverse plane.

DISCUSSION & CONCLUSIONS: To our knowledge, this is the first study that describes the true size architecture of the CS. This novel information on the structure of the CS is relevant for scaffold design in tissue engineering. The protocol provided in this study can be used in future research, for example in a larger number of study objects and/or for extension of analyses.

Ureteral reconstruction in goats using collagen-Vicryl templates after subcutaneous pre-implantation

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INTRODUCTION: Repair of long ureteral defects often requires extensive surgical procedures and graft tissue, which is associated with various complications, e.g. graft site morbidity or lack of suitable tissue. In these cases, tissue engineering may provide an alternative source of tissue.[1] Reinforced collagen templates were evaluated in goats to reconstruct the ureter. In addition, the templates were pre-implanted in the subcutis, which acted as an *in vivo* bioreactor to improve the regeneration outcome.

METHODS: In a cleanroom, tubular templates (l = 7cm, Ø = 6mm) were prepared from 0.7% (w/v) bovine type I collagen and a fast-biodegradable knitted Vicryl mesh (Ethicon), followed by ethylene oxide sterilization. The constructs were pre-implantated in four Saanen goats (aged 2-3 years) one month before functional repair. A silicon tube was inserted in the tubular template and placed in a subcutaneous pocket. For ureter repair a flank incision was performed and the ureter was mobilized, transected, spatulated and anastomosed onto the pre-implanted template. A 6Fr double-J stent was left *in situ* for 3 months until the animals were sacrificed for functional, macroscopic and microscopic analysis.

RESULTS: Templates were successfully implanted in all four goats. One goat was sacrificed prematurely because the humane endpoint was reached as a result of technical complications with stent placement. Two other goats survived complication free for the complete observation period (3 months). One goat is currently two months in the experiment without complications. In the two goats that reached the predetermined endpoint no signs of hydronephrosis or ureteral dilatation was observed. The pre-implanted tissue template was partially degraded and replaced by native tissue. Template remnants could be seen throughout the new tissue. On a histological level the kidneys looked normal, without inflammation. A single layered urothelium covered the template lumen, and muscle ingrowth was minimal.

DISCUSSION & CONCLUSIONS: Using pre-implanted tissue templates, ureteral reconstruction was successfully performed. Technical complications in one animal were attributed to the unfamiliarity with these animals and were avoided in subsequent experiments. Even though tissue regeneration on a histological level was slow for muscle tissue, the goats had normal appearing kidneys. The pre-implantation of the tissue templates, enabled repair of a ureteral defect with good functional outcomes, which is the most critical aspect in tissue engineering. While a larger study is warranted, the promising functional results suggest that this method may offer an alternative approach to current treatment options.

ACKNOWLEDGEMENTS: We would like to thank B.B.M. Kortmann and R.P.E. de Gier from the department of Urology for their help with the surgeries.

DISCLOSURES: The authors have nothing to disclose.

An alternative tissue culture model for the evaluation of biomaterials

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INTRODUCTION: Cytotoxicity and compatibility studies of biomaterials for bone tissue engineering are usually carried out on 2D or 3D cell cultures, while functionality is assessed in animal models with implantation in heterotopic sites or in critical size defects. *In vitro* studies could be improved using models that could provide both a 3D support and a proper microenvironment [1]. For this reason, distal femoral epiphyses were cultured up to 28 days to develop a system able to maintain unaltered bone structural and biological properties. Bone viability and mass were assessed, together with microtomographical (microCT), histological and gene expression analyses.

METHODS: Distal femoral epiphyses were obtained at euthanasia from rats involved in approved experimentation, not affecting the health of bone or joints. Epiphyses were resected and a hole was drilled in the lateral condyle to simulate a defect. The viability of each epiphysis was immediately tested with Alamar Blue assay. MicroCT, histological evaluations or RNA extraction were also performed at T0. Remaining samples were cultured up to 28 days, in vent cap tubes under oscillation using a standard growth medium (DMEM 10% FBS - DMEM), or a medium supporting osteogenic differentiation (DMEM 10% FBS, 50 µg/ml ascorbate 2-phosphate, 7 mM β-glycerophosphate and 0.1 mM dexamethasone - PLUS). Alamar Blue assay and mass assessment were performed again after 1, 7, 14, 21 and 28 days. After 28 days, microCT, histological and gene expression analyses were repeated.

RESULTS: Results showed that the culture system maintained the viability and the mass of bone up to 28 days of culture. The use of PLUS, compared to DMEM, leads generally to a better conservation of mass, probably due to the presence of a phosphate source. Moreover, microCT and histological qualitative evaluations revealed no differences in bone structure throughout the course of the culture. The expression of bone-specific genes, such as *Col1a1*, *Alpl*, *Bglap* or *Spp1*, remained also unaltered from T0 to T28, but a high inter-individual variability was observed.

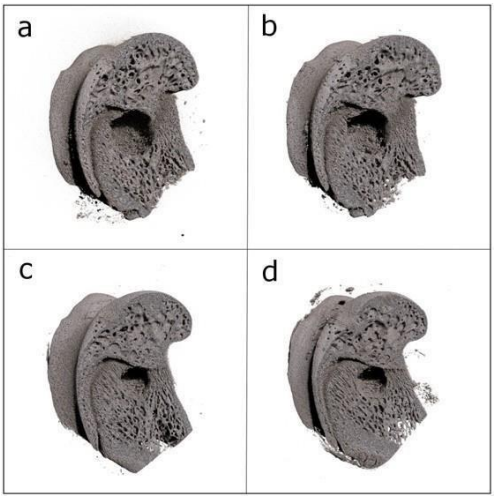

Fig. 1: 3D reconstructions by microCT, virtually cut on the lateral condyle to highlight the internal trabecular structure. a) DMEM at T0; b) DMEM at 28 days; c) PLUS at T0; d) PLUS at 28 days.

DISCUSSION & CONCLUSIONS: The bone segments in culture remained viable and metabolically active, as also shown by bone-specific gene expression. Quantitative microCT and histological analyses, still in progress, are required to confirm the qualitative results. The mid-time point of 14 days should be also evaluated. In conclusion, this culture model seems to be suitable to maintain native bone properties, and it should be validated as a novel method in vitro for the evaluation of biomaterials for bone tissue engineering.

ACKNOWLEDGEMENTS: Funding by Rizzoli Orthopaedic Institute and 5×1000 funds (years 2011-2012).

DISCLOSURES: The authors declare no conflict of interest.

Studying tissue healing by mimicking recruitment in hybrid PEG hydrogels

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INTRODUCTION: The healing of tissues is a complex process in which recruitment of cells into the wound and matrix deposition play essential roles. In order to better understand the underlying biological processes and to test elements influencing regeneration, it is important to dissect the problem into separate approachable questions and develop in vitro assays for each of them. Here we present a poly(ethylene glycol)-based 3D-in vitro healing model that begins to mimic the spatially controlled presentation of biological cues within wounds. In our case, we establish and evaluate the effects of epidermal growth factor (EGF) laden PEG-hydrogel perfused into another PEG-hydrogel pre-cultured with MSCs as it would happen in a wound if PEG is applied as the healing inducting material.

METHODS: MSCs were encapsulated in TG-PEG-hydrogels. Their polymerization progressed while a Tungsten wire was anodically polarized in order to form highly reproducible channels using a localized, electrochemistry-based inhibition of hydrogel polymerization[1]. Hydrogel encapsulated MSCs were cultured for 2 days under standard culture conditions.

The second TG-PEG-hydrogel of same density was tagged with FITC and EGF was mixed in the gel solution. It was then perfused into the channels by removing the tungsten wire. The growth factor diffused radially out of the channel, affecting the encapsulated cells around. Other growth factors known to trigger MSC migration or proliferation (PDGF, FGF)[2] were tested the same way in different combinations.

Confocal imaging was done to assess the presence of the perfused gel and recruitment or not of MSCs in it. Immunohistochemistry was done to stain for extracellular matrix proteins to see the effect of growth factors in the healing process.

RESULTS: We were able to form structured hybrid PEG-hydrogel by injecting a PEG-hydrogel into another PEG-hydrogel previously incubated with stem cells. MSCs showed no response when no growth factor was incorporated in the injected gel. In opposition, hydrogels loaded with EGF and/or PDGF induced cell response and even recruitment into the healing inducing gel. ECM staining was positive and confirmed cell activity by matrix deposition, which is a very important step towards tissue healing.

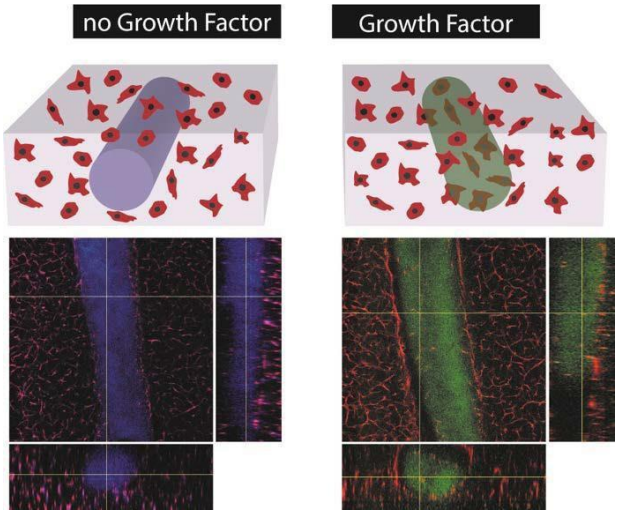

Fig. 1: Schematic and cross sectional view of recruitment gel. MSCs stained for actin (red), perfused gel labelled (blue and green). When PEG-gel is perfused without growth factor, no cell appears to be recruited inside. When the gel perfused contains growth factor (in this case EGF), cells are recruited.

DISCUSSION & CONCLUSIONS: The combination of advanced biomaterials with microfluidic technology enabled us to advance one step towards simulating wound healing mechanism. We believe that this model offers a powerful platform to study cell behavior and recruitment potential of different molecules. It will help increasing our understanding of the role of cell function in the healing mechanism as well as screening for factors inducing it.

ACKNOWLEDGEMENTS: Swiss National Science Foundation for funds; A. Kurmanaviciene for technical support; Ivan Martin for providing cells; University and University Hospital Zurich.

Osteoinductive potential of heparinised calcium phosphates and their inflammatory response

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INTRODUCTION: The regenerative potential of bone grafts is tightly linked to the interaction of the material with the host tissue environment. Hence, strategies to confer artificial extracellular matrix (aECM) cues on the material surface are becoming a powerful tool to trigger the healing cascade and to stimulate regeneration. The use of glycosaminoglycans (GAGs), i.e. heparin, as aECM components has proven to improve osteoblast differentiation[1] and to inhibit osteoclastogenesis[2]. Calcium phosphates (CaP) are extensively used as bone grafts; however no studies have investigated the effect of GAG functionalization on their surface. The aims of this study were to investigate the ability of GAGs to induce osteoinduction as well as their effect on the inflammatory response to various CaPs.

METHODS: Sintered CaP (β-TCP) and biomimetic CaP (CDHA) discs were synthesized at 1100 ºC and at 37ºC, respectively. Heparinisation was achieved though silane coupling (APTES) followed by amidation in the presence of EDC/NHS to covalently link heparin. The effect of heparinised (H) vs. non-heparinised substrates was studied using rat mesenchymal stem cells (rMSCs) and human monocytes. Gene expression of rMSCs (cell density $2.85 \cdot 10^4$ cells/mL) was obtained for CDHA over 6h, 1 and 3 days. Positive controls of tissue culture plastic (TCPS) and TCPS with osteogenic media (TCPS om) were included. The inflammatory response of monocytes (cell density 10^6 cells/mL) was investigated through quantification of reactive oxygen species (ROS) in the presence and absence of 10% wt. of human plasma. A positive control consisting of phorbol myristate acetate (PMA) was used as reference.

RESULTS: Fig.1 shows the RT-qPCR results for alkaline phosphatase, bone morphogenetic protein 2 (BMP-2), collagen and osteonectin. CDHA-H showed higher expression for all markers except BMP-2. Interestingly, BMP-2 levels are higher for CDHA. CDHA-H is capable of sustaining BMP-2 levels over time. Fig. 2 shows the inflammatory response of heparinised vs. non-heparinised CDHA and TCP for monocytes (M). The results suggest that heparin functionalization does not significantly alter in vitro inflammatory response. Sintered materials lead to inflammation similar to control whereas CDHA results in lower values.

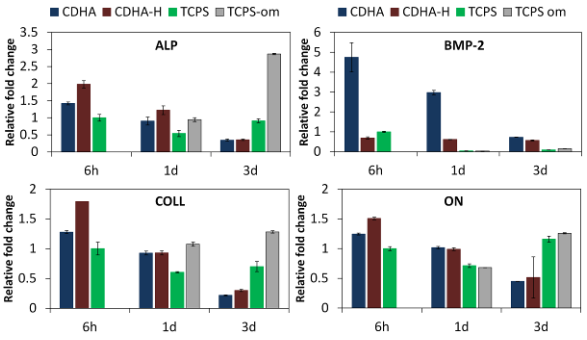

Fig. 1: RT-qPCR for biomimetic CDHA.

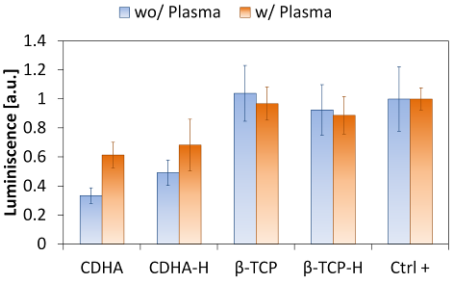

Fig. 2: H_2O_2 generated by the substrates in absence (wo) or presence (w) of human plasma.

DISCUSSION & CONCLUSIONS: Heparinised CDHA enhances matrix deposition and its mineralization (COLL and ON). The sustained expression of BMP-2 emphasizes the direct interaction of GAGs with growth factors. Importantly, the inflammatory response was not substantially affected by heparinisation, although it was affected by the underlying material.

ACKNOWLEDGEMENTS: The authors thank the Spanish Government (MAT2015-65601-R) and Bioiberica S.A for kindly providing the heparin used in the study.

Effects of low level light therapy on endothelial cells and vasculogenesis

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INTRODUCTION: Low level light therapy (LLLT) with light-emitting diodes (LEDs) receives increasing interest in the fields of wound healing and angiogenesis. Recently it has been shown that both red and blue LED light improved wound healing in a single side ligation induced ischemia skin flap model in rats by increased vessel formation and enhanced perfusion [1]. Endothelial cells play an important role in angiogenic processes. The aim of this study was to compare the effects of pulsing LED light of three different wavelengths on endothelial cells and vasculogenic processes *in vitro*.

METHODS: The effects of pulsed LED light on proliferation and migration of human umbilical vein endothelial cells (HUVEC) were investigated in several 2D and 3D cell culture models. Cells were treated with either blue (475 nm), green (516 nm) or red (635 nm) LED light. Control cells were not illuminated. 2D proliferation was determined at given time points by manual counting. 2D migration was assessed by scratch assays, 3D migration was evaluated by Cytodex bead assays. The vasculogenic potential of HUVEC in co-culture with adipose-derived stem cells (ASC) in response to LLLT was determined by analysing the network formation in a 3D fibrin matrix co-culture model after 4 and 7 days [2].

RESULTS: Stimulation with both red and green pulsed LED light significantly increased HUVEC proliferation. Moreover, HUVEC showed increased 2D migration potential with green light stimulation. The 3D migration was significantly enhanced by green and red light. In the 3D fibrin co-culture model, HUVEC elongation as precursor of vasculogenesis was enhanced by green and red light during the first 4 days while green light stimulation led to enhanced vasculogenesis after one week.

DISCUSSION & CONCLUSIONS: Both red and green light enhanced proliferation, migration and vasculogenesis processes while blue light was ineffective. Several parameters showed that green light was even more potent to stimulate regenerative processes than well-established red light therapy. Further studies have to focus on intracellular signalling induced by different wavelengths in order to optimize this promising, alternative application in tissue regeneration and wound therapy.

ACKNOWLEDGEMENTS: Supported by FFG Grant 3925263.

Conductive polymer coatings in 3d, effect of alignment, conductivity and electrostimulation

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INTRODUCTION: Current methods of boosting nerve regeneration in clinical cases have poor success and has been stagnant during a large part of the 20th century [l]. Since the revitalization of electrospinning many have put forth suggestions for its use in development of scaffolds for neural regeneration. At the same time many novel materials with promising properties for the same type of medical applications have been developed. It has been known for a long time that electrostimulation as well as conductive polymers can have positive effects on nerve regeneration [2]. What is now needed is integration of innovation in differing fields to create practical solutions with the combined benefits.

METHODS: Fibrous PCL (polycaprolactone) structures with an average diameter of 1um were manufactured using electrospinning. Two variants of the base fibrous scaffold was made, one randomly aligned and one with a single directional alignment. Conductive polymer coatings based on PEDOT (poly(3,4-ethylenedioxythiophene)) combined with copolymers or in-chain integrated monomers were manufactured. Manufacturing of conductive polymers was performed by a combination of liquid phase and vapor phase polymerization.

The model cell type use was primary cells in the form of dorsal root ganglia isolated from 7-9 day chick embryos. Electrostimulation was supplied using a low voltage variable ac power supply. Evaluation of material suitability for neural regeneration was performed using ICC (immunocytochemistry) as well as metabolic assays (MTS assay). Both cellular morphology and metabolic state was evaluated to determine the combined vs. the separate effect of fibre alignment, coating material and electrostimulation. The general linear model was used for analysis of the experiments.

RESULTS: Preliminary results indicate an effect of material alignment on directionality and efficacy of neurite extension. Electrostimulation has shown an effect on cell attachment and survival.

Table 1. Experimental setup. Sample types of each coating on electrospun PCL.

Caged PEDOT:PSS	PEDOT:Tosylate
Aligned Random Flat sheet	Aligned Random Flat sheet
Uncoated PCL	**Tissue culture plate**
Aligned Random Flat sheet	Untreated surface

DISCUSSION & CONCLUSIONS: Neurite extension is dependent on several variables and we can show that we with these materials can control several of these to get a combined effect larger than can be accomplished with one single method. There is still need to elucidate the source of effect of electrostimulation, whether it comes from a change in oxidative state of the material or from the direct stimulation of cells.

Fetal subcutaneous-derived cells for autologous tissue engineering in congenital malformations

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INTRODUCTION: Congenital malformations affects up to 3% of newborns[1]. Infants with prenatally diagnosed soft tissue defects could benefit from having autologous tissue readily available for surgical reconstruction in the perinatal period. In this study we investigated fetal subcutaneous cells as cellular source for tissue engineering.

METHODS: Fetal subcutaneous biopsies were collected from elective terminations at gestational week 20-21 after informed consent and according to ethical approval. The tissue was placed in a culture dish allowing cells to adhere to the bottom of the culture well and were thereafter expanded and characterised.

To investigate viability and proliferation, cells were stained with a fluorescent nucleotide marker, CSFE, seeded on collagen type I gel and kept in culture for up to 2 weeks. Cell proliferation was measured by flow cytometry at day 0, 3, 7, 10 and 14.

To determine cell regeneration, expansion, migration and reorganisation as well as the expression of protein markers in a 3D construct, cells were seeded in collagen gels with poly(ε-caprolactone) (PCL). The cell containing 3D constructs were kept in culture for 1, 2 and 3 weeks, paraffin sectioned and stained for Ki67 (proliferation), pan-cytokeratin (epithelium), α-SMA (smooth muscle cytoskeleton) and CD31 (endothelium).

RESULTS: Isolation and expansion of cells were 100 % successful with a total mean of 43 population doublings (range 41-45). The cells expressed mesenchymal markers and could be differentiated into adipogenic and osteogenic lineages. In passage 6, the mean fraction of senescent cells was less than 5 % (range 1-5). The cells migrated into the collagen gel and proliferated with sustained viability for up to 14 days (*Fig. 1*). The cells in the PCL constructs were positive for Ki67 and α-SMA (*Fig. 2*) and negative for pan-cytokeratin and CD31 (data not shown).

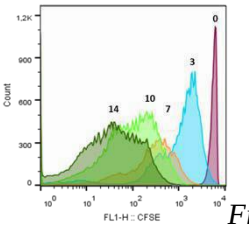

Fig. 1. The cells were stained with CFSE. For each cell division the CFSE divides between the daughter cells and the signal weakens (the peaks shift to the left in the histogram). Flow cytometric assay showed proliferation of fMSC for up to 14 d. The peaks are marked with the time points in days.

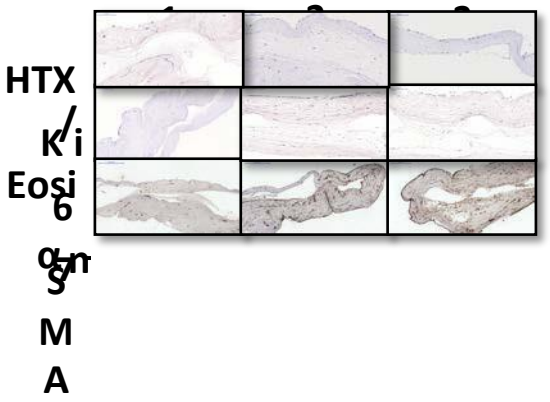

Fig. 2. The cells in the PCL constructs expressed Ki67 and α-SMA, but not pan-cytokeratin or CD31.

DISCUSSION & CONCLUSIONS: The fetal subcutaneous-derived cells demonstrated beneficial characteristics with high potential for autologous tissue engineering for treatment of congenital malformations. In a future clinical setting, autologous cells could be obtained by prenatal skin biopsy during pregnancy and used in the surgical correction of the defect after birth.

ACKNOWLEDGEMENTS: The study was funded by VINNOVA (2010-00501), Stiftelsen Allmänna BB:s minnesfond and Jerringfonden.

PEGylated Wnt-associated liposomes for cell delivery in bone marrow

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INTRODUCTION: Osteoporosis and bone fractures are burgeoning societal and economic problem in ageing populations. The Wnt signalling pathway has recently emerged as a promising target for therapies that augment bone mass or promote fracture healing. Putative new therapeutics may function through the inhibition of circulating antagonists of Wnt signalling or by direct activation of Wnt signalling in target cells through application of Wnt proteins [1]. Wnt proteins are inherently unstable in aqueous solution and must be incorporated with carriers, such as liposomes, to ensure stability [2]. PEGylation (covalent attachment of polyethylene glycol (PEG) polymer chains) preserves the circulation time of liposomes and inhibits uptake and disposal by the reticular endothelial system, but has previously been shown to abolish Wnt activity [3]. Here we test the hypothesis that Wnt protein can be incorporated in PEGylated liposome carriers in active form by modulation of liposome chemistry, and that liposomes then associate with skeletal stem cells.

METHODS: Liposomes were prepared by extrusion through 100 nm filter membranes using combinations of DMPC- and DOPC phospholipids in the presence or absence of 5 molar % PEG-conjugated DSPE. Wnt3A protein was incorporated prior to and post extrusion at a concentration of 2µg/mL. Liposome size and polydispersity was measured by dynamic light scattering. Activity was measured in a Wnt-responsive cell line, and cellular uptake was measured by labelling liposomes with DiI or by coupling of streptavidin conjugated AF647 to biotin linked PEGylated lipid, before analysing using flow cytometry.

RESULTS: PEGylation did not significantly affect liposome size or stability, with mean liposome diameters of ~100 nm in all cases. When Wnt3A was added prior to extrusion, resultant Wnt3A-DMPC-liposomes did not differ significantly in activity compared to Wnt3A alone per unit mass of protein, assuming full incorporation of protein. PEGylation, however, caused a 42.2±6.3% decrease in activity. In contrast, DOPC-Wnt3A-liposomes were less active than Wnt3A (72.5±9.3%) but PEGylation had no effect on activity. When Wnt3A was incorporated into liposomes post-extrusion, the activity of Wnt3A-DOPC was enhanced compared to Wnt3A-DMPC-liposomes (55.6±22.9%) or Wnt3A (83.9±34.0%), and PEGylation had no effect on activity. All liposomes associated rapidly with human bone marrow stromal cells, with half-maximal cellular DiI fluorescence at 3h, but association was significantly higher at 24 hours in PEGylated DOPC-liposomes compared to all other preparations. In mixed bone marrow mononuclear cells, DiI uptake was observed in both stromal and monocyte cell populations (Fig 1).

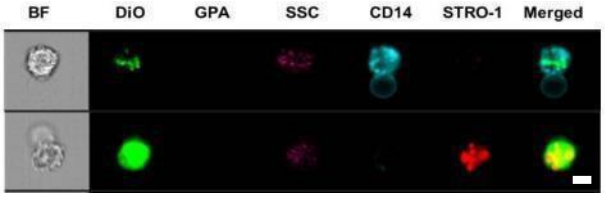

Fig 1: DiI-tagged liposomes associate and label stromal cells of the bone marrow (STRO-1+ cells) to a greater degree than monocytes (CD14+ cells). Scale bar 10 µm.

DISCUSSION & CONCLUSIONS: Stable, circulating carriers such as liposomal or polymer nanoparticles have significant potential for delivery of hydrophobic proteins and molecules for treatment of bone disease and injury. Wnt-carrying liposomes have been shown to have potential as local delivery agents for bone repair, but have limited utility as circulating carriers due their rapid opsonisation and clearance in vivo. Our results show that PEGylated preparations retain Wnt-signalling activity and are rapidly taken up by cells of the bone marrow stroma. These results suggest that this technology may have potential in systemic delivery for bone anabolism or for delivery of other hydrophobic therapeutic molecules.

ACKNOWLEDGEMENTS: We thank Wessex Medical Research and the Medical Research Council UK for supporting our research.

BMP2 and follistatin stimulate skeletal progenitor cell migration and their differentiation into mature osteoblasts

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INTRODUCTION: Tissue engineering is emerging as an alternative to grafting autologous tissues. One bone regeneration strategy comprises of scaffold-based delivery of signaling molecules that stimulate cell migration and osteogenic differentiation of endogenous cells to promote bone repair [1]. BMP2, a TGF-β superfamily member, is the most widely used protein in terms of bone regeneration. However, large doses are needed to produce a significant osteogenic effect. Follistatin (FST) is a glycoprotein distributed in a wide range of tissues and is able to regulate and antagonise members of the TGF-β superfamily, particularly activin A. However, its action on skeletal progenitor cells remains unclear. Since BMP2 and FST are involved in the same signalling pathways, we compared the effect of BMP2 and FST on two cell types, human bone marrow derived stromal cells (MSCs) and simian virus Human Foetal Osteoblasts (SV-HFOs) with regard to cell recruitment and osteogenesis.

METHODS: MSCs migration was assessed using modified Boyden chambers (n= 4 donors in duplicate). The factors were added at a range of doses (FST: 25-175 ng/ml and BMP2: 10-1000 ng/ml) in the lower chamber. After 17h incubation, cells that had migrated were fixed, stained and counted. For osteogenic differentiation, cells were cultured with addition of FST or BMP2 (same concentrations mentioned above). MSCs (n= 3 donors in triplicate) and SV-HFOs (n=3 independent experiments in triplicate) were cultured in osteogenic differentiation medium for up to 16 and 21 days respectively Alkaline phosphatase activity (ALP), calcium deposition, total protein and DNA were measured. For the analysis of the data, a linear mixed model was applied.

RESULTS: FST stimulated MSC migration significantly at all doses tested compared to controls ($P<0.05$, $P<0.01$, $P<0.01$, respectively). However, it did not enhance osteogenic differentiation of MSCs. Interestingly, the addition of FST during SV-HFO culture increased ALP activity at all tested doses. Furthermore, the addition of FST to SV-HFO culture had a positive effect on the mineralisation process ($P<0.01$, $P<0.01$, $P=0.001$, respectively). BMP2 stimulated MSC migration at the two highest doses tested. In contrast to FST, BMP2 induced significant mineralisation by MSCs at the highest dose tested ($P=0.001$), while it had no significant effect on osteogenic differentiation of SV-HFOs.

DISCUSSION & CONCLUSIONS: This work indicates that FST has clear chemoattractant effects upon MSCs. Bone development involves a series of different stages; from proliferation of osteoprogenitor cells at the outset until their differentiation into mature osteoblasts. We used (MSCs) as osteoprogenitors due to their extensive potential to proliferate and differentiate into pre-osteoblasts, and SV-HFOs due to their capacity to become mature osteoblasts. This study has shown that depending on which stage of differentiation the osteoprogenitor cells are at, the addition of BMP2 or FST is able to increase migration and osteogenic differentiation on skeletal progenitor cells. Therefore, their specific addition at different osteogenic differentiation stages could bring several major advantages, as the recruitment of osteoprogenitor cells *in situ* in a cell free therapy and the increase of specificity during differentiation upon skeletal progenitor cells.

ACKNOWLEDGMENTS: BioInspire Marie Curie Action (FP7/2007-2013).

Functionalization of PEEK to maximize the effect of BMPs

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INTRODUCTION: Dental implants are considered a successful treatment option for patients. However, there is still a need to refine implant designs so as to achieve better biological healing and osseointegration. PEEK is a potential alternative to the pure titanium implants. Mechanical properties of PEEK are the most interesting characteristic about this material, having primarily an elastic modulus that closely matches cortical bone [1]. Being bionert, PEEK has to be functionalized to promote healing and improve osseointegration. Here we present a novel bioactive PEEK coating that involves a layer of HA on which a poly ethyl acrylate polymer (PEA) that assembles fibronectin (FN) and promotes high efficient presentation of growth factors is used for cell adhesion, growth and differentiation[2].

METHODS: First PEA polymer (Sigma Aldrich) was solubilized in 8% toluene. This solution was used to spray onto PEEK samples coated with a layer of HA. Then, FN was adsorbed on the sample from a solution of concentration 20 µg/ml. 25 ng/ml BMP2 & BMP7 coatings were performed right before cell cultures. Samples were characterized to determine the surface roughness (with and without PEA) using atomic force microscopy (AFM) and scanning electron microscopy (SEM). Water contact angle was measured before and after FN coatings. A bone marrow human mesenquimal stem cell (hMSCs) culture was performed to proof cell adhesion and study the different morphology on the different surfaces. For differentiation studies, 5 and 21 days hMSCs cell culture were performed and gene expression was analyzed for RunX2, Smad, osteocalcin, osteonectin, osteopontin and BMPR.

RESULTS: Roughness on the PEEK/HA PEA treated decreased compared with the PEEK/HA samples, as we can observe in the AFM and SEM images (Figure 1 & 2). The surface of the original sample was covered partially, leaving some HA available. WCA was studied before and after FN coating on all the samples. PEEK/HA samples coated with PEA and FN had a 60º, while WCA on PEEK/HA after FN coating was 15º (Figure 1). Cell morphology on PEEK/HA samples was more rounded, while on PEA treated samples the cells were more flattened and spread.

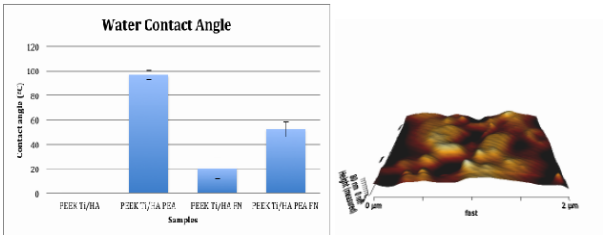

Fig 1. Mean static contact angles (WCA) on the different surfaces (left). 3D AFM height channel images (1x1µm) from PEEK/HA surface treated with PEA

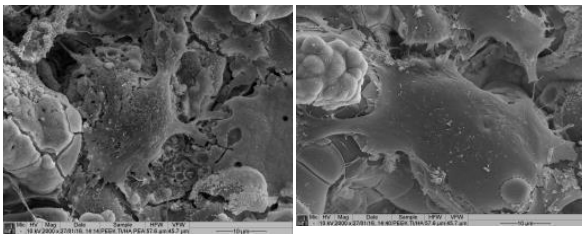

Fig 2. SEM images (2000x) showing hMSCs adhesión onto PEEK Ti/HA surfaces withPEA (left) and without PEA (right).

DISCUSSION & CONCLUSIONS: PEEK has to be modified to promote cellular adhesion and differentiation. Coating PEEK/HA samples partially with PEA and FN have proven to be an innovative and easy surface treatment. The original hydrophobicity and roughness of the PEEK/HA surface was decreased, where cell adhesion and differentiation properties offered by FN fibrillogenesis on PEA are taken in advantage. Here we show an easy translational technique to improve biological PEEK properties for being used in dental implants.

ACKNOWLEDGEMENTS: This research was supported by Senescyt, which funded my studies in The University of Glasgow.

Doxycycline inducible, adenoviral BMP-2 delivery for large bone defect repair

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INTRODUCTION: Gene delivery of BMP-2 offers advantages of localised, sustained and controllable transgene expression in physiologically relevant quantities. Here, we report the use of a doxycycline inducible Tet-on adenoviral vector (AdTetBMP-2) in combination with mesenchymal stromal cells (MSCs), fibrin and a biphasic calcium phosphate ceramic (MBCP®) to repair large bone defects in nuderats.

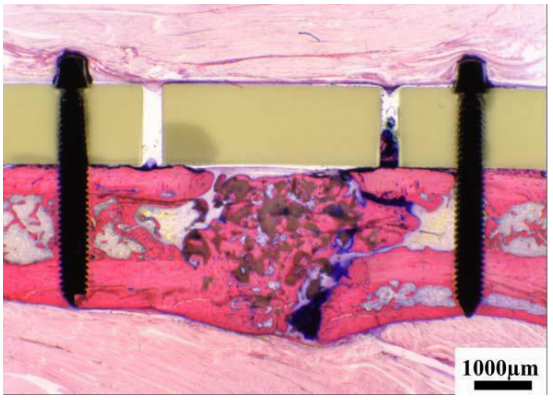
METHODS: Human MSCs encapsulated in fibrin containing MBCP® granules (60% hydroxyapatite and 40% calcium phosphate, Biomatlante SA) were transduced with AdTetBMP-2. 4mm, internally fixated, femoral defects were created in nude rats with full ethical approval. MSCs were transduced, by direct application 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 performedpost-mortem.

RESULTS: AdTetBMP-2 transgene expression was confirmed *in vitro* by ELISA. BMP-2 levels could be effectively tuned by alteration of doxycycline dose. Significantly improved bone defect healing was evident in some but not all animals treated with AdTetBMP-2. Radiograph scores were improved upon direct delivery of AdTetBMP-2 and significantly higher when MBCP® granules were pre-coated with the virus compared to controls ($p=0.0325$). Micro-CT showed a trend towards increased mineralised tissue volume within the defect site in animals that had received direct delivery of AdTetBMP-2 or when MBCP® were pre-coated with the virus.

Bending stiffness EI, determined by micro finite element analysis of micro-CT images, was greater following direct delivery of AdTetBMP-2 compared to (-)doxycycline controls (253.6 ±100.2 vs. 149.9 ±42.94Nmm^2 respectively).

No adverse tissue reaction or ectopic ossification was seen histologically in any specimens 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, although not significantly so, in animals that had received direct application of AdTetBMP-2 +doxycycline (30±10%) or when MBCP® had been pre-coated with the virus (28±7%, Figure 1) compared to their corresponding control groups without doxycycline (both 22±5%). 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: Delivery of BMP-2 using AdTetBMP-2 improved healing induced by the transplantation of MSCs, fibrin and MBCP® in a large bone defect model. Our data suggest this is achievable with low (ng/ml) levels of the growth factor. Effective transgene expression using virally-pre-coated ceramic suggests suitability for an 'off-the-shelf'approach.

ACKNOWLEDGEMENTS: This study was funded by the AO Foundation and the EU -FP7 framework (project GAMBA NMP3-SL-2010-245993 and BIODESIGN NMP- 2010_LARGE-4). Simulations performed at the Swiss National Supercomputing Centre (CSCS).

Photo-grafting of VEGF protein gradients into elastin-like polymer hydrogels for improved vascularization

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INTRODUCTION: Rapid and efficient *in vivo* vascularization of clinical-size tissue-engineered grafts is a key requirement to enable progenitor survival and tissue formation. Vascular endothelial growth factor (VEGF) is the master regulator of vascular growth and is widely investigated to induce therapeutic angiogenesis. However, when VEGF is produced by hypoxic cells, it binds to the surrounding extracellular matrix and forms concentration gradients, which are sensed by endothelial cells and guide the sprouting of new vessels towards the hypoxic areas [l]. This is a key aspect of VEGF physiological function, but under-appreciated in the design of vascularization strategies for tissue engineering. Here we sought to investigate whether the functionalization of a biocompatible biomaterial with VEGF protein in the pattern of a gradient could be more effective than a homogenous distribution to drive rapid ingrowth of vascular networks. Elastin-like polymers (ELP) [2] are an attractive material because they: 1) self-assemble into a hydrogel at body temperature; 2) mimic the elastic properties of natural tissues; and 3) provide a favorable environment for endothelial migration and vascular assembly. Since VEGF does not have any natural affinity for ELP, two-photon absorption was used to induce photo-grafting of the protein in the presence of multi-functional aromatic azides [3], allowing fine control over the spatial distribution of the grafted protein.

METHODS: Recombinant human VEGF-A_{165} was fluorescently labelled with the NT-647-NHS far-red dye and further functionalized with 4,4′-Diazido-2,2′-stilbenedisulfonic acid (DAS) as a photosensitive cross-linker. Bioactivity of the functionalized protein was tested by its ability to induce phosphorylation of VEGF-R2 in endothelial cells (HUVEC) *in vitro*. VEGF was grafted on ELP hydrogel at 2 different concentrations (60 and 240 nM) with 2 mM DAS. After grafting, both DAS and VEGF were visualized by confocal microscopy at 555 and 647 nm wavelengths, respectively. After washing of unbound VEGF, HUVEC were seeded on top of the gel (3000 cells/well in 96-well plate) and their proliferation was assessed after 48 h by the PrestoBlue® assay.

RESULTS: Initial results showed that labelling of recombinant VEGF with both NT-647-NHS and DAS partially reduced its bioactivity, but the modified factor still retained >50% of its ability to induce endothelial VEGF-R2 phosphorylation *in vitro*. Grafting of VEGF to the ELP hydrogel was visualized by confocal microscopy and laser parameters (speed and power) were optimized to enable dose-dependent grafting. ELP hydrogels with photo-grafted VEGF at 3 concentrations over a 400-fold range (0.6, 60 and 240 nM) sustained about 3-fold greater endothelial proliferation than empty ELP. Interestingly, photo-grafted VEGF was significantly more effective than equivalent concentrations of non-grafted soluble VEGF.

DISCUSSION & CONCLUSIONS: These results show that photo-grafting is a feasible strategy to immobilize controlled concentrations of biologically active VEGF on ELP hydrogels with a desired pattern. Current experiments aim at generating 2D VEGF gradients on ELP hydrogels *in vitro* to test their ability to direct migration of endothelial cells in comparison to homogenously distributed VEGF. Finally, these results provide the basis for the generation of 3D constructs functionalized with VEGF protein gradients, enabling the testing of their potential for accelerated vascularization *in vivo*.

ACKNOWLEDGEMENTS: This work was supported by the European Union FP7 grant AngioMatTrain (CP-IP 317304).

Nell1, CCN2 and HMGB1 enhance osteoprogenitors migration and endothelial cell angiogenic activity, but not osteogenesis *in vitro*

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INTRODUCTION: Bone grafting involves many known disadvantages. The goal of bone tissue engineering would be achieved by an off the shelf product capable of promoting tissue regeneration. Using this approach, a cell free material combined with growth factors would induce and instruct the essential processes for bone defect repair using the body as a bioreactor. These processes are: recruitment of osteoprogenitor cells, neovascularisation and bone formation in the damaged tissue. Recently, it has been shown that the combined delivery of osteogenic and angiogenic factors promotes bone tissue regeneration compared with single factor delivery [1]. Here we describe the assessment of the ability of 3 proteins, Nell1, Connective Tissue Growth Factor (CTGF/CCN2) and High Mobility Group Box 1 (HMGB1), to induce migration, osteogenic differentiation and vasculogenic activity upon osteoprogenitors and endothelial cells.

METHODS: Cells were cultured with addition of each factor at a range of doses (Nell1:10-500 ng/ml, CCN2: 10-100 ng/ml and HMGB1:10-100 ng/ml). Human bone Marrow derived Stromal Cells (MSCs) and SV-HFO (simian virus Human Foetal Osteoblasts) pre-osteoblasts were cultured in osteogenic differentiation medium for up to 16 and 21 days respectively. Alkaline phosphatase activity (ALP), calcium deposition, total protein and DNA were measured. Chemotaxis towards each factor was also measured using modified Boyden chambers. After 17h incubation, cells that had migrated were fixed, stained and counted. For tube formation assay, human endothelial cells were loaded on the top of growth-factor reduced matrigel with the addition of each factor at the same range of doses mentioned above. After 8 h of incubation, the number of meshes, nodes and tubes were analysed per condition.

RESULTS: Compared to the control, Nell1 was significantly chemoattractant ($P<0.01$) when the highest dose was provided, in contrast to CCN2, which showed more chemotactic effect at the lowest dose ($P<0.05$). HMGB1 significantly increased the migration of MSCs at 100 ng/ml ($P<0.001$). Moreover, CCN2 and HMGB1 promoted tube formation *in vitro*. The highest peak was found at the lowest concentrations.
No significant effects of Nell1, HMGB1 or CCN2 were found on osteogenesis in pre-osteoblasts or MSCs culture at day 16 and 21, respectively. However, after monitoring the calcium concentration in the medium during culture, the preliminary data suggest that Nell1 promoted the early stages of mineralisation in both, MSCs and pre-osteoblasts.

DISCUSSION & CONCLUSIONS: This work indicates that HMGB1, CCN2 and Nell1 have clear chemoattractant properties upon skeletal progenitor cells. Moreover, our data indicate the potential of CCN2 and HMGB1 to enhance neovascularisation. Nell1 has been described extensively as a novel osteogenic factor in promoting bone regeneration [2]. Our data show that it could have a possible role in kick-starting osteogenesis in both, MSCs and pre-osteoblasts.
Therefore, the combination of these factors with osteoinductive molecules (e.g. BMPs) in a controlled release system could have several advantages; the recruitment of osteoprogenitor cells *in situ* in a cell free therapy, the increase of blood vessels ingrowth and the reduction of the required BMP dose needed. Vascularisation is a critical rate-limiting step in bone regeneration. The use of these factors in a dual release system could overcome this limitation.

ACKNOWLEDGMENTS: BioInspire Marie Curie Action (FP7/2007-2013)

DNA minicircle vector technology limits DNA size restrictions on gene transfer using nanoparticle vectors: Overcoming a translational barrier in neural stem cell therapy

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INTRODUCTION: Genetically engineered neural stem cell (NSC) transplant population offer key benefits for regenerative neurology. NSCs are 'hard-to-transfect' but amenable to 'magnetofection'- a genetic modification approach wherein transfection grade magnetic nanoparticles (MNPs) and DNA complexes are applied to cells in the presence of magnetic fields. Despite the high clinical potential of this approach, the low and transient transfection associated with the relatively large size of therapeutic DNA constructs is a known and critical barrier to translation[1-2].

METHODS AND RESULTS: We demonstrate for the first time that DNA minicircle (mC) vectors (devoid of a bacterial backbone thereby reducing construct size versus conventional plasmids) with MNPs/magnetofection achieve the highest, safe non-viral transfection levels (up to 54%, Fig.1) reported to date for primary NSCs. All daughter cell types of the engineered NSCs (neurons, astrocytes and oligodendrocytes) remained transfected (in contrast to conventional plasmids which usually yield transfected astrocytes only), offering advantages for targeted cell engineering. The differentiated progeny retained reporter protein expression for up to four weeks. For, therapeutic gene delivery, minicircles were engineered to express Brain Derived Neurotrophic Factor (BDNF) (Fig.2), a multifunctional neurotrophic biomolecule involved in cell proliferation, neurite outgrowth and maturation. BDNF expression quantified by ELISA demonstrated up to 24-fold increase compared to untransfected cells, resulting in increased neuronal numbers upon differentiation of NSCs.

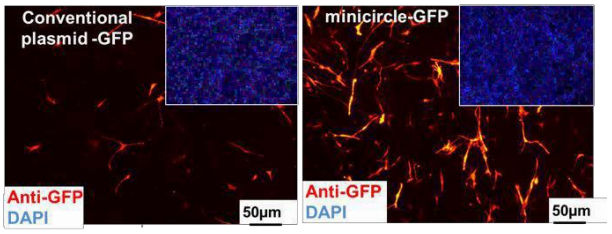

Fig. 1: Efficient GFP expression in NSCs magnetofected with minicircles.

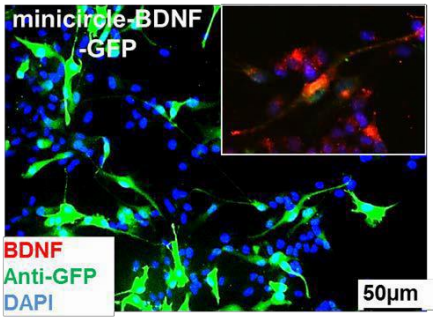

Fig. 2: NSCs magnetofected with minicircles engineered to express BDNF and GFP.

DISCUSSION & CONCLUSIONS: The small size of mCs compared with conventional plasmids enables a key translation barrier associated with larger therapeutic DNA constructs (namely low transfection levels) to be overcome. Additionally, due to their unique structure, mCs offer a host of advantages for clinical translation, such as reduced immune responses, sustained gene expression, versatility for incorporation of a range of therapeutic sequences. Similarly, MNPs have high translational potential as already demonstrated by their utility as clinical contrast agents. Minicircle and MNP production is highly feasible in terms of scale up for human therapies. Further, the methods involved in the genetic modification protocols are simple, cost-effective and reliable. Accordingly, we consider that a fusion of mC DNA vector technology with nanoparticle mediated DNA delivery, could come to represent the genetic modification method of choice for clinical cell therapies.

ACKNOWLEDGEMENTS: This project is supported by a BBSRC grant – BB/JO17590/1.

Modulating blood vessel morphology by combining cell-based gene therapy and tissue engineering approach.

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INTRODUCTION: Angiogenesis induced by direct delivery of Vascular Endothelial Growth Factor (VEGF) is a promising adjuvant treatment to restore micro-circulation and thus blood perfusion in an ischemic tissue. However, a sustained but controlled VEGF release is undoubtedly needed in order to fully exploit its therapeutic potential while avoiding its deleterious effects. In particular, VEGF binds to extracellular matrix (ECM), therefore uncontrolled release of the factor at the microenvironmental level leads to immature capillaries with sparse pericyte coverage and aberrant hemangiomas-like structures[1]. In this study, we achieved a controlled and safe release of VEGF by means of 3D culture of human genetically-modified Adipose tissue-derived Stromal Cells (ASC) on collagen sponge. Our hypothesis is that the collagen cannot bind VEGF and allow its diffusion, avoiding formation of micro-environmental toxic spots. We tested the safety and the angiogenic potential of the engineered tissues in a nude rat subcutaneous pocket model.

METHODS: Human ASC were isolated from healthy donors and transduced with retroviral vectors expressing rat$VEGF_{164}$. Two distinct populations were FACS-purified: one expressing safe and specific (SPEC) and the other uncontrolled (ALL) VEGF levels[2]; naïve cells were used as control. Engineered tissues (patches) were generated with cells seeded and cultured for 5 days on a 3mm-thick either on collagen sponge (Ultrafoam, AVITENE) or on Egg White (EW) scaffold (known to strongly bind to proteins) [4] in a perfusion-based bioreactor. A 7mm-thick cell-free (*empty*) collagen scaffold was sutured beneath the cell-based patch to function as clear model to investigate extrinsic angiogenesis dynamics (time points: 7 and 28 days) in a subcutaneous pocket model in nude rats.

RESULTS: VEGF-releasing patches induced a dense vascularization of the patch already at 7 days, leading to a superior survival of implanted cells compared to control (patches made by naïve ASC). The sustained protein expression led to a fully maturation of the newly-induced blood vessels at 28 days. Moreover, only VEGF-releasing patches induced a functional and mature angiogenesis also in the empty scaffold beneath, with functional capillaries covered by pericytes. No aberrant-like vascular structures were observed when ALL-ASC were used. To test our initial hypothesis, we implanted patched generated by ALL-ASC cultured on EW scaffold and we observed the growth of aberrant vascular structures at 14 days *in vivo* (white arrows, fig.1 A) and a dysfunction in pericyte coverage of capillaries. The aberrant phenotype was completely reverted when SPEC-ASC were used (Fig.1, B)

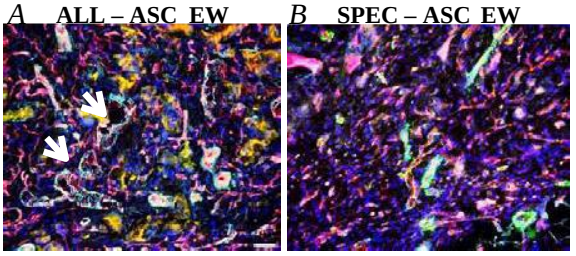

Fig.1: Blood vessel morphology induced by patches made of ALL- (A) or SPEC- (B) ASC and egg white (EW) scaffolds. (DAPI: blu; NG2: green; CD31: red; SMA: cyan; size bar=100 µm)

DISCUSSION & CONCLUSIONS: Taken together our results show that the engineered patches act as safe delivery system inducing mature and functional intrinsic and extrinsic angiogenesis. Until now, the control of VEGF overexpression in cell-based gene therapy was achieved by time-consuming techniques to purify transduced cells expressing only therapeutic doses[2] or by complex co-delivery with PDGF[4]. Our findings open up to a novel and more direct angiogenic approach based by on engineered patches.

ACKNOWLEDGEMENTS: Funding from SNF project (FO132750) to AM.

The synthetic production of cellular derived extracellular matrix for liver tissue engineering applications

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INTRODUCTION: As a result of insufficient treatment options for patients with liver disease[1]; a solution is sought in the form of liver 'organoids'; lab grown devices which can support the survival and function of hepatocytes. While scientists can maintain liver organoids in the lab, decades worth of scientific research has failed to translate this into a fully functional organ[2]. This in part is due to an incomplete picture of the influence of a vital part of the in vivo cellular environment; the extracellular matrix [ECM][3,4]. Synthetic biology and genetic engineering provide methods of manipulating the ECM components, allowing us to establish novel ECM environments for cells. This study aims to demonstrate the effects of a novel 'bespoke' ECM; created via the synthetic overproduction of fibronectin [a key component of liver ECM] in a cell based system[2].

METHODS: A fibronectin vector [PSI:Biology Material Repository] was transfected into two separate cell lines; Chang liver epithelials and 5637 bladder epithelials. Transfections were performed using Lipofectamine 2000 [Invitrogen] on standard tissue culture plastic. Cells were grown in puromycin [Sigma] selection media and stable clones selected using standard methods. The transfected cells were then stripped from the tissue culture surfaces using detergents; leaving behind their ECM. The resulting vector derived fibronectin-rich ECM was reseeded with HepG2s. Scanning electron microscopy [SEM], histology, biochemical quantification and gene expression analyses were performed.

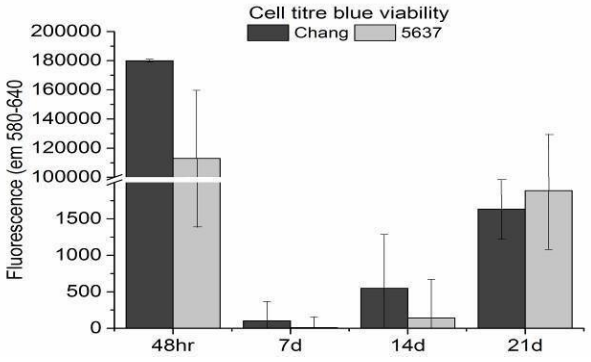

Fig. 1: Survival of transfected cells over 21d, cell titre blue viability test.

RESULTS: While cell survival is reduced by the transfection process [Figure 1], stable clones were obtained. The vector-derived fibronectin is transported outwith the cell, and laid down on the culture surface [Figure 2]. Exposure to the vector derived ECMs influenced the gene expression profile of the HepG2s [data not shown].

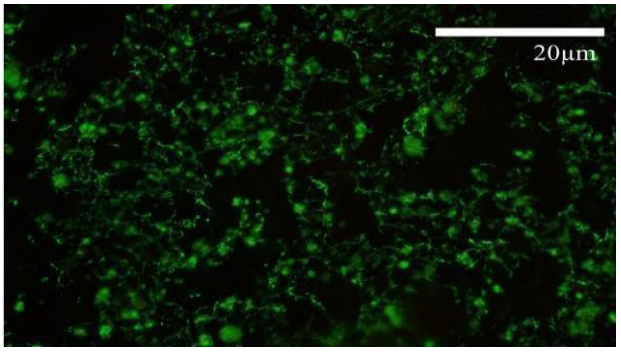

Fig. 2: Decellularized vector-derived ECM, stained using a fibronectin antibody and imaged at 20x

DISCUSSION & CONCLUSIONS: Work is ongoing to establish the effect of this novel, fibronectin rich cell-derived ECM on the behaviour of liver cell lines, and transfer the method to 3D culture. These findings demonstrate a platform for creating bespoke ECMs, and the viability of synthetically controlling ECM production for tissue engineering purposes.

ACKNOWLEDGEMENTS: This work is funded by an Engineering & Physical Sciences Research Council [EPSRC] doctoral training partnership studentship and UK Regenerative Medicine Platform II [RMPII] grant MR/L022974/1.

Human muscle precursor cells overexpressing PGC-1α support early myofiber formation for bioengineering of slow twitch sphincter muscle

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INTRODUCTION: Muscle precursor cells (MPCs) are quiescent muscle cells capable of muscle fiber reconstruction. However, their quality and quantity gradually decline with age. Therefore, autologous MPC transplantation is envisioned for the treatment of muscle diseases, such as Stress Urinary Incontinence [1]. The goal of this research was to explore the possibility of genetically modifying human MPCs to overexpress PGC-1α, a central factor in muscle exercise adaptation [2, 3], in order to enhance skeletal muscle formation and quality for application for urinary sphincter regeneration.

METHODS: hMPCs were harvested from M. rectus abdominis of patients. After genetically modifying the cells to overexpress PGC-1α (or GFP control), viability, proliferation and myogenic phenotype were evaluated in vitro. The expanded cells were suspended in a collagen carrier and *s.c.* injected bilaterally on the back of nude mice. One, two and four weeks later the bioengineered skeletal muscle tissues were harvested and further assessed by histology, WB and RTPCR.

RESULTS: We were able to confirm the sustained myogenic phenotype of the genetically modified hMPCs. Viability and proliferation potential were not significantly different compared to native cells in vitro. Fiber formation capacity and contractility were enhanced in PGC-1α modified hMPCs in vitro. Subcutaneously injected cell-collagen suspensions were harvested after 1, 2 and 4 weeks and histological analysis confirmed the earlier myotube formation in PGC-1α modified samples. Increased contractile protein levels were detected by WB, with a significant initial switch to slow type muscle fibers.

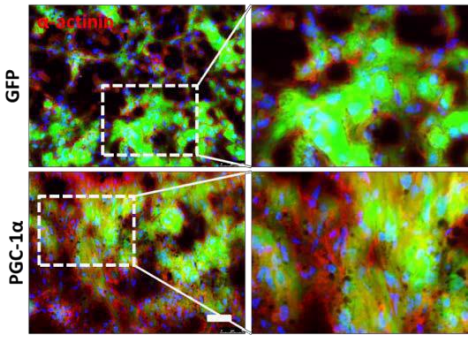

Fig. 1: Engineered ectopic muscle formation is enhanced by overexpression of PGC-1α in hMPCs. The increased differentiation capacity of PGC-1α transgenic hMPCs at early time points (1 week) was visualized by immunostaining for sarcomeric α-actinin (Cy3, red) of the ex situ engineered muscle fibers (green). Scale bar: 50μm.

DISCUSSION & CONCLUSIONS: By genetically modifying hMPCs to overexpress PGC-1α we were able to enhance the early myotube formation in vitro and in vivo, thereby developing a novel strategy for improving sphincter muscle tissue engineering for future application.

ACKNOWLEDGEMENTS: The authors recognize the research efforts of those who have contributed to this field of study and declare no conflict of interest. Special thanks to Damina Balmer for proof-reading. The authors' work is supported by grants from Swiss National Science Foundation, Promedica foundation and Novartis.

Chondrogenic differentiation potential of osteoarthritic chondrocytes under human serum from platelet-rich fibrin (SPRF) and platelet-rich plasma (PRP)

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INTRODUCTON: Articular cartilage is an avascular tissue that is limited by its poor healing capacity to restore tissue damage from sports injuries or trauma, ultimately ensuring an instability and loss of mobility in the joint tissue leading to osteoarthritis. Autologous chondrocyte implantation (ACT) involves a surgical intervention by implantation of autologous cells that are cultivated *in vitro.* Confronting the problem that occurs during *in vitro* expansion onto 2D cell culture substrates is to overcome the state of dedifferentiation [1]. In our current study, we investigated the proliferation rate and the preservation of the chondrogenic lineage of human osteoarthritic chondrocytes in monolayer culture.

METHODS: Human articular chondrocytes were isolated from osteoarthritic (OA) cartilage by enzymatic digestion and expanded in medium containing 10% FCS (control) for 30 days as P0 Cells were passaged and cultured as P1 where the medium is switched to 10% platelet-rich plasma (PRP) or 10% serum from platelet-rich fibrin (SPRF) for a further 3 days and maintained at normoxic (20% O_2) or hypoxic (1% O_2) conditions. Cell proliferation was measured by an XTT assay, and the chondrogenic differentiation potential was determined with RT-qPCR for chondrogenic gene expression markers *COL2A1* (collagen type II, alpha 1) and *COL1A1* (collagen type I, alpha 1) and matrix metalloproteinases *MMP3, MMP13* at 24h and 72h respectively.

RESULTS: SPRF enhanced the proliferation rate of OA chondrocytes in comparison to the control and PRP from 24h to 72h significantly ($p<0.05$) on 2D substrates. mRNA levels revealed that in OA chondrocytes *COL1A1* was downregulated when cultured with PRP than in SPRF or control under normoxic/hypoxic conditions. *COL2A1*, a marker of redifferentiation in chondrocytes was significantly upregulated with a 15 fold increase ($p<0.05$) in PRP under normoxia and hypoxia than in control. *CO2A1* was downregulated in SPRF than in the control from 24h to 72h. MMP3 and MMP13 was upregulated with 1.5 fold increase ($p<0.05$) in PRP under normoxia but downregulated under hypoxia to control and SPRF.

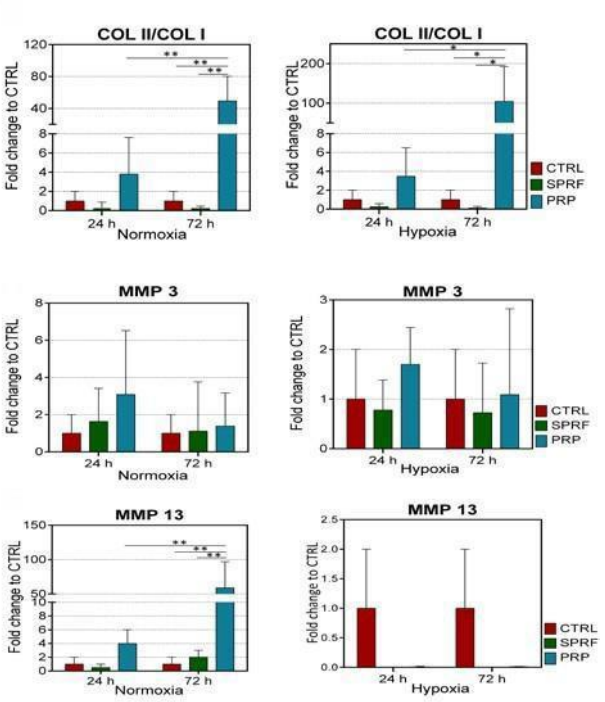

Fig. 1: Chondrogenic gene expression as determined by RT-qPCR for OA chondrocytes cultured in 2D substrates with 10% FCS, PRP, SPRF under hypoxic/normoxic conditions.

DISCUSSION & CONCLUSION: Increased chondrocyte proliferation can be attained *in vitro* on 2D surfaces by addition of PRP to the cells without an increased dedifferentiation. SPRF increases even faster proliferation at the expense of decreased Collagen II production, indicating that the growth factor composition of platelet-rich plasma varieties may have surprisingly different effects on the fate of cultured cells.

ACKNOWLEDGEMENTS: This work was supported by a grant from NFB lower austria.

Pathogen inactivated platelet lysates boost osteogenic and chondrogenic differentiation of MSC and mesenchymal progenitor cells

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INTRODUCTION: Recent advances in tissue engineering and regenerative medicine have made stem cell therapies a realistic possibility. The suitability of mesenchymal stromal cells (MSC) for such therapies has been studied extensively. MSC are particularly interesting due to their multipotent differentiation and immunomodulatory abilities. Embryonic derived mesenchymal progenitor cells (hES-MP) have the same characteristics as MSC and have been thought of as a cell line that can be used therapeutically as MSC. Addition of animal-serum is currently required for the survival and growth of hES-MP and MSC. Using animal-serum to grow cells intended for human medical therapy is not safe and serum-free alternatives need to be found. We have previously demonstrated the efficacy of using platelet lysates from pathogen inactivated platelets on MSC and hES-MP during their growth. Here we show that using human platelet lysates made from pathogen inactivated platelets (hPL-PI) during differentiation of MSC and hES-MP fully supports both chondrogenesis and osteogenesis allowing serum-free growth conditions from expansion throughout differentiation.

METHODS: MSC and hES-MP002.5 were grown and differentiated towards chondrogenic and osteogenic lineages in the presence of fetal bovine serum (FBS) or hPL-PI. Chondrogenic differentiation was evaluated over five weeks and histology and gene expression evaluated weekly. Osteogenic differentiation was performed over four weeks with alkaline phosphatase activity, mineralization and gene expression evaluated weekly.

RESULTS: Histology resembling articular cartilage was observed for MSC and hES-MP at the end of chondrogenic differentiation when differentiated in the presence of hPL-PI (fig 1). *SOX9*, the master transcription factor of chondrogenesis, was significantly upregulated in cell differentiated in hPL-PI compared to FBS. During osteogenic differentiation the activity of alkaline phosphatase, amount of calcium deposits and gene expression was significantly higher in MSC and hES-MP differentiated in hPL-PI compared to FBS.

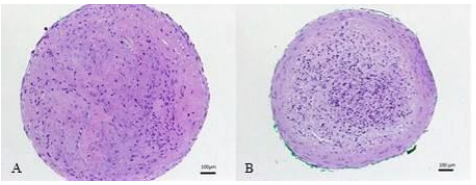

Fig. 1: hES-MP after 5 weeks of chondrogenic differentiation using hPL-PI (A) or FBS (B) as media supplement

DISCUSSION & CONCLUSIONS: Using hPL-PI based differentiation media encourages osteogenic and chondrogenic differentiation of MSC and hES-MP to a greater degree than FBS based media. Using hPL-PI rather than FBS provides a serum-free growth environment for the cells and supports their differentiation. New guidelines advise against the use of animal-serum making hPL-PI an attractive alternative that allows the cells to maintain their biological characteristics.

ACKNOWLEDGEMENTS: The authors want to thank the Landspitali University Hospital Research Fund for partially funding the project.

exogenous electric field using aluminium-silver galvanic patch for split-thickness skin wound dressing in a porcine model.

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INTRODUCTION: We intended to evaluate and discuss the role of exogenous electric field (*Fig. 1 & 2*) using aluminium-silver galvanic patch for split thickness skin wound dressing in a porcine model.

METHODS: Two female Yorkshire pigs weighing 25 to 30 kg were obtained. The proposed wound areas were marked with the aid of flexible plastic frame for making the standardized wounds (5 x 5cm) and the same distance between wounds (2cm). The split thickness of the skin was removed using monopolar cauterization according to the tattooed line. In the control group (C), bare wound was left. In the hydrogel dressing group (H) and the hydrogel with aluminium-silver galvanic group (HG), wound dressing was done with hydrogel and hydrogel with galvanic patch, respectively. Serial analysis of wound was performed. Histological evaluation and immunohistochemistry (IHC) using anti-CD 31 and anti-SMA antibodies were performed. The complete closure of every wound was considered as the end of the study.

RESULTS: The wound healing in the group with exogenous electric field was significantly faster than in the control group (*Fig. 3*).

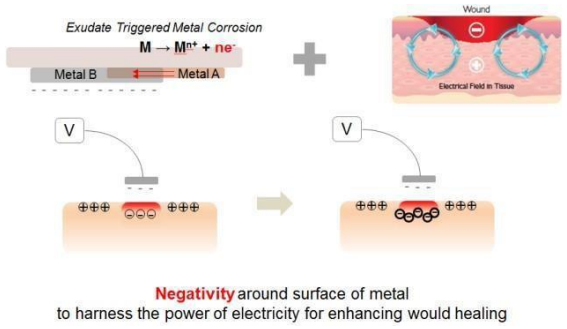

Fig. 1: Concept of exogenous electric field for wound healing.

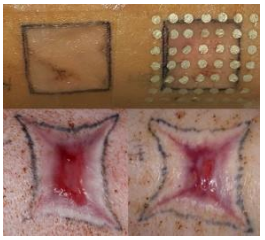

Fig. 2: The hydrogel dressing with/ without galvanic patch

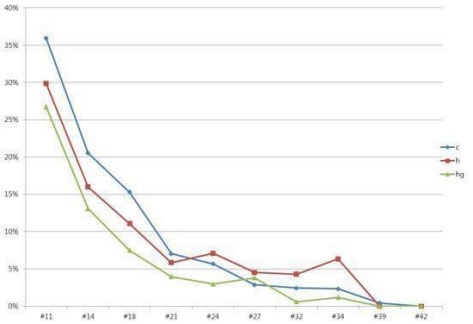

Fig. 3: Wound closure

The length of the new epithelium was significantly longer in the group HG compared to the groups C and H. Neoangiogenesis expressed by CD31 positivity was augmented in the group HG compared to the groups C and H. There was no difference in the myofibroblast differentiation expressed by IHC using anti-SMA antibody between the groups.

DISCUSSION & CONCLUSIONS: Exogenous electric field using aluminium-silver galvanic patch accelerates the wound healing of split thickness skin wound.

Bladder wound healing after mechanical injury

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INTRODUCTION: Surgical interventions of the urinary bladder are common in paediatric urology. In conditions with severe lack of tissue, regenerative medicine using autologous cells has become a field of interest for future cure; however, little is known about normal cell responses in bladder wound healing. In this review, we summarize the current knowledge in wound healing in the urinary bladder after mechanical injury.

METHODS: We searched for articles on PubMed and Web of Science using combinations of the MESH terms: "urothelium", "urinary bladder", "wound healing" and "tissue engineering". The primary selection of articles was based on title and abstract evaluation. Only English publications were selected. A second selection was based on the relevance and quality of the articles.

RESULTS: We found 26 original reports focusing on healing after mechanical injury. Among these, 9 reports investigated a total of 20 signalling and/or growth factors. Most factors were only described in non-validated, single reports. We identified EGF as the most studied factor, based on its stimulating effect on wound healing (6 studies) [1]. Overall, bladder wound healing is similar to that of skin, following the steps; inflammation, proliferation and remodelling. However, proliferation is upregulated sooner than in skin. Moreover, the urinary bladder is capable of a globalized response upon mechanical injury [2], involving also the none-injured bladder half. The effect of exogenous signalling factors has shown to stimulate proliferation and migration. However, most studies used increased area of reepithelization as end-point, without further investigating the separate contribution of proliferation and migration.

Table 1. Current known effects on urothelial wound healing after exposure of exogenous signalling factors.

	Prolifer-ation	Migration	Increased area of re-epithelization
Stimu-late	bFGF, HGF, IGF-I, VEGF, KGF, TGF-β	AR, TGF-α, EGF, bFGF, HGF, IGF-I, VEGF, TGF-β	ATP, HB-EGF, AR, TGF-α, EGF, TRPV-4, bFGF, HGF, IGF-I, VEGF, TGF-β
Inhibit	TGF-β, EGFR-inhibition	TGF-β, EGFR-inhibition	TGF-β, EGFR-inhibition

DISCUSSION & CONCLUSIONS: The healing pattern of the urinary bladder is similar to that of the skin. Moreover, treatment with several signalling factors has shown positive results on wound recovery. However, albeit there are numerous studies addressing wound healing in the urinary bladder, there is a need for studies validating and the results.

ACKNOWLEDGEMENTS: The authors would like to thank the Promobilia Foundation, the Swedish Society for Medical Research, the Crown Princess Lovisa Foundation, the Freemason Foundation for Children's Welfare, the Stockholm City Council and the Swedish Society of Medicine for supporting this work.

Neurotrophic factors conjugated to iron oxide nanoparticles to promote peripheral nerve regeneration

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INTRODUCTION: Tubulization technique with nerve device is a surgical approach widely used for repair of peripheral nerve injured with loss of substance. These nerve devices, already used in clinics, achieve good nerve regeneration only for short gaps. For long gaps the hollow tube does not give satisfying results and implementation are needed [1]. One strategy is to use the tube as a carrier for growth factors that promote cell migration and nerve growth inside the device. Here we present an *in vitro* study using growth factors conjugated with iron-oxide nanoparticles (IONP). The IONP are actually used for serial biomedical application as cell labelling, magnetic resonance imaging, and gene or drug delivery [2]. We investigated the possibility to use conjugated growth factors for engineering nerve device.

METHODS: IONP are nanoparticles of 8.5±0.9 nm diameter with a dextran coating [3]. We selected four neurotrophic factors (NTFs): nerve growth factor (NGF), glial derived neurotrophic factor (GDNF), fibroblast growth factor 2 (FGF2) and neuregulin 1 beta (Nrg1). Each single factor was covalently conjugated to the coating of the IONP (IONP-NTFs). The bioactivity of IONP-NTFs was evaluated analyzing the neurite outgrowth inductive activity in adult or neonatal DRG culture, where factors were added in the medium or mixed in a matrix (NVR gel). For Nrg1 we compared the effects of IONP-Nrg1 and Nrg1 on cell migration. Using the same systems we analyzed the stability of IONP-NTFs. Next, we investigated *in vivo* how the enrichment of a nerve device with NVR gel and IONP-GDNF could improve nerve regeneration in a rat sciatic injury model. The hollow tube or the tube enriched with NVR gel alone or with GDNF were compared with the tube filled with NVR and IONP-GDNF. Functional and morphometric analysis were performed.

RESULTS: We demonstrated that the conjugation with IONP preserves the bioactivity of the factor and increases its stability. Our *in vivo* study confirms that nerve device enriched with a matrix with GDNF gives more successful results in term of regeneration and functional recovery respect to the hollow tube; moreover, there are no detectable differences between free versus conjugated GDNF at the time point evaluated.

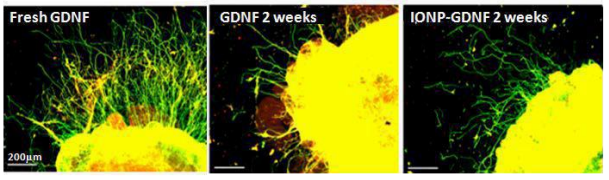

Fig. 1. Representative pictures of adult DRG cultured with fresh medium or with medium containing 2 weeks-aged IONP-GDNF or GDNF.

Table 1. Percentage of cell migration in presence of IONP-Nrg1 or Nrg1 in cell medium (fresh prepared or after 6 weeks at 37°C)

	% cell migration fresh medium	% cell migration 6 weeks medium
IONP-Nrg1	20.2±1.3	14.8±1.7
Nrg1	18.1±4.7	7.8±2.1
No factor	3.1±1.6	4.9±3.5

DISCUSSION & CONCLUSIONS: The conduit enrichment could be achieved by NTF delivery within the nerve conduit. However, the main disadvantage is the short life-time of NTFs. NT-IONPs are more stable and are good candidates for *in vivo* prolonged therapy as suggested by our *in vitro* data. It remains to be elucidated the best strategy for IONP releasing in the nerve device.

ACKNOWLEDGEMENTS: This project has received funding from the European Union's Seventh Programme for Research, Technological Development and Demonstration under Grant Agreement No 278612.

Porous beads to provide regulated BMP-2 stimulation for varying durations: in vitro and in vivo studies for new bone formation

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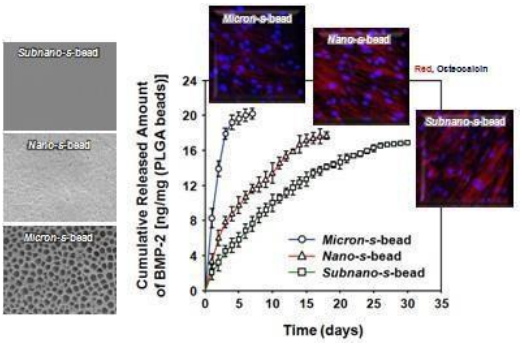
INTRODUCTION: It is commonly accepted that the sustained release of bone morphogenetic protein-2 (BMP-2) can enhance bone regeneration and minimize its safety issues [1]. However, little is known regarding the appropriate duration of BMP-2 stimulation for sufficient osteogenic differentiation and new bone formation. Therefore, the development of a delivery system that offers sustained release of BMP-2 for a designated period of time while providing the concentrations required for bone regeneration, might provide an elegant tool to determine the optimal duration of BMP-2 stimulation for new bone formation. Our main purposes were to develop a BMP-2 delivery matrix that would facilitate the sustained release of BMP-2 for a designated period of time, and to conduct *in vitro* and *in vivo* feasibility studies to determine the appropriate exposure time of BMP-2 for effective new bone formation.

METHODS: The porous beads with different surface pore sizes (*nano-s*-beads, *micron-s*-bead and *subnano-s*-bead) were prepared by spray/precipitation, surface etching, surface welding, respectively [2]. BMP-2 was immobilized in the porous beads by simple adsorption in BMP-2 solution for 3 hrs. The BMP-2 release patterns from each bead were quantified by a ELISA kit. Human periosteum-derived cells (*h*PDCs) were used as model cells to evaluate the effect of the duration of BMP-2 stimulation (i.e., 7, 17, and 30 days) on osteogenic differentiation. The extent of new bone formation facilitated by porous beads that can provide different durations of BMP-2 stimulation was examined in miniature pigs (mandibular defect model).

RESULTS: Porous beads with different surface pore sizes were fabricated by a spray/precipitation method (for *nano*-s-beads), and by subsequent surface etching (for *micron-s*-beads) or surface welding (for *subnano-s*-beads) procedures. The release period of BMP-2 can be controlled by varying the pore size on porous beads (*micron-s*-beads, 7 days; *nano-s*-beads, 17 days; and *subnano-s*-beads, 30 days). For *in vitro* cell culture and *in vivo* animal studies, it was observed that a burst release of BMP-2 within 7 days affects only the initial differentiation of *h*PDCs, and does not significantly enhance their subsequent differentiation into mature functional cells, whereas BMP-2 stimulation for over 17 days can provide a suitable environment for osteogenic differentiation of *h*PDCs as well as for new bone formation (Fig. 1).

Fig. 1: SEM photographs, BMP-2 release profiles,



and immunocytochemical observation of the porous beads with different surface pore sizes.

DISCUSSION & CONCLUSIONS: Our findings may provide a window within which to identify the appropriate stimulation time for BMP-2 released from a delivery matrix for effective new bone formation, even though our system provides just three different BMP-2 release periods.

ACKNOWLEDGEMENT: This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI) funded by the Ministry of Health & Welfare, Korea (HI13C1596).

Ion-complexed lidocaine as a potential strategy for prolonged local anesthesia

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INTRODUCTION: Although local anesthetics are widely used to control perioperative/postoperative pain in the clinical setting, their short duration of action remains a drawback [1,2]. To overcome this barrier, multiple injections, continuous infusion, adjuvant agents, and delivery matrices are commonly utilized in clinical practices. However, the insufficient duration of action (with respect to adjuvants), as well as the inflammation/toxicity caused by the delivery matrix itself or its metabolites, and the fact that anesthetic actions are not reproducible owing to different (uncontrolled) drug release patterns with each fabrication batch remains as huddles for clinical applications. Therefore, our main goals were to fabricate a local anesthetic/multivalent ion complex that would allow sustained release of the anesthetic from the complex and to conduct a feasibility study to determine whether this complex could be part of a strategy to prolong anesthetic action (by extending the release period of the drug) and minimize toxicity (by obviating use of a deliverymatrix).

METHODS: The lidocaine (LD) solution and the K_3PO_4 solution were simply mixed to obtain the ion-complexed LD (*ic*LD). The *ic*LD precipitate created by the complex formed between LD and trivalent PO_4^{3-}. The release behaviour of LD dissociated from *ic*LD was determined by a HPLC. The cytotoxicity of the free LD and of the *ic*LD at different drug concentrations was estimated using rat pheochromocytoma cell line (PC12). To estimate the duration of anesthetic effect and neurotoxicity of the free LD and of the *ic*LD, *in vivo* animal study (SD rat model) were conducted.

RESULTS: We prepared a lidocaine/multivalent ion complex (*ic*LD) by simple mixing of two aqueous solutions containing positively charged LD and a multivalent counter-ion as a possible system for producing prolonged anesthesia. Both the *in vitro* characterizations and the results of the *in vivo* animal study indicated that the *ic*LD offers a prolonged period of LD release (through the gradual dissociation of LD from this complex) and thus provides nerve blockade of longer duration (Fig. 1) with a degree of neurotoxicity that does not exceed that of lidocaine itself in the amount in clinical use.

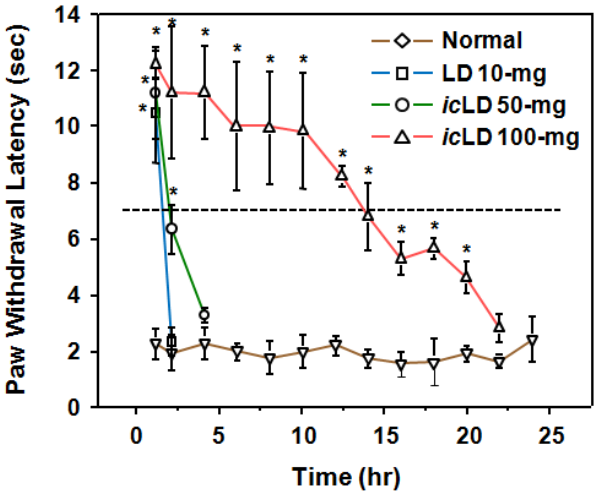

Fig. 1: Changes in the latency of paw withdrawal in the four experimental animal groups at different time points after injection of three drug solutions (n=3). Criterion for determining whether or not the nerve block (dotted line).

DISCUSSION & CONCLUSIONS: Our findings support consideration of the *ic*LD system as a practical strategy for achieving prolonged anesthesia to effectively control perioperative/ postoperative pain.

ACKNOWLEDGEMENT: This work was supported by grants of Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Science, ICT and Future Planning (NRF-2012R1A1A2041836).

Functionalised collagen-MnO_2 fibres inhibit oxidative-induced apoptosis in degenerated IVD

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INTRODUCTION: Intervertebral disc cell apoptosis has been reported as the major factor responsible in promoting disc degeneration. In this study we hypothesize that collagen fibres with manganese dioxide (MnO_2) nanoparticles (NPs) can increase oxygen levels by scavenging ROS species and converting it into byproducts. The specific objective of this study is to fabricate collagen fibres incorporating NPs (Fig. 1), with controlled degradability that are able to scavenge ROS species and generate O_2 while inhibiting annulus fibrosus (AF) cell apoptosis under inflamed conditions.

METHODS: MnO_2 NPs were synthesized and the functionalised fibres were characterised structurally (XRD & IR) and morphologically (TEM) and their charge was measured by DLS. An oxygen sensor was used to measure the oxygen levels after the reaction of NPs with various H_2O_2 concentrations. The NPs were mixed with collagen type I (3.5 mg/ml) and fibres were synthesized by the fibre extrusion method (Fig. 1B). The degradation profile as well as the oxygen levels after reaction in presence of H_2O_2 was measured. Cytotoxicity of the functionalised fibres was carried out on Il-1β induced inflamed AF cells. Changes in the apoptosis rate and intracellular ROS levels in cultured inflamed AF cells were measured by flow cytometry and immunocytochemistry.

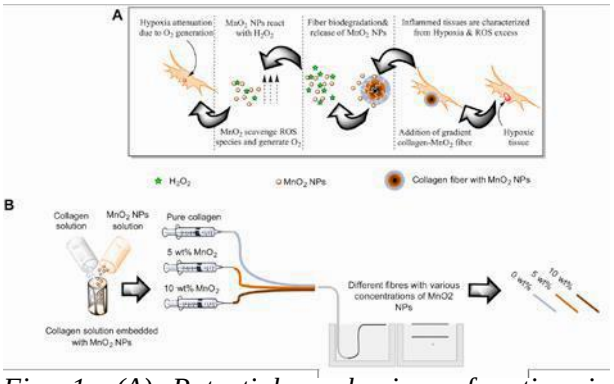

Fig. 1: (A) Potential mechanism of action in inflamed tissues and (B) Schematic representation of functionalised collagen-MnO_2 fibres.

RESULTS: MnO_2 NPs were successfully synthesized and incorporated within collagen fibres. Oxygen was generated following the reaction of the NPs with H_2O_2 (Fig. 2B) and the increasing oxygen levels are proportional to the nanoparticle concentration. *In vitro* studies on show good cellular metabolic activity indicating the scavenging effect of the NPs (Fig. 2C). Flow cytometry results showed that MnO_2 loaded collagen fibres exhibit increased cell viability, decreased ROS production and reduced apoptosis.

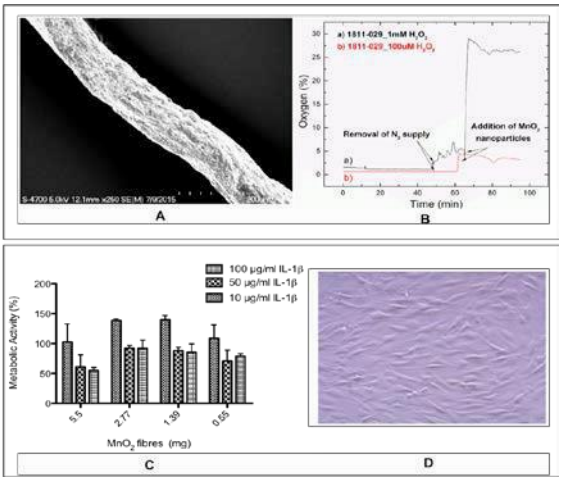

Fig. 2: (A) SEM picture of functionalised collagen-MnO_2 fibres; (B) Oxygen measurements of collagen-MnO_2 fibres after treatment with different concentrations of H_2O_2; (C) Metabolic activity of IL-1β inflamed AF cells after treatment with functionalised collagen-MnO_2 fibres and (D) IL-1β inflamed AF cells

DISCUSSION & CONCLUSION: The functionalised collagen-MnO_2 fibres were able to generate oxygen H_2O_2 by MnO_2 NPs scavenging reaction. Future studies will include measurements of altered mitochondrial membrane potential and molecular markers involved in ECM production of AF cells.

ACKNOWLEDGEMENTS: This work has been co-supported by a research grant from Science Foundation Ireland (SFI), the European Regional Development Fund (13/RC/2073) and also by the Portuguese Foundation for Science and Technology (SFRH/BD/81356/2011).

GDF-5 augmented fibrin glue to improve osteochondral integration of tissue engineering constructs in cartilage defects

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INTRODUCTION: Long-term regeneration of cartilage defects treated with tissue engineering constructs often fails because of insufficient integration with the host tissue. We hypothesize that construct integration will be improved when the implant actively interacts with and integrates into the subchondral bone. Growth and Differentiation Factor 5 (GDF-5) is known to support maturation of chondrocytes and to enhance the chondrogenic differentiation and hypertrophy of mesenchymal stromal cells (MSC). Therefore, we investigated whether GDF-5 is capable to stimulate endochondral ossification of MSC in vitro and in vivo and would, thus, be a promising candidate for augmenting fibrin glue in order to support integration of tissue engineering constructs into the subchondral bone plate.

METHODS: To evaluate the adhesive strength of fibrin glue versus BioGlue®, a commercially available glue frequently used in vascular surgery, an ex vivo cadaver study was performed and adhesion strength was measured via pull-out testing. MSC were suspended in fibrin glue and cultivated in chondrogenic medium with and without 150 ng/mL GDF-5. After 4 weeks, the quality and quantity of the formed cartilage were evaluated and half of the constructs were implanted subcutaneously into immune deficient mice. Endochondral ossification was evaluated after 2 and 4 weeks histologically and by microCT analysis. BioGlue® and GDF-5-augmented fibrin glue were tested for 4 weeks in a minipig cartilage defect model to assess their orthotopic biocompatibility.

RESULTS: Pull-out testing revealed sufficient adhesive strength of fibrin glue to fix polymeric CellCoTec constructs in 6 mm cartilage defects in cadaver knees, however, BioGlue® showed significantly higher adhesive power than fibrin.
In vitro assays revealed that under treatment with GDF-5, MSC embedded in fibrin glue formed cartilage as well as controls. Importantly, significantly increased ALP-activity under treatment with GDF-5 on day 28 indicated enhanced hypertrophic differentiation compared to controls. In vivo, MSC-fibrin constructs pre-cultured with GDF-5 developed significantly more mineralisation on days 14 and 28 compared to control constructs. Constructs showed a significantly higher bone density (bone surface/bone volume coefficient) on day 28 when pre-cultured with GDF-5 than control constructs without GDF-5. This indicated a higher maturity of the formed bone. Orthotopic biocompatibility testing in minipigs showed a good defect filling and no adverse reactions of the subchondral bone plate for defects treated with GDF-5-augmented fibrin glue. Defects treated with BioGlue®, however, remained nearly unfilled and showed considerable subchondral bone lysis.

DISCUSSION & CONCLUSIONS: Thus, BioGlue® – despite its high adhesive strength – should not be used for construct fixation in cartilage defects. GDF-5-augmented fibrin glue is considered promising, because of a combination of the adhesive strength of fibrin with an enhanced osteochondral activity of GDF-5 on MSC. Ongoing work will unravel whether the GDF-5 bioactivity will stimulate endochondral ossification to improve scaffold integration.

ACKNOWLEDGEMENTS: This work has received funding from the European Union's Seventh Framework Programme (FP7/2007-2013) HydroZONES under grant agreement n. 309962.

The role of autophagy during chondrodifferentiation of human mesenchymal stem cells

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INTRODUCTION: Cartilage lesions due to injury heal poorly, leading to early onset of osteoarthritis, chronic joint pain and disability. Mesenchymal stem cells (MSCs) in combination with polymeric carriers can be used to induce differentiation of the cells towards chondrogenesis [1]. Autophagy is a cellular mechanism, required for differentiation of adult stem cells. Influencing autophagy during differentiation of MSC to chondrocytes may alter cell fate [2]. Therefore, we investigated the effect of rapamycin, an autophagy activator, during differentiation of MSC in 3D microtissues to chondrocytes.

METHODS: Cartilaginous microtissues were prepared by mixing droplets of human MSCs in chitosan (sChi) with droplets of oxidized alginate (oxAlg). Microtissues were induced in chondrocyte differentiation media in presence and absence of rapamycin for 10, 14 and 21 days. Changes in gene and protein expression for collagen type II and X and autophagy markers Atg5 and LC3 were investigated by real-time-PCR and immunofluorescence.

RESULTS: Upon chondroinduction, down regulation of key autophagy gene Atg5 and Beclin1 was observed in the non-stimulated group. We observed an increased expression of collagen 2 and hypertrophic marker collagen X starting at day 10 with highest expression on day 21. Rapamycin treated group showed increased Atg5 gene expression with the highest peak at day 14. Treated microtissues exhibited better cell morphology and viability with high expression of Atg5 and punctated immunostaining pattern for LC3 confirming accumulation of autophagosomes.

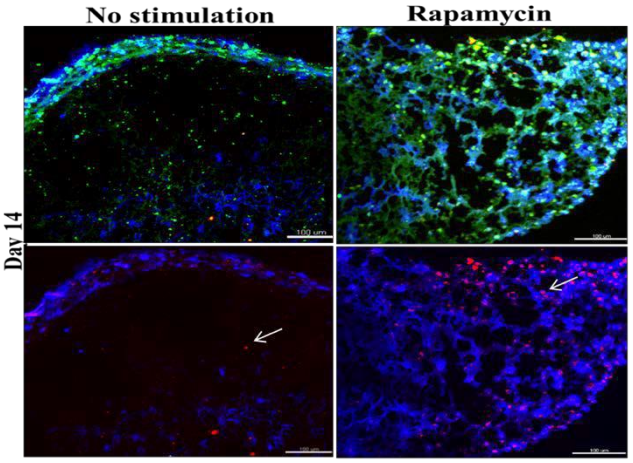

Fig. 1: Immunostaining of non-stimulated and rapamycin (1uM) stimulated microtissues. Treated microtissues exhibited better cell morphology and viability with high expression of Atg5 (green) and punctated pattern for LC3 (red) confirming accumulation of autophagosomes. Nucleus was stained with DAPI (blue).

DISCUSSION & CONCLUSIONS: Our study demonstrates that autophagy plays an important role in chondrodifferentiation of MSC. Administration of rapamycin could serve as novel therapeutic approach for treating cartilage injuries for future clinical applications.

ACKNOWLEDGEMENTS: The authors recognize the research efforts of those who have contributed to this field of study and declare no conflict of interest.

Impact of growth factors combination and timing on early, middle and late chondrogenesis of human mesenchymal stem cells

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INTRODUCTION: Growth factors play an important role during tissue development and repair. Clear advances have been made using them for the regeneration of some tissues e.g. BMP2 and BMP7 for bone. In the case of the enthesis (ligament-to-bone interface), the selection of the adequate growth factor(s) for its regeneration is a very challenging process. Given the multiphasic nature of the enthesis, multiple growth factors and even gradients of those may be necessary to provide the target cell population the suitable differentiation stimuli (Fig. 1) [1]. Among the different zones of the enthesis, mineralized fibrocartilage and fibrocartilage are of primary importance. It has been described that chondrogenesis of mesenchymal stem cells (MSCs) often results in inadequate, hypertrophic differentiation cascade depending on the signalling factors involved [2]. This study aimed to evaluate different growth factor combinations and timing in order to identify potent inducers to achieve chondrogenesis and simultaneously suppressing hypertrophy in adipose-derived MSCs (AMSCs) cultured in 3D matrix.

METHODS: Human AMSCs were isolated from fresh adipose tissue. First, a monolayer cultivation was performed in which the effect of BMP2 and BMP6 was studied. For this, $1.6x10^4$ cells/cm^2 were stimulated with chondrogenic differentiation media (in absence of TGFβ: CM media) supplemented with 50 ng/ml of either BMP2 or BMP6. Two and three weeks after stimulation the induction of PTHrP receptor as well as TGFβ receptor 1 and 2 was analyzed by flow cytometry and qPCR in order to select the BMP that promoted better receptor expression.
Subsequently, a collagen hydrogel was selected as 3D cultivation system ($1.0x10^5$ AMSCs/construct). Next, constructs were cultured in CM media supplemented with different growth factor combinations added at different time points. TGFβ1 (10 ng/ml) was supplemented since day 1 of culture. BMP2 (50 ng/ml) was similarly supplemented since the first day of culture, but only during 15 days. At this point, PTHrP (100 ng/ml) was given to the culture either immediately or 1 week after the interruption of BMP2 stimulation. Gene expression was then evaluated by qPCR for relevant genes e.g. Aggrecan, ALP, BMPR1 and 2, Coll I, II, IIA, III and X, Ihh, MMP-13, PTHrP, PTHrP-R1, RunX2, Sox9, TGFβ-R1 and 2 and Versican. Proliferation under the different stimuli was evaluated by dsDNA quantification. Finally, the occurrence of chondrogenesis was also evaluated histologically (Alcian blue, Safranin O). In addition, collagen deposition was IHC assessed for Coll II, III and X.

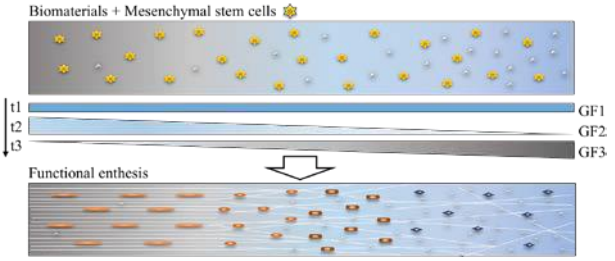

Fig. 1: Stimulation of tissue engineering constructs with a combination of growth factors for the regeneration of the enthesis. Adapted from [1].

RESULTS: The relative expression of TGFβ-R1 and TGFβ-R2 was significantly higher for the BMP2 treated cell when compared to BMP6 treatment for all time of observation. After 3D culture and in the absence of PTHrP stimulation, upregulation of Sox9, Aggrecan and Versican, Coll I, III, X was observed. PTHrP supplementation resulted in upregulation of TGFβ-R1 and Coll II. In addition, a positive effect was also observed for Coll III expression. This one was much clearer when the supplementation of PTHrP was performed 1 week after the interruption of BMP2 stimulation. No effect of PTHrP was found over Sox9 and Aggrecan expression. Interestingly, RunX2, ALP and BMP-R1 were also upregulated after PTHrP addition.

DISCUSSION & CONCLUSION: Our investigation of the different growth factors combination showed the essential role of TGFβ1/BMP2 combination in AMSCs chondrogenesis. No supportive role was found by addition of PTHrP. On the contrary, PTHrP seems to have a positive influence on the osteogenic differentiation of AMSCs.

Controlled delivery of siRNA nanocomplexes from cross-linked gelatin hydrogel matrices

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INTRODUCTION: The successful transient knockdown of hard-to-transfect cells for regenerative purposes requires complex oligonucleotide release and cell carrier matrices. Protein-based hydrogels offer ECM-like structures, which provide cell attachment features. In addition, such gels can be modified in manifold ways according to the favored characteristics. Cross-linking is a useful tool to improve physical properties such as stiffness, storage and loss modulus. These linkers can furthermore adjust chemical characteristic, e.g. charge density. The goal of this study is to combine cross-linked gelatin-based, negatively charged hydrogels [1] with established or novel cell transfection agents to achieve a sufficient silencing of target genes in a reporter cell line. The received RNAi matrix offers perspectives to improve API efficiency by downregulation of antagonists on the mRNA level. This strategy is auspicious in the therapy of poor healing bone defects by a combination of Bone morphogenic protein and siRNA targeting its antagonists [2].

METHODS: For the visualization of oligonucleotide nanoparticle loading efficacy to the cross-linked hydrogels, siRNA was covalently labeled with a fluorophore using the Mirus´ Label IT® Cy™3 labeling Kit. Nanocomplexes were prepared as published previously [3] and pipetted onto sterile hydrogel disks. Samples were frozen and subsequently cut at 10 µm. Sections were pictured at a gel and blot imager.

Release kinetics were determined by agarose gel electrophoresis. Hydrogels were loaded with uncomplexed or complexed siRNA, incubated in HEPES buffered saline at 37 °C and complete supernatant was sampled at predefined time points.

The biological activity of the released complexes was measured by luciferase knockdown in a stably luciferase expressing cell line (SKOV-3 Luc) cultured on the hydrogels for 3 days.

RESULTS: Nanoparticle distribution in the hydrogel was examined by fluorescent image analysis in order to derive hydrogel-nanocomplex interactions. Highly positive charged nanoparticles prepared using PEI F25-LMW as complexing agent, were predominantly accumulated at the surface of the hydrogels. The use of a tyrosine-modified PEI for nano-complexation resulted in a deeper penetration of the nanoparticles. Neutrally charged liposomes added to PEI polyplexes as a further complexation strategy enabled full distribution of the complexes in the gel matrix.

Up to 600 ng (37.5% of initial dose) siRNA was released from the PEI F25-LMW loaded hydrogels within the first 4 hours, which suggests that the highly charged particles tend to be adsorbed in multilayers. In comparison, a maximum of 70 ng was detected from the liposomal enveloped nanoparticles in the gels. All formulations showed a subsequent sustained release over 21 days attributed to matrix degradation.

In vitro, released PEI F25-LMW particles caused a decreased luciferase activity of approximately 20% in SKOV-3 Luc cells independent of the hydrogel matrix formulations. The effect was enhanced on macroporous gels loaded with the tyrosine-modified PEI. A comparably high knockdown efficiency was also observed for the lipopolyplexes. This indicates that the sustained release over the first 3 days, rather than the initial burst release, plays a crucial role for siRNA delivery to cells growing directly on the matrix.

DISCUSSION & CONCLUSIONS: These findings are promising with regard to silencing strategies from a hydrogel scaffold for regenerative applications.

ACKNOWLEDGEMENTS: Financial support by Sächsisches Staatsministerium für Wissenschaft und Kunst (SMWK) and DFG-Transregio 67 A1 is gratefully acknowledged.

Improved wound healing by topical application of APOSEC in a diabetic mouse model

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INTRODUCTION: Incidence of diabetes has risen to an alarming rate. In the European Region about 60 Million people are affected from diabetes and about 3.4 million people die annually from the consequences of increased blood sugar levels. Among patients diagnosed with diabetes the prevalence for diabetic foot ulcer is estimated to range between 4 and 10% and a life time incidence of about 25% [1], however, to date no sufficient therapeutic option exist. APOSEC (an acronym for APOptotic cell SECretoma), the irradiated, virus inactivated supernatant of apoptotic peripheral mononuclear blood cells (PBMCs), has already displayed beneficial effects in tissue regeneration and wound healing in previous studies [2-5]. To assess a possible beneficial effect of APOSEC also in diabetic wound healing, this study was performed in a murine diabetic wound model.

METHODS: Genetically diabetic db/db mice were used to mimic a diabetes type 2 pathology. Homozygous db/db mice exhibit a genetic mutation of the leptin receptor and are characterised by hyperglycaemia, obesity, hyerinsulinaemia and impaired wound healing. 12 mice were assigned per group. A common full-thickness wound model was used in which either APOSEC or control were topically administered to the wound site for the first ten days. To determine a potential dose dependency of APOSEC, three different concentrations were applied. Wound size was measured at day 0, 3, 7, 11, 14, 18 and 25 by tracing the wound on an acrylic foil for planimetry and by using a stereoscopic camera for 3D wound measurement (circumference, surface, average depth).

RESULTS: We showed that local administration of APOSEC enhances wound healing in diabetic mice. Wound circumference, size of wound surface and wound size assessed planimetrically at day 18 was significantly reduced in mice treated with APOSEC. A significant dose dependency of the treatment with APOSEC could be shown for the wound circumference and planimetry.

DISCUSSION & CONCLUSIONS: To the best of our knowledge this is first study on APOSEC in diabetic wound healing. We demonstrated that topical application of APOSEC in diabetic mice significantly enhances wound healing in regard to wound circumference, and wound surface in contrast to a medium control. We further showed a dose dependency where higher doses of APOSEC led to better wound healing. By day 25 regeneration of the wounds had proceeded to a level at which the wound size naturally equalised between groups. Hitherto there is no adequate therapy to address the clinical problem of diabetic ulcers. Utilising the regenerative potential of APOSEC could provide an inexpensive possibility to address this burden in the future. APOSEC is based on PBMCs which belong to leukocytes, that are a side-product of blood donations.

Gelatin and heparin exposure on the surface of electrospun PCL fibers

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INTRODUCTION: Driving of cell type specific adhesion to the surface of electrospun 3D matrices is demanded for rational design of vascular grafts. In contrast to post-production modification of the surface incorporation of the compound directly into fibers is a more simple procedure and potentially provides a longer exposure of the additive[1]. Herein we have investigated the exposure of gelatin (GL) or heparin (Hp) on the surface of the fibers produced from a solutions of polycaprolactone (PCL) with biopolymers in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP).

METHODS: Matrices made from 5÷7% PCL (w/v, %) in HFIP or PCL blended with 0.5÷10% (w/w, % to PCL) GL or 0.5÷3% tributylamine salt of fluorescein labeled Hp in HFIP were produced using the electrospinning setup NF-103 (MECC Co). Fluorescein moiety was introduced into Hp by means of EDAC activation of the carboxyl groups. Fiber structure of all matrices was confirmed by scanning electron microscopy (SEM) on JSM-6460 LV (Jeol). Hp release was evaluated using CLARIO Star (BMG Labtech) fluorimeter. Surface-exposed biopolymers were measured using X-ray photoelectron spectroscopy (XPS) performed in a SPECS equipped with a PHOIBOS-150 MCD-9 analyzer and MgK source (SPECS GmbH). Ratios of basic elements (C,O,N, in GL and C,O,S in Hp) were obtained by XPS to calculate ratio of biopolymers in the surface area to PCL.

RESULTS: All matrices consisted of fibers 0.3÷0.9 um, with a wall thickness of 150 ± 30 um. Concentration of GL in the surface area of 10% GL-PCL matrices was shown to be 25%, decreasing to 21% after washing in water and to 13,5% after proteinase K treatment, demonstrating lasting binding of GL with PCL. Matrices with 0,5% Hp slowly released up to 2% of the biopolymer during 48h, whereas in matrices with higher Hp content it was quickly released from the fibers: 90% of potentially released Hp exited the fibers in the first 30 min. XPS demonstrated complete removal of Hp from the surface of EP fibers after 48 hours of incubation in water.

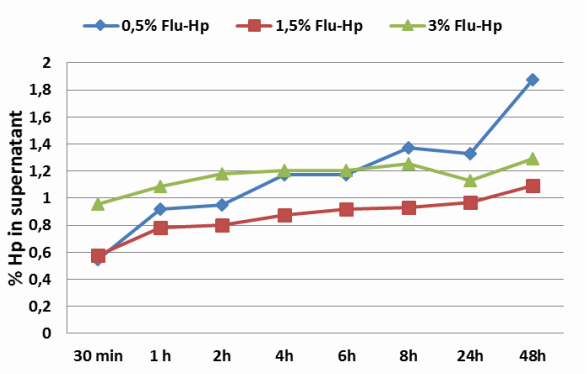

Fig. 1: Release of Flu-modified heparin from the matrices depending on the initial concentration and time of incubation.

DISCUSSION & CONCLUSIONS: The incorporation of GL into fibers was shown to increase tensile strength and biocompatibility of the matrices[2] whereas surface modification with Hp increased hemocompatibility of the materials[3]. Efficient and lasting exposure of GL at the fibers demonstrates the potentialities of this method to facilitate the anchoring of the cells. Complete release of Hp from the fibers demonstrates that simple introduction of Hp in starting solution can't be used to successfully produce hemocompatible matrices.

ACKNOWLEDGEMENTS: This work was supported by a Russian Science Foundation grant (№ 14-15-00493).

Site-specific immobilization of BMP-2 modified with an unnatural amino acid as a tool for improved regeneration of bone defects

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INTRODUCTION: The high regenerative potential of the bone tissue is strongly impaired in non-union bone defects. To support bone regeneration various scaffolds functionalized with osteogenic growth factors, mainly from the BMP family, have been developed. Standard approaches of scaffold functionalization lead to an initial burst release phenomenon, bone overgrowth, osteolysis and inflammation. To overcome these side effects we created a BMP-2 variant containing one unique non-canonical amino acid substitution created by genetic code expansion. Such a mutation creates a tool for a site-specific and covalent immobilization of BMP2-plk onto a solid scaffold. The advantages of this approach over other immobilization techniques are much lower and controlled scaffold loading, optimal orientation towards BMP2 receptors, preserved bioactivity and longer osteogenic activity of such modified scaffolds.

METHODS: A BMP2 mutant was cloned and expressed in E.coli in medium supplemented with a non-canonical amino acid. BMP-2 monomers were isolated from inclusion bodies and refolded to form biologically active dimers. Incorporation of the unnatural amino acid was confirmed by mass spectrometry. To verify biological activity of the mutant the interaction with BMP receptors was analyzed in *vitro* by SPR and in cell-based assays. The BMP2-plk mutant was successfully coupled to functionalized beads by a copper catalyzed azide-alkyne cycloaddition (CuAAC) either directly or via a short PEG linker.

RESULTS: The site-specifically coupled BMP2-plk variant was detected on the beads using fluorescent labelling and its interaction with main BMP receptor was positively verified. We showed that already sub-nanograms of BMP2-plk conjugated to beads were able to trigger differentiation of progenitor cells. While exposing the beads to myogenic progenitor cells, osteogenic differentiation appeared locally restricted to close proximity of the beads showing, that site-specifically coupled BMP2-plk was able to trigger differentiation of cells into osteoblastic lineage.

DISCUSSION & CONCLUSIONS: We developed a system which potentially eliminates the necessity of high-dose scaffold loading with BMP2. It additionally enables a favored orientation of the immobilized growth factor within the scaffold and provides a tight control over the saturation of scaffolds with BMP2. Biocompatible scaffolds functionalized with our method might be in the future used for bone regeneration in the region of cervical spine, which is extremely difficult to treat with the standard implantation technology which is based on free diffusion of BMP2 from the implanted scaffold.

ACKNOWLEDGMENTS: This project has been supported by the program BayernFIT.

The role of molecular mechanisms of the cell attachment and actin tension in human adipose stem cell differentiation

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INTRODUCTION: The cell adhesion, which is mediated primarily through integrins and focal adhesions (FA), is known to regulate differentiation of MSCs[1]. Focal adhesion kinase (FAK) is a central mediator of integrin-activated signalling. FAK is interconnected with various cellular signalling proteins including extracellular signal-regulated kinase (ERK) and cytoskeletal regulator, Rho- (Rho-associated protein kinase) ROCK[1]. However, the role and interplay of these molecular mechanisms in regulation of human adipose stem cells (hASC) differentiation is unclear. The aim of the present study was to assess the significance of FAK, ERK and ROCK signalling on the morphology, proliferation and differentiation of hASCs.

METHODS: Small molecule inhibitors targeted to above-mentioned signalling proteins were used to diminish the signal transduction in hASCs. The inhibitory effect of these molecules were analysed by Western Blotting (WB) and morphological impact by fluorescent phalloidin staining of actin cytoskeleton. The osteogenic differentiation was evaluated by alkaline phosphatase (ALP) activity and matrix mineralization assays. Adipogenic differentiation was analysed by assessing the fat vacuole formation with Oil red O staining, and the expression of adipogenic markers LEPTIN and aP2 by RT-PCR.

RESULTS: Our results indicated that the inhibition of FAK, ERK and ROCK function suppresses both cell proliferation and osteogenic differentiation, dose-dependently. Adipogenic differentiation was enhanced as a result of ROCK inhibition and this inhibition also modulated cell morphology leading to reduced tension of the actin cytoskeleton. Our WB results also suggest functional interplay between studied signalling proteins. FAK and ROCK inhibition modulated p-ERK1/2 levels and ERK inhibition diminished p-FAK levels.

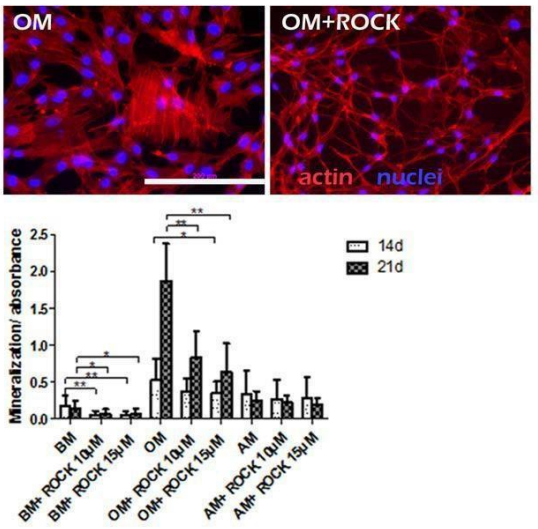

*Fig. 1: ROCK inhibition in hASCs induces morphological changes and reduced matrix mineralization. BM=basic medium; OM= osteogenic medium; AM=adipogenic medium scale bar =200µm; *p< 0.05; **p< 0.001*

DISCUSSION & CONCLUSIONS: These results suggest that the cell adhesion and morphology modulated by FAK, ERK and ROCK signalling are relevant regulators of the hASC differentiation. The functionality of these molecules is essential for the full osteogenic potential of hASCs. Their inhibition, on the other hand, supports adipogenic potential of hASCs.

ACKNOWLEDGEMENTS: The work was supported by TEKES, Jane and Aatos Erkko foundation and the Doctoral Programme in Biomedicine and Biotechnology, University of Tampere.

DISCLOSURES: Authors declare no conflict of interest.

New insight into the structure-property relationship of physically cross-linked elastin-like hydrogels

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INTRODUCTION: In recent years, numerous thermally gelling polymers have been developed due to their exceptional applications as injectable devices in tissue engineering and regenerative medicine. A large proportion of physically cross-linked hydrogels are those that are obtained from amphiphilic multiblock copolymers, which self-assemble into organized nanostructures because of the interaction of the hydrophobic blocks. In such way, elucidating the influence of the hydrophobic blocks is decisive to improve the properties of these smart devices.

This work is focused on amphiphilic multiblock elastin-like corecombinamers, which combine the stimuli-responsive behavior of the natural tropoelastin with the advantages of the recombinant materials [1]. Based on the ability of self-organization demonstrated by a previous amphiphilic tetrablock ELR [2], three new copolymers have been developed varying the individual block size of the original ELR.

METHODS: All the biopolymers were designed and constructed by recombinant DNA technology and produced by *Escherichia coli* fermentation. Nano and macroscopic properties of the different thermoresponsive recombinamers were studied by Cryo-TEM and Dynamic Light-Scattering (DLS) in order to compare the physically self-assembled nanostructures in a range of concentrations (1, 3, 5, 7, 10 mg/mL) in ultrapure water and phosphate buffered saline (PBS). The mechanical properties of the hydrogels were determined through rheological analysis in a controlled stress rheometer. Viscoelastic properties of the hydrogels were evaluated in the concentration range of 25-30 wt % as a function of temperature and time.

RESULTS: Dynamic Light-Scattering, Cryo-TEM and rheological analysis have revealed that the size proportion between the individual hydrophobic and hydrophilic blocks in amphiphilic self-assembled elastin-like block co-recombinamers is a factor that predetermines the supramolecular organization of the physical hydrogels and consequently their mechanical properties. Those

tetrablock recombinamers that maintain the proportion between the individual blocks self-assembled into more stable nanoparticles and into hydrogels with superior viscoelastic properties.

DISCUSSION & CONCLUSIONS: Injectable hydrogels as scaffold for tissue engineering have become increasingly studied. Mechanical properties as well as thermal behaviour are crucial parameters in the ability of a matrix to provide suitable environment for the accommodation of living cells, but the lack of structural studies hinder the production of these smart devices. In this work, we aim to shed light on the influence of the hydrophobic content and the physico-chemical properties of tetrablock amphiphilic elastin-like hydrogels, with the final goal of setting the basis to perform a rational design from the sequence level. Thus, increasing the hydrophobic content and maintaining the individual block proportion a new recombinant material with potential applications as injectable scaffold for tissue regeneration or as controlled drug delivery device in regenerative medicine has been developed with the ability to self-assemble into stable physical hydrogel with improved thermal behavior.

ACKNOWLEDGEMENTS: Authors would like to thank to the European Union for providing financial support through the European Social Fund (ESF), the European Regional Development Fund (ERDF) and THE GRAIL project. Also to other Spanish institutions, namely MINECO (PRI–PIBAR-2011–1403, MAT2012–38043, MAT2013–42473-R and MAT2013–41723-R), the JCyL (projects VA152A12, VA155A12 and VA313U14). Also to the CIBER-BBN, the JCyL and the Instituto de Salud Carlos III under the "Network Center of Regenerative Medicine and Cellular Therapy of Castilla and Leon".

Long-term degradation of brushite cements in three different liquids

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INTRODUCTION: Calcium phosphate cements (CPCs) can be used as bone void filling material as well as a part of fracture stabilization treatments. In order to determine when it is appropriate to use CPCs it is important to assess how their mechanical as well as chemical properties are affected by degradation. However, there is a lack of data on how the in vitro degradation affects brushite cements, and in particular high-strength such, over longer periods of time, i.e. longer than it takes for a bone fracture to heal. This study aimed at evaluating the long-term in vitro degradation of a high-strength brushite CPC in three different solutions: water, phosphate buffered saline (PBS) and a serum solution.

METHODS: Brushite cements were prepared in accordance with a previous study [1] The composition of the powder phase was a 45:55 molar ratio mixture between monocalcium phosphate monohydrate (< 75 µm) and beta tricalcium phosphate, with the addition of 1 wt% sodium pyrophosphate. A 0.5 M citric acid solution was used as liquid phase. The set cements were soaked in the three different solutions and the degradation was evaluated over 25 weeks. The porosity of the cements was assessed by micro computed tomography (micro-CT) and a solvent exchange method. The mechanical properties were studied by quasi-static compressive testing. The phase composition of the cements was evaluated by XRD and Rietveld refinement. The cements were also studied by SEM.

RESULTS The long-term degradation study showed that the loss in both volume and mass was the lowest for the specimens kept in serum solution, compared to those kept in water and PBS. The cements had an open porosity of approximately 13 % after they had set and following 25 weeks of degradation a significant increase in porosity could be noted for all solutions, on average 0.3-0.8 percentage points per week. A concomitant significant decrease in quasi-static compressive strength could be seen, from an initial strength of approximately 44 MPa with an average strength decrease of 0.8-1.0 MPa per week. The cements mainly contained brushite at the start of the study, but over time the phase composition changed, especially for specimens kept in PBS. Micro-CT and SEM revealed that a porous layer was formed on the surface of the specimens, see Figure 1, and XRD analysis revealed that this layer consisted of octacalcium phosphate.

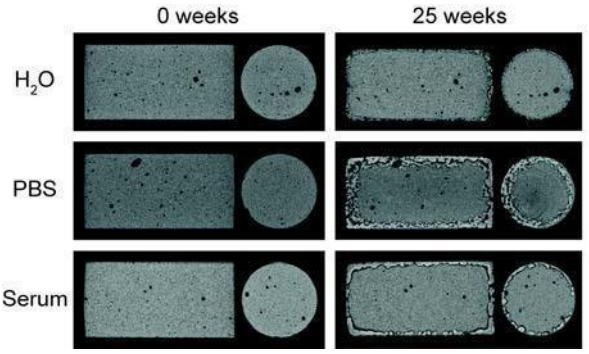

Fig. 1: Micro-CT cross sections of cements at start of the study and after 25 weeks degradation in water, PBS and a serum solution.

DISCUSSION & CONCLUSIONS: The porosity increase was lower than in a previous finding (on average 3.8 percentage points per week, as determined by helium pycnometry [2]) and by the end of the study, the strength of the cements was still higher than reported trabecular bone strengths (1-23 MPa) [3-5]. Micro-CT was found to be useful to assess the degradation of the cements in 3D, however, due to limitations in resolution, it was also important to complement this analysis technique with other porosity measurement methods. This study demonstrated the importance of performing long-term studies as important changes in the physical and chemical properties of the cements were observed after 10 weeks of incubation time.

ACKNOWLEDGEMENTS: The Swedish Research Council is gratefully acknowledged.

PHA-PLA blend wet spun scaffolds coated with ELP-REDV for bone tissue engineering

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INTRODUCTION: In the present study, wet spun scaffolds using poly(hydroxyalkanoate)-poly(lactic acid) (PHA-PLA) blends were prepared, coated with elastin-like proteins (ELP) carrying REDV amino acid sequences (attractive for endothelial cells) and studied *in vitro* for bone tissue engineering applications.

METHODS: PHA-PLA scaffolds were produced via wet spinning. Mechanical properties of the scaffolds were determined under compression, and their fiber morphology and porosity were studied using SEM and micro CT. The polymeric construct was coated with ELP by treating with oxygen plasma (50W, 2 min) and then dip coating in ELP solution. The attachment of the protein on the surfaces was studied with FTIR-ATR. AFM was used to study the surface roughness of the blend films. Finally, rabbit BMCs were seeded on the wet spun scaffolds. After 7 days, the scaffolds were introduced to a differentiation medium. Proliferation was evaluated using Alamar Blue Cell Viability Assay. Differentiation of cells was studied by scanning laser confocal microscopy (CLSM) after labelling for osteopontin.

RESULTS: Diameters of PHA-PLA fibers of the scaffolds were about 100 µm. µ-CT showed well defined, interconnected 3D structure with the closed porosity 0.03% and a total porosity of 78%.

Mechanical properties of PHA-PLA scaffolds were tested in dry state (n=6). Young's modulus was found to be 1.25±0.10 MPa indicating that PHA-PLA scaffolds produced by wet spinning were not mechanically strong and could only be used at non-load bearing areas and as a bone filler.

Oxygen plasma treatment was applied both to improve the hydrophilicity of the scaffolds and also for activation of the surface for ELP coating. Contact angles decreased from 83° to 63° after oxygen plasma treatment ($p \leq 0.05$). After plasma treatment, rms values of the surfaces showed that roughness of the surfaces of the untreated films (60.76 nm) was lower than the films treated with oxygen plasma (82.11 nm). Peak-Valley value of the films treated with oxygen plasma (463.00 nm) was significantly higher than untreated film (398.80 nm). These results showed that surface roughness of scaffolds increased upon oxygen plasma treatment.

The attachment of ELP-REDV was checked with FTIR-ATR searching for the presence of amide I (1640 cm^{-1})[4] and amide II (1550 cm^{-1})[4] peaks because neither PHA nor PLA have amine groups. The spectra showed the presence of a small amount of ELP-REDV.

Alamar Blue Assay showed that O_2 plasma treated scaffolds had higher cell proliferation than untreated and ELP coated.

Immunofluorescence revealed abundant cytoplasmic expression of Osteopontin osteogenic cultures after 28 days of induction for all scaffolds (*Figure 1*).

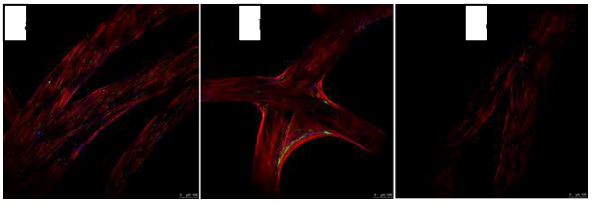

Fig. 1: Osteopontin immunofluorescence. a) untreated, b) O_2 plasma treated PHA-PLA scaffold, c) O_2 plasma treated and ELP coated PHA-PLA scaffold. Stains: osteopontin-blue, DRUQ5: nuclei-green, FTIC: actin-red.

DISCUSSION & CONCLUSIONS: The results indicate that wet spinning could be used to obtain highly porous fibrous scaffolds using PHA-PLA blends. Probably due to this high porosity the samples were suitable for use only in non-load bearing applications. Oxygen plasma modification increased the hydrophilicity and made them more suitable for cell growth. Cell proliferation trend on O_2 plasma treated scaffolds was lower than others starting from 14th day. Also, osteopontin labelling of 3 week culture in the differentiation medium exhibited expression of osteopontin supporting the osteogenic differentiation of the rabbit bone marrow cells.

Biocompatibility of magnetic core-shell nanocomposites for tissue engineering applications

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INTRODUCTION: Magnetic field-responsive engineered tissues are made of magnetic nanoparticles (MNPs) embedded in biopolymer matrixes. For large enough MNPs (*i.e.*, diameter ~ 50-100 nm), the mechanical properties of the tissues can be tuned in a reversible way by the application of external magnetic fields [1]. Here we report magnetic bio-artificial tissue-like constructs using nanocomposites consisting of a polymer core and a magnetic shell that could be used for the generation of tissue substitutes of the cornea, oral mucosa, cartilage or nerve.

METHODS: Polymer particles (diameter ~ 500 nm) were coated with a first layer of MNPs and a second layer of polyethylene glycol (PEG). Human fibroblasts were used to elaborate bioengineered human stromas based on fibrin-agarose (FA) hydrogels [2] containing the nanocomposites. The *in vivo* biocompatibility of constructs was studied by surgical implantation in the interscapulum of mice. *Ex vivo* and *in vivo* histological and histochemical analyses were conducted. Constructs were mechanically characterized by using a shear stress rheometer.

RESULTS: Perls' method showed that the core-shell nanocomposites appeared homogeneously distributed over the FA network (Fig.1a). Fibroblasts showed their characteristic elongated shape with large filopodia along the fibrin fibers with prominent cell nuclei. The *in vivo* studies showed that host cells were able to invade the implanted constructs (e.g. Fig.1). Nanocomposites appeared homogeneously distributed over the tissue. Histological analyses showed that there was only a localized and transitory acute inflammatory reaction (Fig.1), without affecting distal organs.

The storage modulus of the artificial tissues containing the nanocomposites twofold increased with respect to FA constructs without nanocomposites in the absence of field. The application of a magnetic field of 25.6 $kA \cdot m^{-1}$ resulted in an increase of G' of ~ 10%.

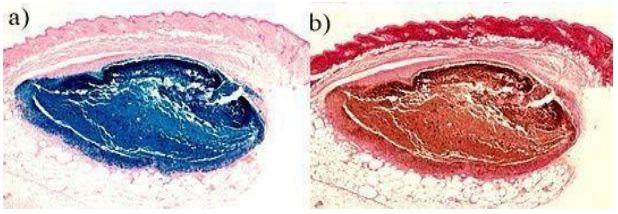

Fig. 1. Ex-vivo Perls' (A) and Picrosirius (B) staining of the constructs after 3 weeks.

DISCUSSION & CONCLUSIONS: We have generated magnetic field-responsive engineered tissues using core-shell nanocomposites loaded into FA hydrogels. The FA matrix promoted the interaction with the host tissue when the constructs were implanted *in vivo*. The implantation only resulted in a localized and transitory acute inflammatory reaction, without affecting distal organs. The mechanical properties of the constructs were enhanced by the application of a magnetic field. Finally, these novel magnetic tissue-like constructs could be used for several tissue engineering applications, including oral mucosa, cornea, cartilage or nerve substitutes.

ACKNOWLEDGEMENTS: This study was supported by the Spanish Plan Estatal de Investigación Científica, Desarrollo e Innovación Tecnológica (I+D+I) 2013-2016 from the Spanish Ministry of Economy and Competitiveness (Instituto de Salud Carlos III), grants FIS PI14/0955, FIS PI14/1343, FIS PI14/2110 and FIS PI15-2048 (co-financed by FEDER funds, European Union), by grants FIS2013-41821-R and FISPI14-1343 (Ministerio de Economía y Competitividad, Spain) and by Programs Contratos Puente and Fortalecimiento de Doctores (University of Granada, Spain).

Generation of bioactive SMARTiScaffolds by Wharton's jelly cell self-assembly

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INTRODUCTION: Several models of human oral mucosa have been developed by tissue engineering strategies using different types of biomaterials including collagen, chitosan, alginate, and fibrin, among others. In this context, Human Wharton Jelly Stem Cells (HWJSC) are especial stem cells in terms of proliferation, differentiation capability and regeneration potential of human tissues[1].

In this study, we have designed and elaborated a novel scaffold based on the capability of HWJSC cells to spontaneously synthesize a rich extracellular matrix (ECM) in which cells become self-assembled. This new smart cellular scaffold termed SMARTiScaffold should be highly biocompatible and may have great potential for clinical applications in cornea, palate and oral mucosa regeneration.

METHODS: First, human umbilical cords were obtained from full-term newborns with informed consent of the parents. Then, the Wharton's Jelly tissue was surgically dissected and sectioned into small pieces, which were then digested with type I collagenase and a trypsin-EDTA (0.5 g/L-0.2 g/L) solution. Once cell isolation was performed, HWJSC were cultured in high cell density using Amniomax cell culture medium during seven days until cells spontaneously formed cell-ECM rich SMARTiScaffold membranes in vitro. Then, HWJSC were grafted intramuscularly in Wistar laboratory rats to determine the in vivo biocompatibility and biosafety properties of this natural biomaterial. Histological evaluation of the SMARTiScaffold was carried out by hematoxylin-eosin (H&E) staining for tissue structure and morphology and by picrosirius red histochemistry for the analysis of collagen content.

RESULTS: First, the ex vivo analysis of the SMARTiScaffold bioartificial tissues using hematoxylin-eosin staining showed a regular biomaterial surface with abundant cells scattered throughout the membrane (Figure 1). When these tissues were grafted in vivo, numerous cells and well-oriented fibers were detected, and abundant blood vessels repopulated the membrane. The analysis of collagen content as determined by picrosirius red histochemistry showed a large amount of collagen fibers in the SMARTiScaffold membranes kept ex vivo and grafted in vivo, although orientation of the fibers was much more evident after in vivo grafting (Figure 1).

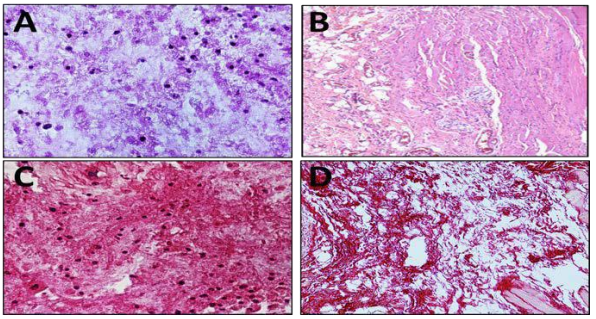

Figure 1: Histological analysis of the SMARTiScaffold membranes generated ex vivo (A and C) and grafted in vivo (B and D) stained with H&E (A and B) and picrosirius (C and D).

DISCUSSION & CONCLUSIONS: HWJSC have been previously used for numerous tissue engineering applications with promising results [2]. Here, for the first time, we uses these cells in the form of SMARTiScaffold membranes synthetized by the own cells, and results demonstrate that these biomembranes can be easily grafted in vivo and show bioactive remodelation and maturation properties. These results support the use of these bioartificial tissues for tissue engineering purposes.

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ACKNOWLEDGEMENTS: This study was supported by the Spanish Plan Estatal de Investigación Científica, Desarrollo e Innovación Tecnológica (I+D+I) 2013-2016 from the Spanish Ministry of Economy and Competitiveness (Instituto de Salud Carlos III), grants FIS PI14/0955, FIS PI14/1343, FIS PI14/2110 and FIS PI15-2048 (co-financed by FEDER funds, European Union).

strontium and zinc based phosphate glass beads as osteogenic tissue engineering material

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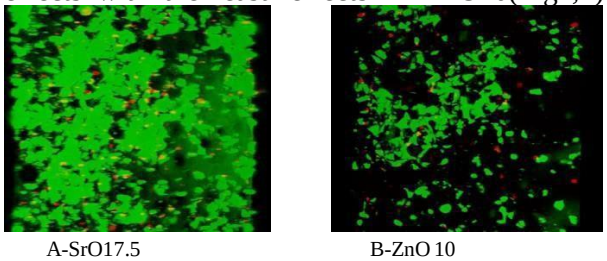
INTRODUCTION: Degraded phosphate-based glass beads with the formula of (P_2O_5)–(Na_2O)–(TiO_2)–(CaO)–(SrO) or (ZnO) have developed to examine its ability to work as scaffold material for bone repair. These were made in different compositions according to previous studied data .These were furtherly studied in term of cell culture aspect. This has involved some of their biocompatible charact-eristics by using MG63 osteoblast like cells. **METHODS:** Four compositions of glass beads were prepared by melt –quench technique followed by spherionization which were identified according to the percentage of calcium oxide mole replacement with either zinc or strontium oxides. These glass compositions were SrO17.5%, SrO35%, ZnO5 % and ZnO10% where the glass composition was ($50P_2O_5$ - $10Na_2O$ - $5TiO_2$ –(35-x)CaO – x (SrO or ZnO)), in which x was the percentage of zinc and strontium oxides . These glass compositions were studied according to different chemical and physical aspects, followed by biological effects on Mg63 by doing CCK assay and live and dead stain and SEM pictures, all of these were made in three time points which were days 1,4,7. Glass beads were weighted to made 0.5mm height multilayers scaffold on 6.5mm polyster membrane transwell in 24 well plates flask. **RESULTS:** Both CCk essay data and Live& Dead 3D confocal images showed that all the glass compositions displayed part of biocompatibility effects with the least effects in ZnO10(Fig1,2)



A-SrO17.5 B-ZnO 10

Fig1: Confocal images for two different multilayer glass beads in day7

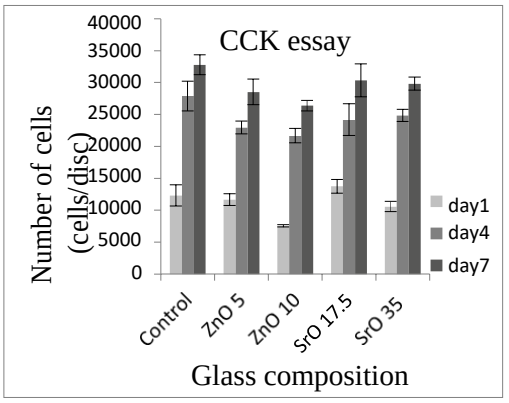


Fig2: CCK assays that shows cell number estimation per each glass disc composition

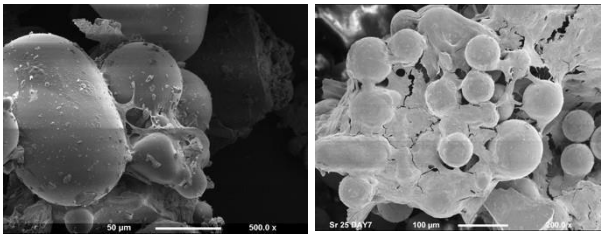


Fig 3: SEM image show the attachment of MG63 cells on glass beads

DISCUSSION & CONCLUSIONS: The aim of this study was development of phosphate glass beads(Fig 3) that may be able to work as osteoconductive material. This was already preceded by previous studies on glass discs. Glass beads preparation was successful as a step to make glass in suitable features that can be used suitably as scaffold material. All the glass beads showed acceptable results on cell number proliferation in comparison to the control group. However, ZnO10 showed slightly decrease in cell number which may be due to the cytotoxic effects of high zinc percentage as it was shown in previous studies.

ACKNOWLEDGEMENTS: This study was funded by the Higher Committee of Education Development in Iraq.

Bioactive peptides may be covalently coupled to functionalised hydroxyapatite

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INTRODUCTION: Bone tissue loss often results in reduced quality of life, pain, poor aesthetics and impaired functionality for millions of people throughout the world. Surgeons use a variety of alloplastic biomaterials as bone graft substitutes to regenerate tissues and so restore function and health. Calcium phosphates such as hydroxyapatite (HAP) are arguably the most successful group of bone graft substitutes, but clinical performance is more limited in ageing or otherwise compromised patients [1]. While some progress has been reported in the field of orthobiologics (e.g. the use of bone morphogenic proteins), this approach is both expensive and faces a more complex regulatory pathway [2]. New strategies are therefore being investigated to enhance bone tissue regeneration without the costs and risks associated with orthobiologic substances, with one promising route described here being the coupling of bio-functional peptides to implant surfaces.

METHODS: Sintered HAP discs were produced via gelcasting and sintering at 1160°C. The surface was then functionalised using acrylic acid plasma polymerisation. The carboxyl groups generated at the surface were then activated for peptide attachment using carbodiimide hydrochloride and n-hydroxysuccinimide (EDC/NHS). Finally the HAP discs were exposed to a heparin binding peptide KRSR [3]. X-ray photoelectron spectroscopy (XPS) was used for surface characterisation at each stage of treatment. The release profile of the KRSR peptide was studied in water for 14 days and analysed using reverse-phase high performance liquid chromatography (HPLC).

RESULTS: When analysed using XPS, survey scans from the sintered gelcast HAP discs showed a calcium/phosphate ratio of ~1.63 which was close to stoichiometric HAP (1.67). The XPS survey scans also indicated only very low carbon contamination at the surface of ~ 7 at%. Following plasma polymerisation, only carbon and oxygen were detected at the surface of the HAP scaffolds, indicating successful generation of the desired carboxyl functional groups. After full treatment XPS survey scans detected ~7 at% nitrogen at the HAP surface indicating the presence of peptides and therefore successful functionalisation. Fig. 1 summarises some results from the releasestudies, which showed peptide retention was significantly improved on fully treated HAP samples when compared to controls.

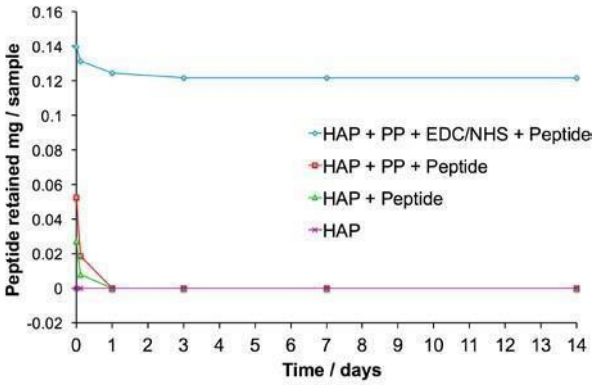

Fig. 1: Graph indicating the amount of peptide retained at the surface of HAP scaffolds, partially or fully functionalised, in the presence of water for 14 days.

DISCUSSION & CONCLUSIONS: The results indicated that the gelcasting and sintering procedures used here were successful in generating near pure HAP scaffolds. As seen from the XPS and HPLC results, the plasma polymerisation process successfully generated functional groups that were then used to couple peptides to the surface of the HAP scaffolds. Further work is underway to determine the biocompatibility and bioactivity of the functionalised HAP. It was concluded that peptide coupling provided a highly promising route to produce bio-functional modifications to calcium phosphate biomaterials.

ACKNOWLEDGEMENTS: The authors would like to thank JRI Orthopaedics Ltd, Ceramisys Ltd and the EPSRC (White Rose DTC in Tissue Engineering & Regenerative Medicine) for providing financial support for this project. In addition, the authors would also like to thank the National EPSRC XPS User's Service at Newcastle University.

Tuning mechanical features of chitosan-gelatin scaffolds to enhance differential biological responses of dental pulp stem cells

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INTRODUCTION: Chitosan-gelatin (Ch-Gel) scaffolds have been already proposed for several biomedical applications, including cartilage, skin and peripheral nerve regeneration [1, 2]. Aim of this study was to evaluate attachment, viability, and osteo/odontogenic differentiation potential of Dental Pulp Stem Cells (DPSCs) seeded in Ch-Gel scaffolds crosslinked with various concentrations of glutaraldehyde (GTA), to assess ideal scaffold properties for targeted dentin/bone regeneration.

METHODS: Scaffolds were prepared by dissolving 2% (w/v) chitosan in a 1% (v/v) acetic acid and 2% (w/v) gelatin in water at 50 ^{o}C. Solutions were poured at 40%-60% Ch-Gel and stirred for 2 h at 50 ^{o}C. GTA was added at either 0.1% (GTA-0.1) or 1% (GTA-1) (w/v) under agitation at 50 ^{o}C until gelation and then cast into 24-well plates and lyophilized for 24 h at -20 ^{o}C. Scaffolds were neutralized with 0.1 M NaOH, followed by washing steps with ultrapure water, PBS and culture medium. DPSC cultures were established from healthy donors and characterized with flow cytometry [3]. Scaffolds were spotted with 10^6 DPSCs in 100 µl Complete Culture Medium (=CCM: a-MEM with 15% FBS, 100 mM L-ascorbic acid and antibiotics/antimycotics), incubated for 2 h at 37 ^{o}C and 5% CO_2 and then fully covered with CCM. Scanning Electron Microscopy-SEM, live/dead staining (Calcein AM/Eth1) and MTT assays were used to evaluate cell attachment and viability; Real time PCR for expression of osteo/odontogenic markers (BSP, DSPP, BMP-2, ALP, Osterix, Runx2 and BGLAP, and B2M and SDHA as housekeeping controls).

RESULTS: Stable 40%-60% Ch-Gel scaffolds with interconnective pores with average size of 100 µm were prepared. Higher degradation rate was recorded for cell-free GTA-0.1 scaffolds (44 %) as compared to GTA-1 (17% after 15 d). Both GTA-0.1 and GTA-1 scaffolds supported initial cell adhesion and spreading inside their structure. Cell viability was initially (up to 72 h) higher for GTA-0.1 compared to GTA-1 scaffolds, as shown by MTT assays. Live/dead staining confirmed that the majority of cells (> 90%) remained viable throughout the entire culture period in both types of scaffolds. Real time PCR revealed pronounced upregulation of mineralization markers in GTA-0.1 scaffolds at day 7 (ALP: 3.5-, BMP-2: 5.2-, DSPP: 25.2-, BSP: 36.1-, Osterix: 36.4-, RunX2: 2.1-, BGLAP: 5.2-fold), followed by significant downregulation at day 14. In contrast, GTA-1 scaffolds showed much lower but sustained over time expression of DSPP (up to 2-fold), BSP (5.5-fold) and Osterix (2.5-fold), while expression of BMP-2 (3.2-fold), ALP (3.7-fold) and BGLAP (6.3-fold) was significantly higher compared to GTA-0.1 scaffolds only at day 14.

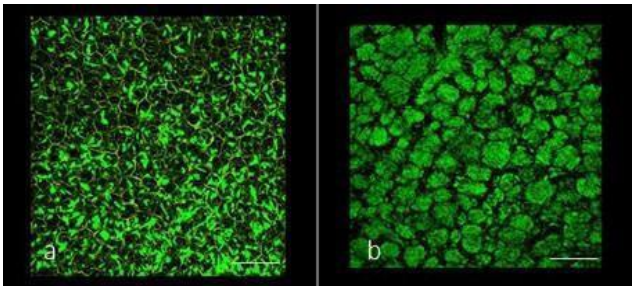

Fig. 1: Confocal microscopy images (live/dead staining) of DPSCs seeded in GTA-1 (left) and GTA-0.1 (right) Ch-Gel scaffolds after 72h.

DISCUSSION & CONCLUSIONS: Ch-Gel hybrid scaffolds supported attachment, viability and osteo/odontogenic differentiation of DPSCs. Lower GTA concentration (0.1% w/v) enhanced - despite higher degradation rates- biological responses, revealing that "fine tuning" of the scaffold mechanical properties can be critical for targeted mineralized tissue (dentin/bone) engineering.

ACKNOWLEDGEMENTS: This study was funded by the Greek General Secretariat for Research and Technology grant Aristeia II 'Osteobiomimesis 3438', the European Union(EU) and National Resources.

Development, characterization and biological properties of nano-hydroxyapatite scaffolds for bone engineering

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INTRODUCTION: Bone tissue has a good regeneration capacity. However, larger defects may appear as result of trauma, infections or tumors. In this case, the native regeneration capacity of bone tissue may be compromised. Different bone graft substitutes have been used for decades to replace and/or regenerate bone tissue. Ceramic materials are among the most widely used for bone regeneration as they feature osteoconductive properties. In addition, their composition may be tailored to mimic the mineral composition of the native bone. Currently, great efforts are being done on increasing the porosity of ceramic scaffolds. A porous scaffold with interconnected porosity is desired in order to increase osteoconductivity, cell migration and cell-cell interaction among others. Therefore, the main aim of our study is the development of biocompatible, highly porous 3D scaffolds using hydroxyapatite (HA) nanoparticles.

METHODS: HA nanoparticles were synthesized using $Ca(OH)_2$ and H_3PO_4 as precursors for calcium and phosphorous. The HA nanoparticles were characterized using FTIR, FE-SEM, DRX and BET superficial area. Subsequently, the nanoHA scaffolds were prepared by means of foam replication method [1]. In this process, polymeric foam is commonly used as a template and dipped into bioceramic slurry followed by drying and sintering. In our study, the effect of different solid contents (20-40 wt.%) of the slurry and of the sintering temperature (800-1200ºC) on the grain growth of the HA nanoparticles and nanoHA scaffold porosity were studied. The porosity of the different nanoHA scaffolds obtained was evaluated by µCT.

The biocompatibility of the nanoHA scaffolds was evaluated *in vitro* by culturing human adipose mesenchymal stem cells (AMSCs) for a period up to 14 days. Cell viability was evaluated by MTT and LDH. Cell proliferation was evaluated by PicoGreen assay. Experiments were performed in triplicate.

RESULTS: The HA nanoparticles showed needle-like morphology. The size of the needles was approximately 200 nm and the superficial area was 93.0 m^2/g. FTIR and DRX showed characteristic HA bands and peaks, respectively. All nanoHA scaffolds obtained showed high porosity and good interconnectivity of pores. The total porosity of the scaffolds as determined by µCT was in the range of 74-87%. The porous diameter for the scaffolds with 20% HA was 458 ± 70 µm. By increasing the HA content, a significant decrease in the porosity was observed. *In vitro* studies showed good cell viability and proliferation for all nanoHA scaffolds tested. The proliferation of AMSCs seeded on the scaffolds increased up to 14 days, with clear preference for the nanoHA with higher porosity.

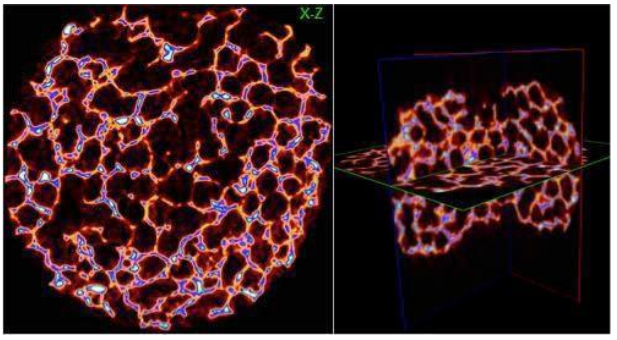

Fig. 1: Representative µCT image of a nanoHA scaffold (20%HA, produced at 1150ºC sintering temperature).

DISCUSSION & CONCLUSIONS: highly porous and biocompatible nanoHA scaffolds were successfully fabricated. The porosity of the scaffolds was greatly influenced by the density of ceramic slurry and sintering temperature. An increase in the HA content in the ceramic slurry enhanced the strength of the scaffold and resulted in smaller pore size.

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ACKNOWLEDGEMENTS: LM thanks the University of Guadalajara and FAPERJ/Brazil for funding her research.

Biodegradable ureteral stents for the treatment of urothelial tumors of the upper urinary tract cancer

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INTRODUCTION: Biodegradable ureteral stents have proven to be a useful alternative to conventional stents. Furthermore, they can be functionalized as drug eluting stents, especially for urothelial tumors of the upper urinary tract. In these cases, the conventional method of drug administration is via drug instillation. This has several drawbacks, such as high concentration dosage, increased side effects, short residence time and low bioavailability [1]. To avoid these problems, biodegradable ureteral stents impregnated by supercritical fluid CO_2 (SCF) with four different anti-cancer drugs were produced.

METHODS: Biodegradable ureteral stents were produced according previous work [2]**.** Four types of drug-eluting biodegradable stents based on natural polymers were prepared. The stents were impregnated by SCF (40°C, 100 bar, 90 min), with paclitaxel, epirubicin, doxorubicin and gemcitabine. The release kinetics of the impregnated drugs from the anti-cancer drug-eluting stents was evaluated in artificial urine solution (AUS) for 9 days. The anti-tumoral effect of the developed stents in transitional cell carcinoma (T24) and HUVEC primary cells, used as control, was evaluated.

RESULTS: The *in vitro* release study in AUS showed a sustainable release in the first 72h for the four drugs impregnated, after this time a plateau was achieved and finally the stent degraded after 9 days. To determine the sensitivities to each drug (IC50), a T24 cancer cell line was exposed to graded concentrations (0.01 to 2000 ng/ml) of the four drugs for both 4h and 72h. Additionally, toxicity as a result of both direct and indirect contact of the cell lines with the different material conditions of biodegradable stent were studied. The four anti-cancer drugs studied showed a concentration-dependent inhibitory effect on the T24 and (control) HUVEC cell lines with IC50's for paclitaxel of 7.30 ng and 501.50 ng, respectively. The T24 cancer cell line was shown to be more sensitive than the control HUVEC cell line for all the anti-cancer drugs tested.

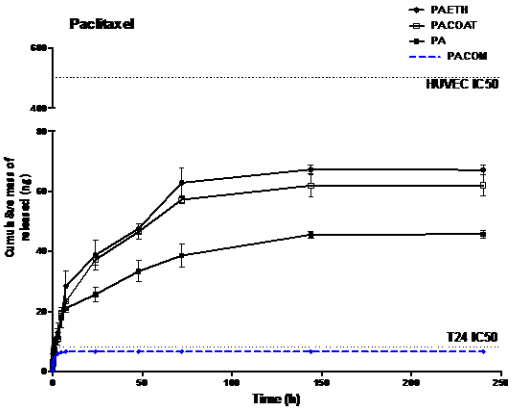

Fig. 1: *Release profile of the different stents impregnated with paclitaxel. Comparison with IC50 concentration for T24 and HUVEC cells.*

DISCUSSION & CONCLUSIONS: The direct and indirect contact of the anti-cancer biodegradable stents with the T24 and HUVEC cells confirm the anti-cancer effect of the stents impregnated with the four anti-cancer drugs, reducing approximately 75% of the viability of the T24 cell line after 72h and no negative effect in the HUVEC cells for the total drug release from stents. This study has thus shown the efficacy of the anti-cancer drug eluting biodegradable stents *in vitro* for the T24 cell line, with no toxicity observed in non-cancerous HUVEC cells.

ACKNOWLEDGEMENTS: Healy lab for the work was performed at UC Berkeley. FLAD Grant for internship in UC Berkeley 2015/CON5/CAN8. FCT doctoral grant SFRH/BD/97203/2013. APU Grant Jaba Recordati 2015.

The fabrication of synthetic three dimensional niche environments designed to modify *in vitro* stem cell behaviour.

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INTRODUCTION: Tissue engineering research commonly utilises 3D scaffolds as a supportive environment in cell culture, and several have been translated into clinical use. The majority of existing 3D scaffolds are homogenous, with relatively little attention directed at the preparation of controlled topographies that have the potential to modify cell behaviour. Researchers at Sheffield recently described the fabrication of a 3D synthetic stem cell niche environment for delivery of limbal stem cells in opthalmology, demonstrating the significant therapeutic potential of this technology [1]. There are numerous other stem and stromal cells with clinical value, and the aim of this research was therefore to investigate the preparation of 3D micrometer-scale features in electrospun scaffolds using metallic custom templates prepared using additive manufacturing. The organisation of fibres into random or aligned structures was also investigated [2].

METHODS: Polycaprolactone (PCL) was chosen as a scaffold material as it is a slowly degrading polymer with good biocompatibility [3]. PCL (10% w/v) was dissolved in a solvent mixture based on dichloromethane (DCM 92.66 wt%) and dimethylformamide (DMF 7.34wt%), and this was stirred until a uniform dope was produced (> 4 hours). The solution was electrospun onto a flat collector to produce random fibres or a rotating drum to produce aligned fibres. Custom templates made of commercially pure titanium were attached to the flat collector by carbon tabs to spin scaffolds with different structural features. All scaffolds were spun at an infuse rate of 2.5 with a 1 ml plastic syringe and a needle using a total of 2 m of the solution. The scaffolds were characterised using scanning electron microscopy (SEM), and preliminary *in vitro* biocompatibility experiments were performed using a range of stem and progenitor cells (mesenchymal stromal cells and α-tanycytes).

RESULTS: Scaffolds were produced with different fibre orientation and/or micron-scale topographical features. The structural organisation of the fibres was investigated using SEM (Fig 1.). The metallic templates successfully generated reproducible features within the 3D scaffold (Fig 1 c-e), and the microenvironments thus created have the potential to act as niches to modify cell behaviour. Preliminary studies of mechanical properties and *in vitro* biocompatibility are ongoing, but initial results are encouraging.

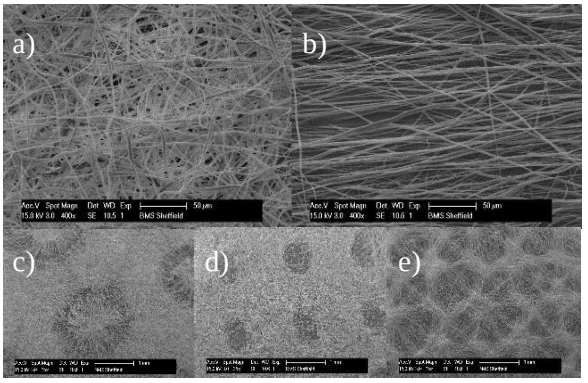

Fig. 1: SEM images of 10% PCL scaffolds – a) random b) aligned c) template 1 scaffold d) template 2 scaffold e) template 3 scaffold

DISCUSSION & CONCLUSIONS: Electrospun scaffolds were successfully fabricated using a custom metallic target to generate reproducible 3D features. These novel scaffolds have considerable potential as research tools to study and control stem cell behaviour *in vitro*, and ultimately they could be translated for clinical use in a variety of regenerative applications.

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ACKNOWLEDGEMENTS: This work is associated with MeDe Innovation (the UK EPSRC Centre for Innovative Manufacturing in Medical Devices, grant number EP/K029592/1).

Collagen scaffolds with tailored pore geometry for building 3-dimensional vascular networks

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INTRODUCTION: Vascularisation is critical for the survival of thick engineered tissues. In this context, co-cultures of endothelial cells (EC) and osteoblasts (OB) within scaffolds with customised pore architecture were used to create capillary-like networks and to study the spatial aspects of micro-vascular assembly and lumen formation.

METHODS: Scaffolds with tailored pore geometries were produced by manipulation of the freeze-drying conditions [1,2] and characterised by scanning electron microscopy and X-ray computed tomography. Endothelial cell 3D organization was assessed by histology and immunohistochemistry.

RESULTS: *Figure 1* shows scaffolds with isotropic (randomly aligned) and anisotropic (uniaxially aligned) pore geometries. EC seeded on anisotropic scaffolds infiltrated deeper than cells seeded on isotropic ones - see *Figure 2*.

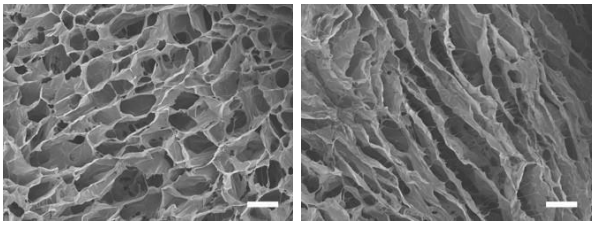

Fig.1: Scanning electron microscopy micrographs of the longitudinal sections of isotropic (left) and anisotropic (right) scaffolds. Scale bars = 200 µm.

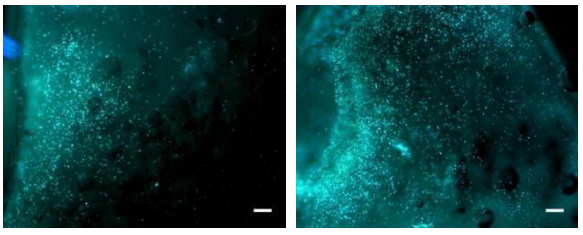

Fig.2: ECs migration into isotropic (left) and anisotropic (right) scaffolds after 1 day in culture. Cell nuclei stained in blue. Scale bars = 200 µm.

Figure 3 demonstrates that endothelial cells retained cobblestone morphology when kept in monoculture. The presence of osteoblasts promoted organisation of the cells into elongated multinucleated structures on top of the scaffolds. Endothelial cells cultured within anisotropic scaffolds formed capillary-like structures around the pore walls. Co-culture conditions improved this self-organisation significantly.

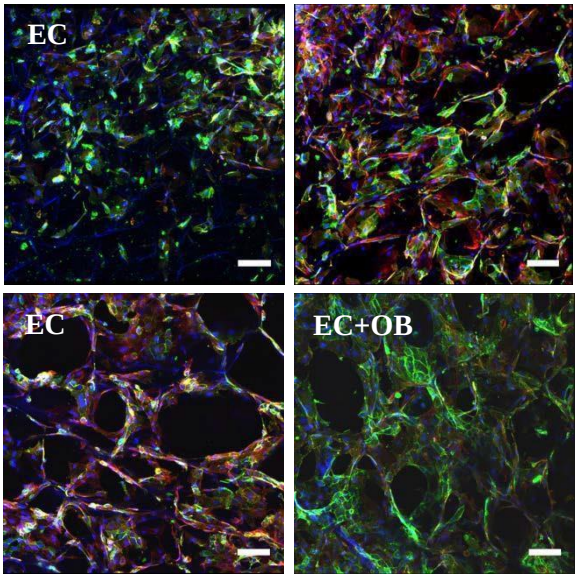

Fig.3: Endothelial cells seeded alone or with osteoblasts on isotropic (upper) and anisotropic (lower) scaffolds after 14 days in culture. EC marker CD31 is shown in green, actin in red and nuclei in blue. Scale bars = 100 µm.

DISCUSSION & CONCLUSIONS: We have demonstrated for the first time that scaffold pore architecture significantly affects the invasion, morphology and organization of the endothelial cells into capillary-like networks, in both mono- and co-cultures. Our studies suggest that deeper cell penetration, along with more efficient medium perfusion within the anisotropic scaffolds may account for these findings. The exact mechanism and conditions for optimal 3D capillary network formation have yet to be established.

ACKNOWLEDGEMENTS: SB is supported by the Blavatnik Family Foundation. RAB gratefully acknowledges funding from the NIHR.

Development of hyaluronic acid hydrogels with possibility of non-invasive monitoring of their degradation by MRI

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INTRODUCTION: Injectable hydrogels have found extensive use in tissue engineering and regenerative medicine. Unfortunately, they lack the possibility of following their degradation pathway in vivo after implantation. Our goal was to prepare injectable hyaluronic acid (HA) hydrogel with possibility to track it in vivo in non-invasive manner. For that purpose, HA hydrogel with covalently linked gadolinium complex was prepared in situ. This allowed longitudinal monitoring of its degradation by magnetic resonance imaging (MRI).

METHODS: Two types of hyaluronic acid (HA) derivatives have been used to prepare labelled hydrogels. The first one contained hydrazide and thiol groups [1]. DTPA gadolinium complex was subsequently linked through thiol groups. The second HA derivative contained aldehyde groups [2]. Hydrazone hydrogels were prepared by mixing of aqueous solutions of the two derivatives.

RESULTS: DTPA gadolinium complex has been successfully modified with "clickable" dithiopyridyl groups. Conjugation of the gadolinium complex was performed through thiol-disulfide exchange reaction with HA containing thiol and hydrazide groups. The obtained Gd^{3+}-labelled HA derivative bearing hydrazide groups was cross-linked after addition of aldehyde containing HA. Figure 1 shows MRI images of the labelled hydrogels (derived from Gd^{3+}-labelled HA-hydrazide derivative) and non-labelled hydrogels (derived from HA-hydrazide derivative).

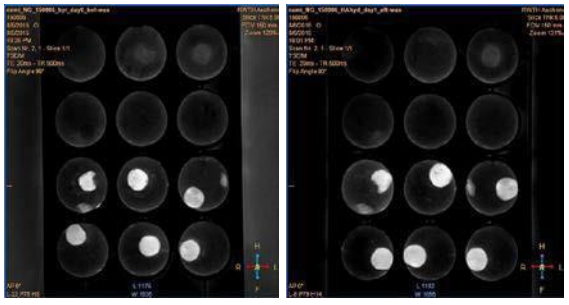

Fig. 1:T1-weighted images of Gd-labelled HA hydrogels (two lower rows) and non-labelled hydrogels (two upper rows) before (left image) and after (right image) swelling.

The images were taken after hydrogels setting as well as after the hydrogels swelling in PBS to remove all compounds that were non-linked to the matrix. We observed that MRI images of the labelled hydrogels were not changed after washing step which proved that gadolinium complex was covalently linked to the matrix. In vitro enzymatic degradation of hydrogels (using hyaluronidase) was followed by non-invasive MRI technique (Figure 2). The degradation was complete in three days and the final compositions after degradation showed no MR image.

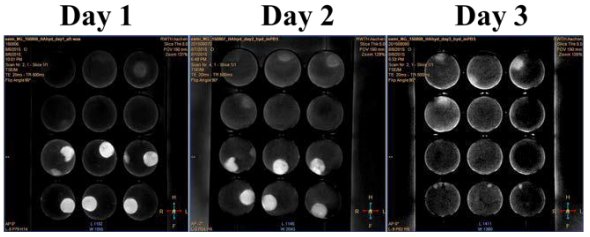

Fig. 2: MRI images of non-labelled (two upper rows) and Gd-labelled HA hydrogels (two lower rows) taken at different time points of the hydrogels degradation by hyaluronidase.

DISCUSSION & CONCLUSIONS: It was demonstrated that in vitro degradation of injectable gadolinium labelled HA hydrogels can be followed by MRI. This observation indicates that the developed gadolinium labelled hydrogels can be applied for longitudinal tracking in vivo.

ACKNOWLEDGEMENTS: This research was funded by the European Commission via the Marie Curie Initial Training Network (ITN) iTERM (Image materials for Tissue Engineering and Regenerative Medicine) Grant agreement no.:607868.

Non-mulberry silk fibroin grafted poly (Є-caprolactone)/nano hydroxyapatite nanofibrous scaffold for osteoconduction and osseointegration

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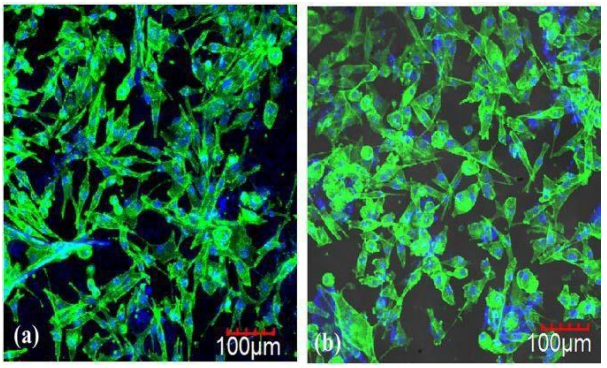
INTRODUCTION: Nanofibrous scaffolds of mulberry silk fibroin are widely accepted as bone scaffolds. Nonmulberry silk protein fibroin of *Antheraea mylitta* has better mechanical properties over mulberry silk with improved cell proliferation due to built-in RGD sequences in fibroin, which could unveil new horizons for its application in regenerative medicine[1]. This study aims at developing biodegradable, mineralized, nanofibrous scaffolds for use in bone regeneration.

METHODS: Nano-hydroxyapatite (nHAp) was synthesized by precipitation reaction. To prepare nHAp doped poly (Є-caprolactone) (PCL)(reported as osteogenic material) nanofibrous scaffold, a defined amount of nHAp (25wt%and 50wt %) was suspended in chloroform by ultra-sonication and vigorous stirring before adding to the 10 wt% polymer solution prepared in chloroform. Dioctyl sulfosuccinate sodium salt served as a surfactant for stabilizing the suspended nHAp in the polymer solution. E-spinning was carried out using at 11 kV (DC) supply with flow rate set at 1 ml/h at 30°C maintaining 15 cm distance from the capillary tube to collector. These matrices were stripped off the aluminum foils upon reaching a thickness of about 1 mm. Immobilization of 2 wt% non-mulberry silk fibroin (NSF) on fabricated nHAp doped PCL nanofibrous scaffold was carried out using aminolysis[2]. After grafting of protein the matrices were finally dried off and treated with ethanol for a couple of minutes before carrying out further studies. The NSF grafted PCL nanofibrous matrix (without nHAp) was also prepared to act as a control.

RESULTS: The nHAp particles showed rod like characteristics with length and width of ~70 and 24 nm, respectively. Analysis of FESEM images using Image J®, diameter of the different nanofibrous scaffold were estimated to be 456nm (NSF-PCL/n25) and 462 nm (NSF-PCL/n50). As per expectation, adding nHAp slightly increased the diameter of fibers. The TEM micrographs showed the nHAp particles evenly distributed in the polymer fibers. XRD, FTIR and TGA analysis revealed the effective blending of nHAp and grafting of NSF on nanofibrous matrix. The results of contact angle measurement showed that incorporation of nHAp reduced the hydrophobicity. Mechanical strength of the nanofibrous matrices was increased with addition of nHAp; NSF-PCL/n25 (16.06 MPa, 168.42%) exhibited greater strength over NSF-PCL/50 (12.7 MPa, 159.03%). Confocal microscopy (Fig.1) of the constructs after 7days *in vitro* culture exhibited more intensive and homogenous distribution of actin filaments for nHAp doped scaffolds along with better ALP activity and promoted significant expression of genes related to bone growth over 2 weeks of cell culture.

Fig. 1: *The actin organization of MG-63 cells grown on (a) NSF-PCL/n25 and (b) NSF-PCL/n50 nanofibrous scaffolds at day-point 7.*



DISCUSSION & CONCLUSIONS: The results indicate that incorporation of nHAp on NSF grafted PCL nanofibrous matrix are suitable as scaffold for bone tissue engineering; while the matrix with a high nHAp loading density is found to be weaker than one with low nHAp loading density due to reduction of dissipate energy of nHAp particles. The current *in vitro* study serves as a starting point for design and testing of clinically relevant orthopedic grafts *in vivo*.

ACKNOWLEDGEMENTS: Supported by DBT and ICMR, Govt. of India.

one-pot synthesis of α,β-unsaturated polyaldehyde of chondroitin sulfate

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INTRODUCTION: There is an increasing need for *in situ* crosslinkable materials driven by the applications in tissue engineering and regenerative medicine. Both synthetic and natural polymers are available, but the use of natural polymers especially polysaccharides is more attractive because of biocompatibility issues, cost-effective, eco-friendly production and ease of chemical modification. One of such advantageous biopolymers is chondroitin sulfate (CS), a glycosaminoglycan, highly abundant in the extracellular matrix of all vertebrates [1,2]. In this study, one-pot synthetic strategy as well as a novel derivative of CS, namely α,β-unsaturated aldehyde, were invented [1,2]. The utility of this valuable precursor for both pH-responsive conjugation and chemical crosslinking was demonstrated by several model compounds under physiological conditions.

METHODS: Chondroitin sulfate sodium salt (Mw = 47 kDa) was isolated from bovine trachea and purchased from Aldrich as a mixture of chondroitin sulfate A (C4S) and chondroitin sulfate C (C6S) in relative ratio 3/2. A co-oxidation approach based on 4-Ac-Tempo/NaOCl/NaOBr was used for chemical modification of CS. A degree of substitution and structural analysis was determined by means of solution-state NMR spectroscopy carried out on a Bruker Avance III 500 MHz. The surface morphology of the freeze-dried gels was analysed by scanning electron microscope Zeiss Ultra Plus using SE2 detector. Fibroblast viability in the presence of α,β-unsaturated aldehyde of CS with a degree of substitution 20% was tested in 1000, 500, 100 and 10 μg mL^{-1} range of concentration and evaluated spectroscopically by means of MTT assay in 24, 48 and 72 hours.

RESULTS: CS was successfully modified to α,β-unsaturated aldehyde in one-pot (Fig. 1). The derivative was treated with various N-nucleophiles to demonstrate pH responsive bonding to a polymer chain. The chemical crosslinking with biocompatible hexanedihydrazide allowed rapid sol-gel transition under physiological conditions (Fig. 2). The obtained hydrogels were stable in PBS and possessed a well-organized, interconnected, porous structure with average pore diameter 68.5 μm.

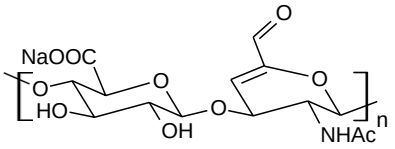

Fig. 1: The structure of α,β-unsaturated aldehyde of CS.

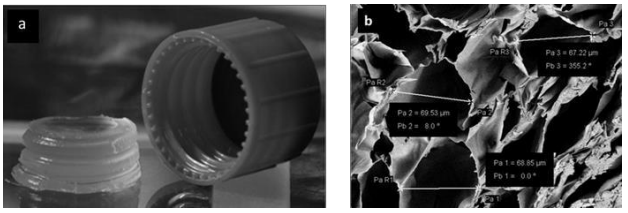

Fig. 2: The crosslinking of α,β-unsaturated aldehyde of CS with hexanedihydrazide: (a) a moulded hydrogel, (b) SEM cross-section view of a freeze-dried sample with a porous structure.

DISCUSSION & CONCLUSIONS: It is possible to synthetize α,β-unsaturated aldehyde of CS in one-pot, thanks to its structural benefits [1,2]. A big advantage of this approach, compared to those in state of the art [3], is addressed to one-pot set up and to a higher degree of substitution. In addition, there is no need for an elimination agent, an organic solvent, increased reaction temperature and isolation of an intermediate - saturated C-6 aldehyde in the process. All above mentioned attributes are very important for cost-effective technology. The derivative readily underwent crosslinking and the hydrogels might be considered as functional scaffolds for tissue engineering and regenerative medicine.

Intensified decellularization confers a higher biocompatibility of equine carotid arteries after subcutaneous implantation in rat

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INTRODUCTION: Xenogeneic decellularized arteries are a promising alternative to alloplastic materials for the generation of vascular grafts. Decellularization is necessary to reduce immunogenicity towards xenogeneic proteins of the tissue. However, it has been previously shown for equine carotid arteries that conventional decellularization using detergents and nucleases produced scaffolds (dEAC-con) with a multitude of cellular residual proteins evolving antibody formation in mice [1]. Coating with the matricellular protein CCN1 nevertheless improved significantly inflammation, reseeding and neovascularization after implantation in sheep [2]. To further improve the scaffolds, an intensified decellularization protocol was developed resulting in non-cytotoxic and mechanically stable [3] scaffolds (dEAC-intens) with a reduced antigenicity when extracts were injected into mice [4]. However, dEAC-intens have not been tested *in vivo* so far and also its coating with CCN1 was not evaluated. We here demonstrate for the first time the subcutaneous implantation of dEAC-intens with and without CCN1-coating in a rat model.

METHODS: Equine carotid arteries were decellularized with 0.5%SDS and 0.5% sodium deoxycholate for either 40h in 100 mL solution (dEAC-con) or 72h in 300 mL solution (dEAC-intens). Coating was performed by immersing the scaffolds in CCN1 (100ng/mL) for 24 h. Three pieces (5x5 mm) of each scaffold were implanted per Wistar rat subcutaneously in the back skin (n=3). After 1, 4 and 12 weeks scaffolds were explanted and analyzed histologically. Acute and chronic inflammation, fibrosis and neovascularization were evaluated semi-quantitatively using a score system (0-3).

RESULTS: Acute inflammation as assessed by neutrophile infiltration was increased at early time points independent of scaffold or coating, whereas only in dEAC-intens a decrease of 67.8% by CCN1 after 12 weeks was observed. The time course of chronic inflammatory infiltration was identical in all explants showing a normal wound healing. However, in dEAC-con the infiltrated scaffold area after 4 weeks was considerably higher than in dEAC-intens showing only peripheral infiltration. CCN1 reduced infiltration in dEAC-con to a level comparable to dEAC-intens, where CCN1-coating had no further effect. Moreover, tissue integration after 12 weeks was more complete in dEAC-intens and comparable to dEAC-con with CCN1-coating. Likewise, neovascularization was enhanced in dEAC-intens after 4 weeks independent of CCN1, whereas the coating exerted a clear antifibrotic effect again only with dEAC-intens.

DISCUSSION & CONCLUSIONS: Intensified decellularization significantly improves biocompatibility of equine carotid arteries in terms of reduced inflammation, increased integrative potential and neovascularization. CCN1-coating seemed not to be necessary to further improve biocompatibility of the scaffold after subcutaneous implantation which is a great advantage under regulatory aspects. However, since dEAC-intens seem to be particularly susceptible for the antifibrotic and anti-inflammatory CCN1-effect which may also enhance cellular repopulation functional animal studies are necessary to further evaluate the potential for remodeling and reseeding of intensified decellularized equine carotid arteries with and without CCN1-coating.

ACKNOWLEDGEMENTS: This project was funded by the Else Kröner-Fresenius-Foundation (Project 2012_A58).

Monocyte subset in blood can predict obesity related response of macrophages to biomaterials in vitro

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INTRODUCTION: Macrophages play a key role in the foreign body response to implanted biomaterials and they obtain a pro-inflammatory phenotype in response to the low-grade inflammation in obesity[1]. Obesity also often results in adverse surgical outcome[2]. The aims of this study were to investigate to what extend obesity affects the acute response of macrophages to biomaterials in vitro and whether this can be predicted with biomarkers measured in blood.

METHODS: Obese (n = 20, BMI 43.8 ± 6.5) and age and gender matched lean persons (n = 20, BMI 22.9 ± 2.6) were included. Peripheral blood was collected to isolate CD14+ monocytes and to analyses classical, intermediate and non-classical subsets based on the presence of CD14/CD16 surface markers. Monocytes were seeded on polypropylene (PP), polylactic acid (PLA), polyethylene terephthalate (PET) monofilament, and PET multifilament (mPET) and cultured for 3 days. IL-6, TNF-α, CCL18, and IL-1ra were measured in the culture medium. C-reactive protein (CRP) was measured in serum.

RESULTS: Macrophages from obese patients produced more IL-6 (on PET, p = 0.022) and less CCL18 (for all materials) and IL-1ra (on PLA, p = 0.026) than macrophages from lean subjects. TNF-α was unchanged.

Percentages of classical monocytes ($CD14^{++}/CD16^{-}$) in the blood negatively correlated with IL-6 production by macrophages cultured on biomaterials. Percentages of intermediate monocytes ($CD14^{++}/CD16^{+}$) positively correlated with IL-6 production of macrophages cultured on PLA, and negatively with CCL18 production on PET and mPET and IL-1ra production on PET. A positive correlation was also seen between CRP and IL-6 on PET and mPET.

DISCUSSION & CONCLUSIONS: Monocyte-derived macrophages of obese patients respond predominantly pro-inflammatory to biomaterials in comparison to macrophages from lean subjects. Interestingly, variations were seen among the behavior of macrophages from obese patients, which correlated with percentages of monocyte subsets in peripheral blood or serum CRP levels of the patient. This knowledge and culture model can contribute to personalized medicine pre-operatively.

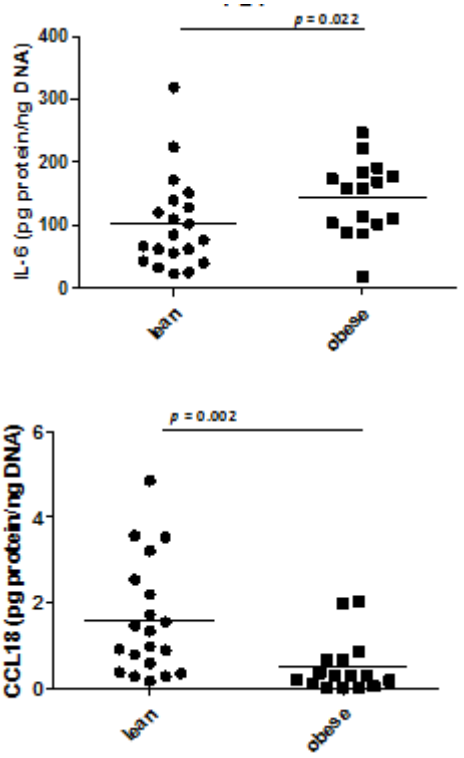

Fig. 1: Examples of IL-6 and CCL18 production by macrophages seeded on PET, lean vs. obese groups. Every dot represents a single donor.

ACKNOWLEDGEMENTS: This study is supported by Medtronic-Sofradim, but they were not involved in the design, conduct or data analysis of the study. The authors declare no conflict of interest.

Tissue engineering approaches to optimize the decellularization of equine tendons as biological grafts for the replacement of damaged tissues

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INTRODUCTION: Tendon ruptures and large losses remain a clinical challenge in orthopaedics. Biological scaffolds have been increasingly involved in regenerative medicine because there is no better way to replace a tissue with its homologous structure [1]. The ideal tendon graft is a nonimmunogenic, readily acquired matrix with mechanical properties resembling the native tendon features [2]. Thanks to the limited risks of disease transmission, the appropriate dimensions and biomechanical characteristics, equine tendon xenografts meet the requirements, even if further evidences need to support the use of these grafts in clinics. This study aims to identify a novel decellularization protocol for large equine tendons to obtain a bioengineered graft for the replacement of injured human tendon.

METHODS: Equine superficial digital flexor tendons were treated with 1% tri (n-butyl) phosphate (TBP) associated or not to multiple concentrations of peracetic acid (PAA) - 1, 3 and 5%. The complete decellularization of the tendon matrix was assessed by means of histological, biochemical (DNA, glycosaminoglycan GAG and collagen content) and biomechanical analyses. The best decellularization protocol was employed to decellularize tendons, then statically reseeded and cultured with murine fibroblasts. The construct biocompatibility was then evaluated through MTT and Live&Dead assays, biochemical (DNA content) and histological analyses.

RESULTS: Tendons treated with TBP+3%PAA showed a significantly reduced DNA content with respect to the native tissue ($p<0.001$), TBP+1%PAA ($p<0.01$) and TBP alone ($p<0.001$), coupled with an accurately preserved GAG content and biomechanical properties (elastic modulus, failure stress and strain) when compared to the native tissue. All the tested treatments similarly affected the collagen content by inducing a decrease compared to the native tendon. Differently from the cytotoxicity of the TBP+5%PAA decellularized matrix, the absence of cytotoxicity of the matrix decellularized with TBP+3%PAA allowed fibroblasts to homogeneously colonize the construct surface. Indeed, a dense fibrous extracellular matrix produced by the viable cells was histologically detected (Fig. 1).

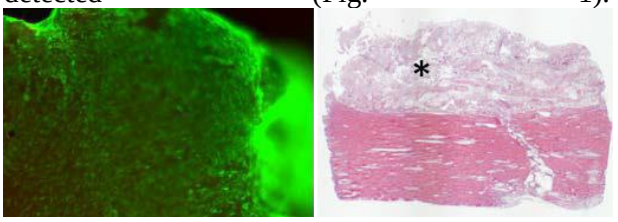

Fig. 1: Evidences of reseeded tendon matrix – representative images of Live&Dead assay and histological analysis. indicates the deposed cellular matrix over the decellularized tendon.*

DISCUSSION & CONCLUSIONS: Given the growing need to regenerate injured tendons, in this study, we propose for the first time an effective method able to properly decellularize large equine tendons. According to the achieved results, the TBP+3%PAA treatment is the most suitable decellularization protocol, being highly effective in donor cell removal, while maintaining both the native matrix structure and cytocompatibility. To restore the native collagen content after the decellularization process, the cell seeding and colonization is mandatory. Thus, the biocompatibility of the decellularized tendon allowed the cell distribution throughout the construct surface producing novel collagen matrix. Our protocol - based on a never tested combination of decellularization agents - leads to the generation of biocompatible, acellular tendon scaffolds that will be suitable for tendon tissueengineering.

ACKNOWLEDGEMENTS: The present study was funded by the Italian Ministry of Health (RF-GR-2011-02348899). The authors have nothing to disclose.

Thermoresponsive magnetic colloidal gels via surface-initiated polymerisation from functional microparticles.

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INTRODUCTION: The emerging field of regenerative medicine is critically dependent on materials to support cell culture, differentiation and proliferation.[1] Colloidal gels derived from reversibly associating microparticles are of potential value in these applications, for example as building blocks for scaffolds which can support tissue growth or as microcarriers for cell delivery.[2-4]

METHODS: thermoresponsive poly(chloromethylstyrene)-gr-poly(diethyleneglycolmethylmethacrylate) (PCMS-PDEGMA) microparticles have been synthesized by surface initiated atomic transfer radical polymerization. Reversible gelation of Aquods suspension of these particles has been tested by rheometer. Cell culture assays have been performed to confirm the feasibility of prepared colloidal gel for invitro expansion of cells.

RESULTS: Novel magnetothermally responsive core-shell microparticles have been synthesized. The aqueous suspensions of these particles exhibit fast thermoreversible fluid-to-gel transitions and retain good magnetic properties. Rheological measurements demonstrated that the viscoelasticity of the prepared particle gels can be tuned, enabling these gels to have the mechanical properties that should facilitate their applications as 3D cell scaffolds for *in vitro* expansion of cells. Also, it was found that the responsive particles could be used in repeated heating-cooling cycles without marked changes in gel elasticity. Presto Blue viability assays of 3T3 fibroblasts and human mesenchymal stem cells cultured within the colloidal gel showed that the cells remained viable and proliferated, with significant increases in cell numbers over extended culture times. Confocal microscopy images of 3T3 cells cultured within the colloidal gel demonstrated that cells adhered, spread and retained their normal morphologies during proliferation. Furthermore, magnetic separation allowed efficient recovery of cells after their expansion *in vitro* without need for enzyme-mediated release steps. Trypsin-free cell passages were performed allowing multiple growth, separation and reloading of cells within the colloidal gels.

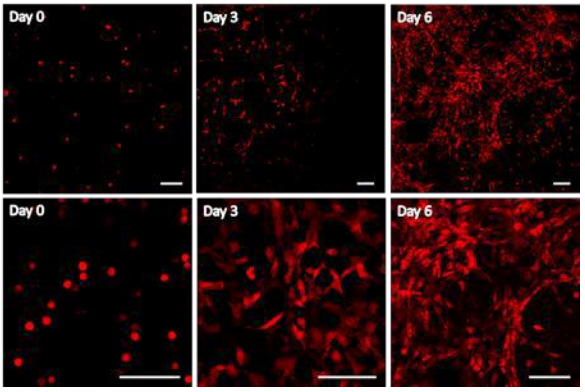

Fig. 1 Confocal Microscopy Images of RFP-3T3 cells in 3D scaffold on day 0, 3, and 6 post-seeding. Images were taken at low (10) and high (40) magnification for the same samples. Scale bars – 100 mm.

DISCUSSION & CONCLUSIONS: Overall, the results suggest this colloidal gel has potential as a 3D scaffold for *in vitro* expansion of a variety of cell types and for enzyme free cell harvesting.

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

the transitory extracellular matrix as a template for biomaterial-based tissue regeneration

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INTRODUCTION: The restoration of tissues of the musculoskeletal system such as bone, muscle, tendon and cartilage after injury is a critical step to restore mobility and mechanical functionality. After trauma, the transient extracellular matrix formed at the onset of healing is, next to biochemical triggers, believed to play a fundamental role in guiding cellular migration and differentiation [1]. Here we studied the formation of cell-produced collagenous extracellular matrix (ECM) and its influence on mesenchymal stromal cell (MSC) behaviour inside a 3D biomaterial via a decellularization approach.

METHODS: To mimic the initial healing scenario, primary human dermal fibroblasts (hdFs) were cultured in macroporous collagen scaffolds with channel-like pores (Matricel GmbH) in DMEM (2% FBS, 1.36 mM ascorbic acid) for 3, 7 and 14 days. De-cellularization was performed in 1% TritonX-100/0.1% SDS solution and subsequent DNase treatment. For geometrical description, the micro-tissues were scanned and the cross-sectional area/height was calculated. Amount and orientation of newly formed fibrillar collagen I was visualized by second harmonic generation (SHG) under a confocal microscope. For migration assay scaffolds were placed on top of a confluent layer of human MSC (hMSC) for o/n adherence and incubation in DMEM (0%FBS, 1% Nutridoma) for 3 days. The median migration distance of cells was determined by analysis of cryo-sections along the migration direction.

RESULTS: Confocal microscopy at 3,7 and 14 days revealed increasing cellular alignment resulting in the deposition of highly directional fibronectin and collagen fibrils in the centre of the scaffold pores (Fig.1A). Contraction of micro-tissues was observed to increase over time up to 14 days. After decellularization at 3 and 7 days, the scaffolds expanded to their original size indicating that cell-induced contraction was not stabilized by new ECM. However, after 14 days of culture a strong collagen network carrying mechanical tension was formed (Fig.1B) and prevented tissue expansion during decellularization. The involvement of the collagenous ECM in tissue formation and mechanical stabilization was verified by depletion of ascorbic acid from culture medium where only minor contraction was observed. Finally, repopulation of decellularized matrices showed preferred adhesion (Fig.1C) and more directional migration of hMSC on hdF-produced tensioned collagen-fibres than on the collagen walls of the scaffold.

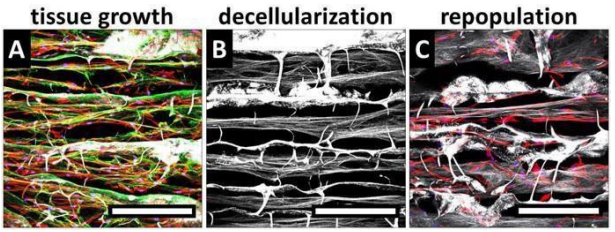

Fig. 1: confocal images of: (A) micro-tissue after 7 days of culture, (B) decellularized sample after 14 days of culture. (C) sample of (B) re-seeded with human MSC. Colours: green: fibronectin, red: actin, blue: DNA, white: SHG. Scale bar 250µm.

DISCUSSION & CONCLUSIONS: We have analysed the process of tissue formation inside a macro-porous collagen scaffold acting as an efficient template for cell and ECM organization. Our data point to a process in which matrix formation is driven by a cell-force dependent compaction and tensioning process that is stabilized by a final transfer of load from the cells (hdFs) towards fibrillar collagen-I in the ECM. Enhanced hMSC adhesion and migration indicated favourable physico-chemical properties of this early cell-derived ECM, including tension and high alignment. Our observations highlight the importance of the properties of the transitory matrix in guiding cell behaviour and might hold cues for the future design of instructive biomaterials for tissue regeneration.

ACKNOWLEDGEMENTS: This work has been partially funded by the DFG research group "FOR 2165 – Regeneration in Aged".

The implications of electrospun scaffold morphology in kidney tissue engineering

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INTRODUCTION: Polymer based scaffolds, such as polycaprolactone (PCL), have been highlighted as a potential avenue for tissue engineered kidneys, but there is little investigation down this stream[1]. Electrospinning is a fabrication technique used in tissue engineering (TE) producing fiber diameters from 10's of nanometers to 10's of microns. This variation in morphology has been shown to effect the way cells behave and integrate with the scaffold[2]. With larger fibers allowing greater cell integration, nanofibers representing the natural ECM and aligned fibers guiding cell growth. The aim of this work is to examine the cellular response to fiber diameter and morphology on novel electrospun scaffolds for kidney TE.

METHODS: Two sets of parameters were used for electrospinning PCL to yield 'large' and 'small' fibers. Large fibers were created using 14 wt% PCL in chloroform/methanol (5:1) and parameters: 4 ml/hr, +15kV, -4kV, 23 cm working distance and 0.8mm needle bore. Small fibers were made using 6.5 wt% PCL in HFIP and parameters: 0.8 ml/hr, +14kV, -4kV, 12cm working distance and a 0.4mm needle bore. Traditional random fibers were spun onto a mandrel rotating at 250 rpm, aligned at 2000 rpm with novel cryogenic fibers spun onto a mandrel loaded with dry ice rotating at 250 rpm. SEM was used to confirm fiber diameter and morphology.

RC-124 human kidney epithelial cells used to assess the scaffolds, validated by cell viability assay (CellTitre-Blue), DNA quantification assay (Pico Green), RT-qPCR (GAPDH, AQP-2,KRT-8, KRT-18, E-CAD) and histology.

RESULTS: Scaffolds had average fiber diameters of 1.1±0.18 µm and 4.0±0.32 µm. Cryogenic fibers has a significantly larger porosity and aligned considerably less, fig 1. This resulted in a considerable difference in mechanical properties.

Aligned scaffolds presented with considerably less cell attachment compared with random and cryogenic. Random fibers showed greatest cell viability after 3 days for both small and large fibers. Although after 14 days large random and small crogenic fibers presented with similar results, fig. 2.

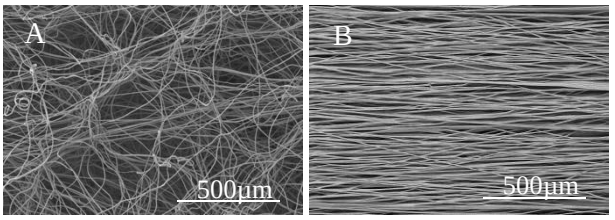

Fig. 1: SEM image of Cryogenic (A) and aligned fibers (B).

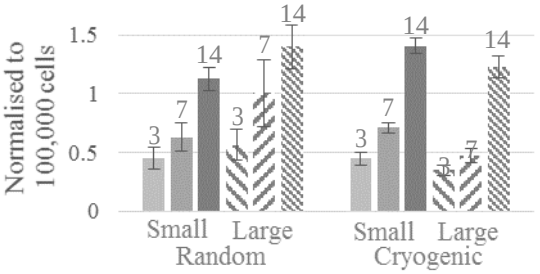

Fig. 2: Cell viability assay at 3, 7 and 14 days of large and small fibers.

DISCUSSION & CONCLUSIONS: The differences between random, aligned and cryogenic scaffolds is a result of the ability of the cells to penetrate the scaffold. The far greater porosity of the traditional large random and novel cryogenic fibers results in an increased cell penetration into the scaffold, shown in previous studies[3].

The viability and expansion of cells, particularly on the traditional random and novel cryogenic scaffolds, shows the potential for electrospun scaffold in kidney TE.

ACKNOWLEDGEMENTS: This research is funded by an Engineering and Physical Sciences Research Council (EPSRC) Doctoral Training Partnership (DTP) Studentship.

In vitro modelling of young and aged bone through polymeric coated glass-ceramic scaffolds

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INTRODUCTION: The improved world population average age has increased the importance of understanding aging associated conditions. Particularly, osteoporosis is an aging-related pathology, characterized by a loss of bone mass which results in an increased risk of fractures. This research work aims at developing *in vitro* models of both normal and osteoporotic bone for the investigation of the influence of those different architectures on cell response, in order to gain new knowledge to support future regenerative approaches. The normal and pathologic bone models were obtained by fabricating 3D glass-ceramic scaffolds, where the porosity, pore dimension and trabecular thickness were varied in a controlled way to mimic normal and osteoporotic bone. The scaffolds were surface functionalized by a newly designed bioartificial blend, made from collagen and a hydro-soluble polyurethane (PUR), obtaining biomimetic and biocompatible constructs, as assessed by *in vitro* tests using rat mesenchymal stromal cells (BM-MSCs).

METHODS: The glass-ceramic scaffolds were fabricated from a SiO_2–P_2O_5–CaO–MgO–Na_2O–K_2O bioactive glass, CEL2, by sponge replication technique. The different architectures were obtained by changing some process parameters, as described elsewhere [1]. The scaffolds were coated with a bioartificial blend based on type I collagen and a newly synthetized water soluble PUR. PUR was synthetized from poly(ethylene glycol), 1,6-hexamethylene diisocyanate and N-BOC-serinol. The coating on glass-ceramic scaffolds was carried out by surface-silanization, followed by further blend chemical grafting, using genipin as a crosslinker. Scaffold structure was investigated by micro-computed tomography. The biocompatibility was assessed with BM-MSCs. Cell viability, proliferation and morphology were evaluated using Presto Blue® according to the manufacturer's instructions after 1, 3 and 7 days of culture on both coated and uncoated 3D scaffolds.

RESULTS: The data from micro-CT characterization on the scaffolds are reported in Table 1. No differences in pore microstructure were found between coated and uncoated scaffolds.

Table 1. Microstructural characteristics of normal, (N) and pathologic (P) scaffolds.

Sample	Porosity [vol.%]	Strut thickness [µm]	Pore Size [µm]
N	63	92	388
P	82	214	745

The viability of BM-MSCs are shown in Figure 1.

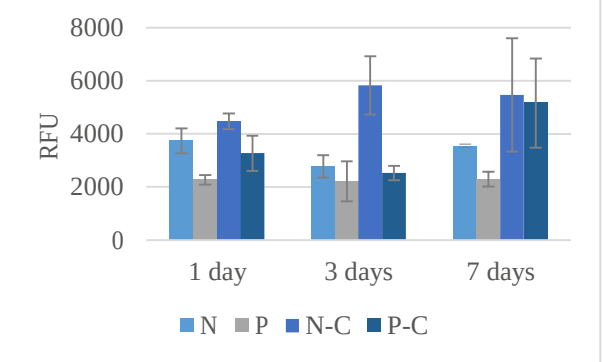

Fig. 1: Metabolic activity of BM-MSCs cultured on the uncoated (N, P) and coated scaffolds (N-C, P-C) modelling normal (N) and pathologic (P) trabecular bone, with time in culture.

DISCUSSION & CONCLUSIONS: The scaffolds had microstructural characteristics closely matching those of healthy [2] and osteoporotic bone [3]. The biocompatibility tests showed that the presence of the coating resulted in an improved cell viability. Further experiments are on-going in order to evaluate the osteogenic potential of the developed models.

ACKNOWLEDGEMENTS: This work was financially supported by the projects MIND (PRIN 2011) and MEDE.

Novel ultrasound-based seeding method for adventitial progenitor cells onto composite scaffolds.

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INTRODUCTION: Limb Ischemia (LI) is one the most common manifestation of peripheral artery disease (PAD). In cases of critical LI, patients are unsuitable for traditional surgical techniques and different approaches are now under investigations. Injection of Adventitial Progenitor Cells (APCs) [1] demonstrated therapeutic effects stimulating the angiogenic process. Despite the shown beneficial effects, many works showed the importance to have a 3D construct able to support a multidimensional growth of cells and ensure the homing of the cells to support the healing of diseased tissue. In this work we propose the application of a novel seeding technique for APCs combined with the development of composite scaffolds [2] with the aim to create a complex structure with specific angiogenic pathways of revascularization. The proposed seeding method is based on acoustic radiation forces. In fact, in order to have a defined homing of cells we exploit the interaction between ultrasonic waves which create an acoustic field able to trap cells in its pressure nodes. This method of manipulating particles in two dimensions allows us to produce alignment of cells on the surface of the scaffolds. Composite scaffolds were designed to have a Polycaprolactone (PCL) structure with the specific geometry of channels, fabricated by extrusion-based system (PAM^2), to provide a precise spatial organization and a mat of gelatin electrospun nanofibers to give an adhesion surface with molecular binding sites.

METHODS: APCs cells were isolated and expanded from leftovers of saphenous vein of coronary artery bypass graft surgery. PCL (Sigma Aldrich) was solved in chloroform (Sigma Aldrich) 10% w/v and processed with PAM^2 system to fabricate squared grid scaffolds. Solution of gelatin (12% w/v) from porcine skin (type A, 300 bloom) in acetic acid-deionized water (60/40) was crosslinked by GPTMS was electro-spun onto the channel structure made with PAM^2 system. The ultrasonic device for cell seeding has two piezoelectric transducers (lead zirconate titanate) (NCE51, Noliac, Kvistgaard, Denmark) which are able to generate a planar standing waves in a culturing chamber. The pattern of the acoustic field is strictly related to the shape of the device; the use of two parallel transducers allows the 2D trapping of the cells in lines. Paired T-test with a p-value of 5% was used for statistical analysis. In order to assess the effects of the geometry and the seeding, the *in vitro* tests were performed on a planar surface coated with gelatin nanofibers and on both composite scaffolds with and without the seeding by ultrasound device. Viability assay (MTS), proliferation (EdU) and arrangement onto the surface of the scaffold were evaluated.

RESULTS: The most challenging step of the work was the trapping of the cells on the surface of the scaffold, due to the structure complexity. Tests showed that viability of cells was not be affected by the ultrasonic manipulation and that the seeding method has a higher orientation ratio compared with classical seeding on fibers and scaffolds.

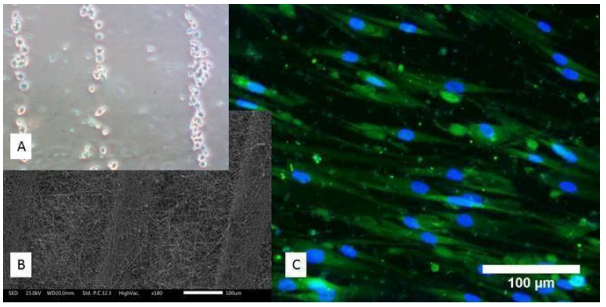

Fig. 1: (A) alignment of APCs by ultrasounds; (B) SEM picture of composite scaffold; (C) APCs seeded on scaffold.

DISCUSSION & CONCLUSIONS: The shown seeding approach onto a composite scaffold could be applied in stimulating vascular tissue regeneration following specific growth pathways.

Biomimetic collagen type I gels for enhanced stem cell mediated osteogenesis

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INRODUCTION: Each year, 9 million fractures require hospitalization worldwide and an estimated 200 million women are at risk of bone fracture due to osteoporosis, needing regenerative treatment [1]. Despite the fact that material science technology has resulted in clear improvements and breakthroughs in the field of bone tissue engineering, challenges remain that need to be addressed. Most of the outstanding properties of the bone are related to its matrix constitution. The non-collagenous proteins that predominate the bone matrix have been investigated because of their potential influence on cell attachment, Ca^{2+} and hydroxyapatite binding, and the mineralization of bone tissue [2,3]. Together with collagen, the non-collagenous proteins form a scaffold for the deposition of hydroxyapatite. Non-collagenous proteins may be used for the production of biomimetic scaffolds, since they may control cell adhesion and enhance mineralization. This study was specifically designed to evaluate the effect of non-collagenous proteins on the mineralization response of human bone marrow mesenchymal stem/stromal cells (hBM MSC) on collagen gels.

METHODS: Different concentrations of non-collagenous proteins (R&D Systems,MN; Millipore,MA) were added to purified bovine collagen type-I (Advanced BioMatrix,CA) solutions. Collagen type-I gels were used as control specimen. hBM MSC were seeded onto these collagen type-I gels and cultured for 21 days using osteogenic differentiation medium. Mineralization assays were performed after 21 days in culture.

RESULTS: After 21 days, mineralization assays suggested that gels supplemented with non-collagenous proteins demonstrated greater hBM MSC activity. In fact, these biomimetic collagen gels demonstrated a significant increase in calcium deposition, compared with the collagen gels without any protein supplementation (Fig.1). However, the mechanism of increased mineralization has not been completely understood and is being investigated.

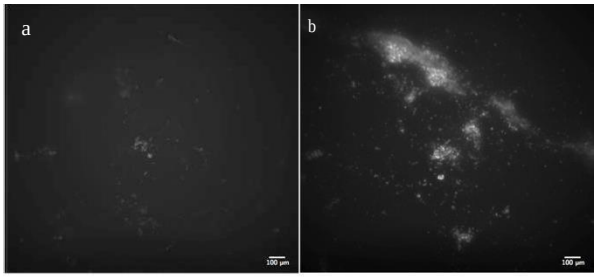

Fig. 1: Mineralized nodules of hBM MSC after 21 days of culture using osteogenic differentiation medium (a) collagen gels without supplementation; (b) biomimetic collagen gels. Bar = 100 μm.

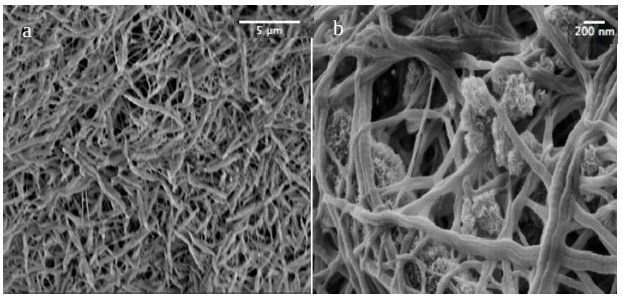

Fig. 2: Microscopic analysis of hBM MSC in biomimetic collagen gels (a) Scanning electron microscopy (SEM) of control acellular collagen gels. Bar = 5 μm; (b) SEM micrograph of a cell-seeded 21-day biomimetic collagen gel. hBM MSC mineralization on biomimetic collagen gel is possible to be observed. Bar = 200 nm.

DISCUSSION & CONCLUSIONS: These results suggest that non-collagenous proteins may promote bone tissue regeneration, by enhancing mineralization, being effectively applied as a new biomimetic scaffold. Their effect on stem cells and other bone cells thus warrants further investigation.

ACKNOWLEDGEMENTS: This work was supported by CBIS-Rensselaer Polytechnic Institute and Fundação para a Ciência e Tecnologia (Portugal) – SFRH/BD/52478/2014 granted to MC.

Numerical simulation of PCL-Collagen tissue engineering constructs

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INTRODUCTION: Scaffolds are used in diverse Tissue Engineering (TE) applications as hosts for the development and proliferation of cells. The selection of the appropriate scaffold depends mostly on geometry and mechanobiological properties [1]. Collagen is a hyper-poro-viscoelastic natural biomaterial, frequently used as scaffolding material and/or cell substrate [2]. The motivations for this study are the understanding of tissue differentiation and molecular structures involved in TE applications, in conjunction with the optimization of scaffold design and performance [3]. The Finite Element (FE) method is the tool used here to evaluate the biomechanical behaviour of such TE systems.

METHODS: A FE model of a construct constituted by a PCL scaffold embedded in a collagen cylindrical layer (Fig. 1) was simulated under 8% confined and unconfined uniaxial compression loading profiles. Compression was imposed progressively during 10s and then hold for 100s. The scaffold model was based on the CAD model presented in [1]. The material properties of PCL scaffold and collagen were extracted from in-house experimental tests (Table 1). Both materials were modelled as poro-hyperelastic with a Neo-Hookean model and strain-dependent permeability [2], using FE software Abaqus® (Dassault Systèmes, USA). Quadratic 10-node tetrahedral elements were used.

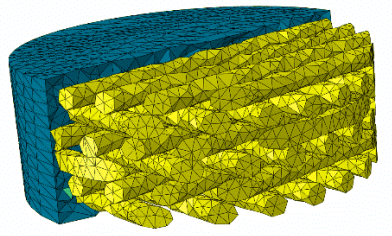

Fig. 1: Section cut of the scaffold+collagen construct (yellow and blue, respectively).

Table 1: Material properties.

Material	K_0^* (m^4/Ns)	M	Void Ratio	C10 (kPa)	D1 (kPa)
PCL	1.00×10^{-14}	-	0.786	4.17	0.0144
Collagen	1.70×10^{-10}	1.8	499	0.0131	2312

RESULTS: Figure 2 shows the minimum principal strain levels on the peak stress point. Strain is uniformly distributed over the interior of collagen layer, which is in direct contact with the scaffold (not shown). The effects of the 8% compression at the macroscopic level of this construct will be significant for the equivalent microscopic strain sensed the cells [4]. Differences to the unconfined case are noticed particularly on the lower overall strain magnitudes. The pore fluid effective velocity is also higher on the confined compression case, being predominantly negligible in the scaffold for both situations.

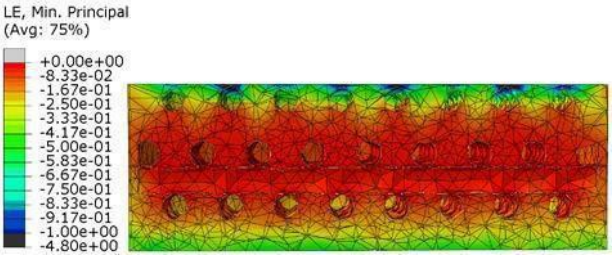

Fig. 2: Distribution of Minimum Principal Strain over a section cut of the collagen layer under confined compression (t=10s).

DISCUSSION & CONCLUSIONS: The results of this study are encouraging for the mechanobiological analysis of complex TE strategies. Numerical simulation through the FE method provides an insight on the mechanical environment that cells will sense when they are distributed over the PCL-collagen construct [3]. It is also important to evaluate and prevent plastic deformation of the PCL scaffold, which may happen at higher strain levels. This study also suggested that confined compression tests allow better control over the construct, but they also cause higher fluid velocities and strain levels, which are determinant to cellular activities[3].

ACKNOWLEDGEMENTS: Financial support from the European Research Council (258321).

DISCLOSURES: Nothing to disclose.

Mechanically-tuned alginate gels as new 3D breast cancer models

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INTRODUCTION: Breast cancer is the second leading cause of cancer death among women. Cancer research effort is addressed to tackle this social problem by developing new tools for improvement of therapies. Recent studies revealed that traditional 2D in vitro models are largely insufficient for drug screening, since they inadequately reflect tumor microenvironment [1]. New 3D in vitro models, more similar to the in vivo environment and ethically preferable to animal models, are thus necessary. Recently, the tissue engineering approach has focused on cancer modelling. In this scenario, hydrogels have gained attention due to their tunable properties, which offer valuable opportunities to study fundamental biological mechanisms [2].

METHODS: Three different sodium alginate solutions (0.5%, 1%, 2% w/v) were obtained by mixing Alginate powder (Kelco Co) in 0.154 M NaCl solution. Cylindrical molds (diameter 5 mm, height 3 mm) of agar gel (1% w/v of DIFCO Agar powder in 0.154 M NaCl enriched with 0.2 M or 0.5 M $CaCl_2$, as alginate cross-linker) were prepared. 150 µL of alginate solution were introduced into the mold, and gelification was allowed to take place for 1 h to ensure calcium diffusion from agar to alginate. Gels elasticity was obtained by AFM (Agilent Technologies 5500 ILM). MCF-7 breast cancer cell line was expanded in complete EMEM medium (Sigma-Aldrich), and 3D cell seeding was achieved by suspending 0.8×10^5 cells/sample in alginate solution and by following the described gelification protocol. Cells viability was analysed at day 7 by Live/Dead assay (Sigma Aldrich). Cells morphology was evaluated after fixing (4% paraformaldehyde) and staining cells with DAPI (1 µg/ml) for nuclei and with Alexa Fluor 488 phalloidin (100 µM) for actin (Sigma-Aldrich). 2D cultures were carried out as control. After 1 and 2 weeks, gels were processed for histological analysis, cross-sectioned (7 µm) and stained with haematoxylin–eosin (H&E).

RESULTS: A remarkable elasticity range (20–4000 kPa) was obtained by varying Alginate and $CaCl_2$ contents. Cells number and viability after 1 week was observed to significantly decrease with the progressive increase of hydrogel mechanical properties.

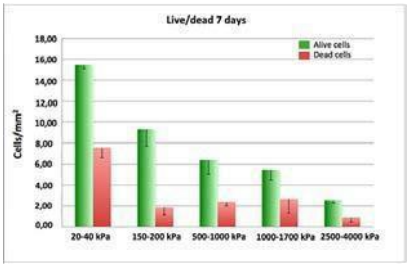

Fig. 1: Alive and dead cells in gels after 7 days.

Alarming morphological differences were observed between cells in 2D and 3D: cells within the gels were characterized by round shape and cluster organization, in contrast to the flat morphology assumed in Petri dish. Clusters of 100 µm-diameter appeared after 7 days in softest alginates (20-40 kPa), while only few cells composed them in stiffer gels. Cluster size increased over the second week of culture, reaching 300 µm in the same soft alginates.

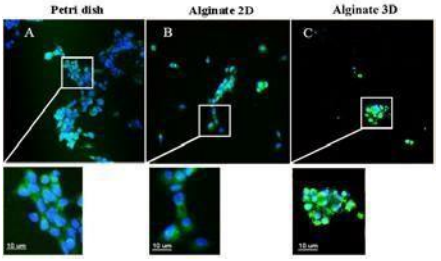

Fig. 2: Cells show different morphology in relation to microenvironment complexity. Scale bar 10 µm.

DISCUSSION & CONCLUSIONS: Breast cancer cells activity was investigated in 3D mechanically controlled gels environments, correlating cell fate to substrate mechanical properties. Deeply differences in viability and proliferation were observed: gels characterized by 20-40 kPa elasticity promoted duplication capacity and formation of clusters up to 300 µm in two weeks. This cluster-like organization was compared with 2D standard cultures, showing a greatly more representative structure of a solid tumor. Substantial efforts still need to be made; however, the improvement of similar 3D bioengineered tumors could conduce in future to very functional in vitro cancer models.

Golden hydrogel - hydrogels visible for µCT imaging in tissue engineering

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INTRODUCTION: When employing imaging modalities to evaluate in vivo response to implanted biomaterials, it is crucial to make quantifiable diagnostic conclusions about the biomaterial-host interactions and degree of tissue repair. MicroCT (µCT) is a commonly used imaging technique that provides high resolution and non-invasive visualization for hard tissue, especially the microarchitecture, volume, surface area, tissue thickness and spatial distribution[1]. In our study, we aim to create µCT visible hydrogels for longitudinal tracking of scaffold degradation and tissue formation.

METHODS: *Synthesis of GelMA:* GelMA was synthesized as described previously[2]. Briefly, type A porcine skin gelatin was mixed at 10% (w/v) into PBS. Methacrylic anhydride was added to gelatin solution and allowed to react for 3h. The mixture was dialyzed against distilled water to remove unreacted methacrylic acid. The solution was lyophilized and stored at -80 °C until further use. *Integrating Au NPs to GelMA scaffolds:* Commericially available Au NPs were dispersed in 10% GelMA solution containing 0.5% photoinitiator (Irgacure 2959). 100 µL of GelMA-Au NP solution has been transferred into molds and crosslinked with UV light. *Synthesis of thiol functionalized GelMA:* Due to the strong interaction between Au and –SH groups, GelMA has been functionalized with thiol groups[3]. Thiolated GelMA was synthesized by covalent modification of amino groups of GelMA by the addition of sulfhydryl moieties. 1% of GelMA solution was prepared and varying amounts of thioglycolic acid added to the solution. Any unreacted thioglycolic acid residue was removed by repeated dialysis steps. The purified thiolated gelatin was freeze-dried and stored at -80 °C for further use[4]. The –SH modified GelMA has been mixed with Au NPs and scaffolds have been prepared as described before.

RESULTS: After the Au NPs blended with GelMA solution, the solution has been transferred into molds and UV crosslinked. Following this step, the hydrogels were scanned via µCT in wet state. Further on, the gels were freeze-dried and rescanned in dry state. Tomograms of the hydrogels and Au NP carrying hydrogels were analysed in Image J. The histograms of each sample have been plotted according to the grayscale values in chosen region of interest (Fig.1).

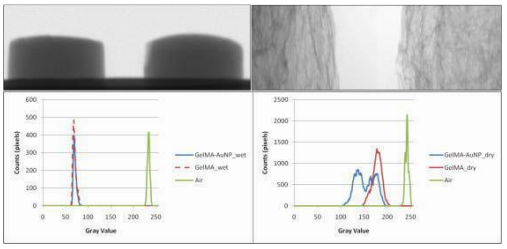

Fig. 1: µCT scans and contrast histograms of GelMA and GelMA-Au NP hydrogels in wet and dry state

We have observed a complete overlay on attenuation values on tomograms when GelMA is on wet state regardless of Au NPs. However in dry state, the shift to higher attenuation has been observed due to the presence of the Au NPs.

DISCUSSION & CONCLUSIONS: In our study we have observed the desired contrast for GelMA hydrogels can be achieved by using Au NPs in dry state, however, the dilution of Au NPs, due to the high water content of the hydrogels, eliminates the contrast difference. More effective binding of Au NPs to GelMA is going to be further studied.

ACKNOWLEDGEMENTS: The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement No. 607868 (iTERM).

3D printed biodegradable polycaprolactone scaffolds with antibacterial effect for bone tissue repair

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INTRODUCTION: Implant-associated infection has been reported as a major source of morbidity in orthopaedic surgery [1]. Aim of this study is the development of bi-functional 3D scaffolds from PCL with incorporated silver nanoparticles (Ag-NPs) as an antibacterial component aiming to (i) promote the osteogenic response of the scaffold and (ii) provide an antibacterial protection by preventing biofilm formation. We aim to evaluate the cellular responses of pre-osteoblasts cultured on these bi-functional scaffolds in terms of cell adhesion, growth and osteogenic differentiation potential, as well as their antibacterial effect.

METHODS: A new injection printing head was developed for a low-cost, open-source Fused Filament Fabrication device, turning it into a powerful printing unit, facilitating the integration of nanoparticles into printable biopolymers. In this context, nanocomposite 3D scaffolds with pore size of 100 μm were fabricated using PCL as matrix and Ag-NPs as impregnated material at a concentration of 30 ppm. Scaffolds were visualized by means of scanning electron microscopy (SEM).

Ag-NPs were produced by an environmentally friendly method based on plant extracts [2] and characterized physicochemically. Antibacterial effect was assessed by means of a broth micro-dilution method and visualized by SEM.

Pre-osteblastic MC3T3-E1 cells are used to investigate cell adhesion onto the scaffold, viability and growth, and osteogenic differentiation by means of alkaline phosphatase activity, collagen and calcium produced in the extracellular matrix [3]. Scaffolds are loaded with pre-osteoblastic cells and visualized by SEM following fixation and dehydration in increasing ethanol concentrations, critical point drying and gold sputtered prior observation. Additionally, we evaluate the antibacterial potential of the Ag-NPs incorporated in scaffolds by SEM.

RESULTS: Reproducible 3D scaffolds are printed in dimensions of 7 mm x 7 mm x 2 mm, consisting of a PCL matrix, in which Ag-NPs with diameters of 20-50 nm are incorporated. Kinetic investigations show a MIC of the Ag-NPs on E.coli at 15 μg/ml, and a bacteriocidal effect observed by SEM at 75 μg/ml after 5 h in culture. Scaffolds loaded with pre-osteoblastic cells depict good cell adhesion after 2 days in culture, as shown by SEM in Figure 1. Cell morphology data are confirmed by the visualization of the cytoskeleton by means of confocal fluorescence microscopy. Cell proliferation increases after 7 and 14 days in culture. Ongoing osteogenic differentiation experiments indicate an elevation of the osteogenic markers such as the alkaline phosphatase activity, collagen secretion and calcium biomineralization in the ECM. Antibacterial testing of the bi-functional scaffolds is in progress.

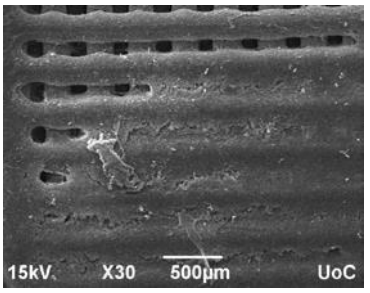

Fig. 1: SEM image of pre-osteoblasts covering a 3D PCL-Ag-NPs scaffold after 48 h in culture.

DISCUSSION & CONCLUSIONS: Polycaprolactone scaffolds functionalized with antibacterial Ag-NPs compounds support attachment, viability and osteogenic differentiation of pre-osteoblastic cells and demonstrate potential applicability in bone tissue engineering.

In vitro cytotoxicity of dental cements on odontoblast cells

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INTRODUCTION: Glass ionomer cements (GICs) have been widely used in dentistry as restorations, luting cements, bases and liners for more than thirty years [1]. We have recently reported a modified GIC based on the strategy to mix glass ionomer cements and calcium silicate ceramics [2]. Compared to conventional GIC, the calcium silicate modified GIC showed comparable setting time, mechanical properties and enhanced bioactivity. However, the cytotoxicity of the modified GIC is still unknown. Thus the aim of this study was to evaluate the cytotoxic effect of the modified GIC on odontoblast cells. The ion concentration and pH of the materials were measured to correlate these properties to the cytotoxicity study.

METHODS: Four groups of cement samples were prepared in this study: (1) Conventional glass ionomer cement (GIC); (2) Mineral trioxide aggregate (MTA) (3) Wollastonite-modified GIC (W-mGIC); (4) MTA modified GIC (M-mGIC). Odontoblast-like MDPC-23 cells were chosen as cell model. An indirect contact assay was used to evaluate the cytotoxicity of the cements. The study consisted on culturing the cells with cement extract. Cement extracts were prepared by immersing a set cement disk (∅ = 12 mm, h = 2 mm) in 1 ml of complete media. The surface-to-volume ratio chosen was 3 cm^2/ml, according to the ISO standard ISO-10993-11 [3]. Cells were seeded (10,000 cells/cm^2) and after 24h extracts were added as obtained (100%), diluted 2-fold (50%) and diluted 5-fold (20%). The concentrations of calcium, aluminium, strontium and silicon ions in extracts were measured by inductively coupled plasma-atomic emission spectroscopy (ICP-AES, Spectro Analytical Instruments, Kleve, Germany). The pH was measured with a pH meter.

RESULTS: At 24h most samples showed a similar number of cells ($p > 0.05$), except for GIC-100% ($p = 0.001$) and positive control ($p = 0.000$). From 24h to 72h, a large increase in the cell number was observed ($p < 0.05$) when cells were incubated with extracts diluted 20% or with undiluted MTA extracts. When undiluted extracts were used to culture the cells, the number of cells present at day 3 showed a clear pattern depending on the types of cement used to prepare the extract: MTA > W-mGIC ≈ M-mGIC > GIC. In terms of ion concentration, MTA extract showed the highest Ca^{2+} concentration (467.3 mg/L), followed by M-mGIC extract (112.2 mg/L). The concentration of Si^{4+} in the extracts followed the trend: GIC (19.9 mg/L) > W-mGIC (14.9 mg/L) >M-mGIC (10.4 mg/L) > MTA (3.2 mg/L). All the cements extracts showed low concentration of Al^{3+}, ranging from 2.4 mg/L (W-mGIC) to 7.2 mg/L (GIC). In the extract, GIC caused the highest Sr^{2+} concentration (100.3 mg/L), followed by W-mGIC (83.5 mg/L), M-mGIC (54.6 mg/L) and MTA (16.9 mg/L).

DISCUSSION & CONCLUSIONS: The cytotoxicity of conventional GIC can be moderated by incorporating calcium silicate based ceramics. In addition, we found the cytotoxicity of GIC was unlikely caused by the release of inorganic ions or the low pH. It may be speculated that the incorporation of calcium silicate based ceramics reduced the total amount of organic compound present in the cement and thus decreased the cytotoxicity of the modified GICs.

ACKNOWLEDGEMENTS: We gratefully acknowledge support from VR (Swedish Research Council (2011-3399)) and China Scholarship Council (CSC). GM thanks Marie Curie Actions FP7-PEOPLE-2011-COFUND (GROWTH 291795) for funding via the VINNOVA program Mobility for Growth and Lars Hiertas Minne Foundation (project n. FO2014-0334).

HSA release from and exposure at the surface electrospun PCL fibers

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INTRODUCTION: Mechanical design of the implant and prediction of its interaction with surrounding tissues and cells are the main targets of tissue engineering. Thus, study of protein release from 3D matrices and exposure of proteins on fibers surface are the urgently needed. Previously, protein release from 3D matrices loaded with minor amounts of proteins was studied [1]. In the current study scaffolds produced from a solution of polycaprolactone (PCL) and model protein - human serum albumin (HSA) in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) were investigated. Protein release from scaffolds as well as the exposure of HSA on the fiber's surface was studied.

METHODS: Matrices made from pure 6% PCL or PCL blended with 0.4÷30% HSA (% relative to PCL) in HFIP were produced using the NF-103 setup (MECC Co). Scanning electron microscopy (SEM) on a JSM-6460 LV (Jeol) and transmission electron microscopy (TEM) on a JEM 1400 (Jeol) with osmium tetroxide/uranylacetate contrasting were used to characterize matrices and fiber's cross-sections. HSA release from 3D matrices was measured by ELISA. X-ray photoelectron spectroscopy (XPS) study was performed with a SPECS equipped with PHOIBOS-150 MCD-9 analyzer and MgK source (SPECS GmbH). Small-angle X-ray scattering (SAXS) data were acquired using a S3 MICRO (Hecus X-Ray Systems GmbH) with point collimation and Cu Kα radiation.

RESULTS: All studied 3D matrices consisted of fibers 0.3÷0.9 um, with a thickness of 150 ± 30 um. SEM and TEM (magnification ×50000 and ×120000) showed a smooth fiber surface, granular inner structure and granules enriched subsurface area. Up to 10% of the protein (varied from 0.4 to 10%) was released from the fibers in the first 30 min, remaining protein remained bound for no less than a week. SAXS demonstrated ~6, ~12 and ~20 nm HSA particles in the fibers. Wetting of the matrices with toluene decreased 6 nm particles to ~5 nm and drastically decreased the number of larger particles. For scaffolds produced from a low HSA (0.4-10%) solution XPS demonstrated enrichment of the outer surface layer of fibers with HSA as compared to the initial solution. It was shown that exposure to water further increased the amount of surface-exposed protein; proteinase K removed ~20-35% of surface-exposed HSA (*Fig. 1*). HepG2 cells, which express receptors for albumin, readily adhered and proliferated on HSA/PCL matrices in a dose-dependent manner in relation to HSA concentration as it was shown by AlmarBlue viability test.

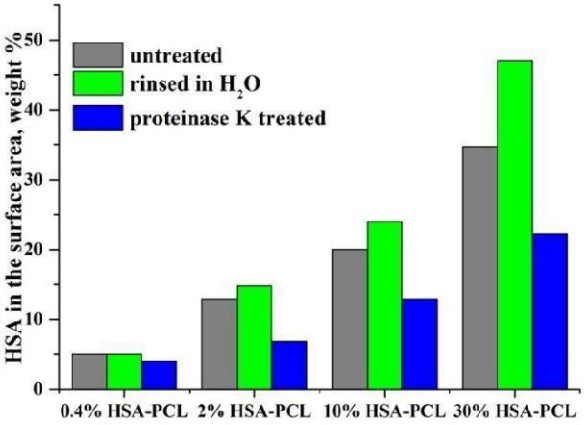

Fig. 1: Quantification of HSA in the surface area of electrospun produced 3D matrices by XPS.

DISCUSSION & CONCLUSIONS: The incorporation of the protein into fibers is not only a simple procedure, but it also increases the tensile strength of the 3D matrix and enables the delivery of the proteins [2]. The fast release of HSA from PCL fibers correlating with the dissolution limited mechanism [3] and uneven distribution of the protein in the electrospun fibers should be considered when matrices for cell growth or protein-mediated drug delivery are designed.

ACKNOWLEDGEMENTS: This work was supported by a Russian Science Foundation grant (№ 14-15-00493).

Biomimetic functionalisation to enhance endothelialisation of bioabsorbable stents

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INTRODUCTION: In-stent restenosis and late thrombosis are the major clinical failures of cardiovascular stent implantation. Fast stent endothelialisation was proven to prevent both thrombosis and restenosis [1,2]. The combinations of antifouling molecules with extracellular matrix peptides are promising strategy to design antithrombogenic surfaces able to promote fast endothelialization [3,4]. The REDV fibronectin-derived peptide is recognized by $\alpha_4\beta_1$ integrins and selectively promotes endothelial cell adhesion and spreading over smooth muscle cells and platelets [5]. In this study carboxylic groups generated by plasma treatment were employed to covalently graft PEGylated-REDV and polyethylene glycol (PEG) at different molar ratio to selectively promote endothelial cell adhesion avoiding platelet activation.

METHODS: Acrylic acid was polymerised via plasma treatment onto different polymeric substrates. Argon plasma activation was carried out by setting a flow rate of 20 sccm, power of 50 W and time of 5 min. Acrylic acid vapors were diluted in Argon (flow = 20 sccm). Polymerization was performed by a pulsed plasma discharge applying a discharge RF power of 200 W, a duty cycle of 10% (on time = 10 ms, off time = 90 ms) for 10 min [6]. After plasma treatment, samples were dipped into an EDC/NHS solution (ratio 4:1, pH 5.5) for 1 h at 4°C and subsequently dipped in PEGylate-REDV and PEGylate-REDV:PEG solutions at different molar ratio (100 μg/mL in PBS) for 20 h at room temperature. Surface modification was characterized through physico-chemical analyses and *in vitro* cell tests.

RESULTS: Physico-chemical characterisation, performed after plasma treatment, confirmed the successful of AAc grafting/polymerization on different polymeric substrates. Plasma treated surfaces were functionalized with PEGylate-REDV peptide and different ratios between PEGylate-REDV and PEG. The evidence of successful grafting on the sample surfaces has been obtained through X-ray Photoelectron Spectroscopy (XPS), contact angle measurement and FTIR-ATR spectroscopy. Different molar ratios of PEGylate-REDV and PEG were covalently grafted on polymeric films to obtain both antifouling and biomimetic properties. Results showed that PEG introduction as spacer molecule induces changes into surface wettability, as surfaces become more hydrophilic with increasing ratio of PEG. The REDV-functionalisation resulted in high endothelial cell adhesion with adequate cell spreading.

DISCUSSION & CONCLUSIONS: An antifouling coating able to promote a new endothelial cell layer on polymer stent surface was developed. AAc was polymerised on the polymer surface to expose –COOH groups needed for biomolecule grafting. PEGylate-REDV and PEG were successfully grafted on the substrates. The presence of the REDV peptide increased the endothelial cell adhesion on the materials. Fast formation of a healthy endothelial cell layer could enhance the hemocompatibility *in vivo*.

ACKNOWLEDGEMENTS: This work was funded by the EU Commission 7FP - grant agreement number 604251- ReBioStent (Reinforced Bioresorbable Biomaterials for Therapeutic Drug Eluting Stents). Authors thank ReBioStent partners for their support in this work.

Characterization of hyaluronan acyl derivatives by Raman spectroscopy

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INTRODUCTION: Hyaluronan (HA) is a linear polysaccharide with several important roles in the human body. This makes both native HA and chemically modified HA suitable for various cosmetic and medical applications. One promising HA modification is the grafting of aliphatic acyl side-chains, yielding acyl-HA derivatives [1]. These can be used for drug delivery [2] or in applications requiring stability in aqueous environments [3,4]. The properties of acyl-HA can be tailored by the acyl length and the degree of substitution (DS), which is thus one of the most important characteristics of acyl-HA. In this contribution, we present a fast and simple method for evaluating the DS of acyl-HA using Raman spectroscopy.

METHODS: Raman spectra were collected on an in-house developed confocal system equipped with a 633 nm He-Ne excitation laser, dispersive spectrograph and multichannel CCD detector. Samples were measured in the form of pellets.

RESULTS: The Raman spectra of native HA and acyl-HA derivatives (palmitoyl and lauroyl HA) with various DS were measured and compared. We observed that the intensity of several bands was sensitive to HA substitution. These bands were identified as mostly belonging to various CH_2 vibrations of the aliphatic acyl side-chain. For evaluation purposes, we chose the spectral region from 2800-3050 cm^{-1} (Fig. 1) that contained both bands sensitive to DS (2850 cm^{-1}) and suitable for normalization (2930 cm^{-1}).

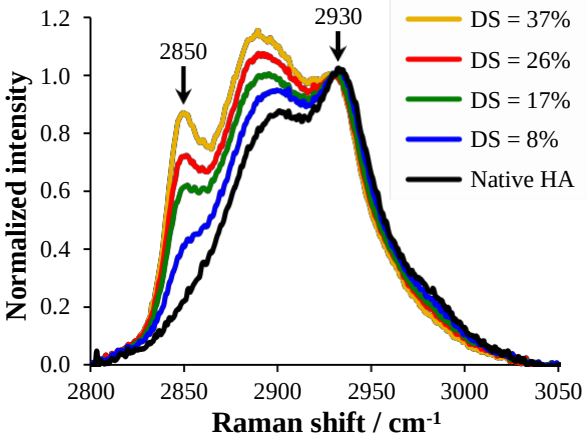

Fig. 1: Raman spectra of native HA and selected palmitoyl-HA derivatives with various DS.

After applying baseline correction, the intensity ratio I_{2850} / I_{2930} was calculated for each sample. To enable quantitative evaluation of DS from the Raman spectra, a calibration series of acyl-HA oligosaccharides with known DS was measured and processed in the same way as the acyl-HA spectra, providing a linear dependence between DS and I_{2850} / I_{2930}.

DISCUSSION & CONCLUSIONS: The DS obtained from the Raman spectra were compared with values measured by ^{1}H NMR and GC-MS. The GC-MS and Raman results were in a good agreement (Fig. 2), with the average relative deviation below 10%. This suggests that Raman spectroscopy is suitable for evaluating the DS of HA substituted with aliphatic side-chains. On the other hand, liquid-phase ^{1}H NMR provided significantly higher DS than the other methods, which was attributed to solvation issues.

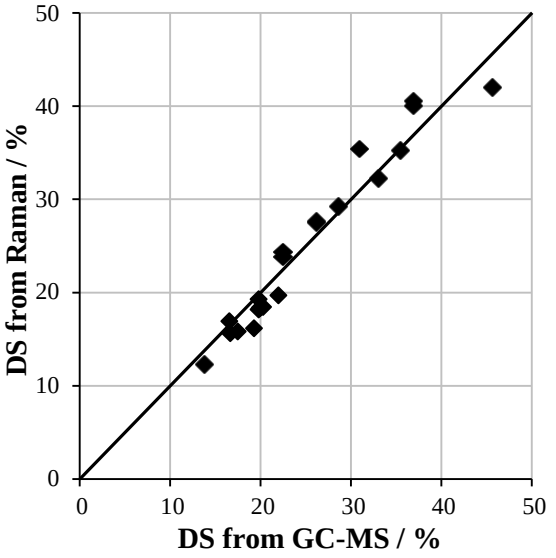

Fig. 2: Comparison of DS measured by GC-MS and Raman spectroscopy.

A novel bioreactor for the perfusion of electrospun fibre scaffold supporting 3D engineered tissue production – Application of human skeletal satellite muscle cells and human embryonic stem cells differentiated towards neural cells

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INTRODUCTION: Electrospinning fibre creates fibrous scaffolds of the size of the extra cellular matrix highly suitable for the 3D culture. To fully benefit of this advantage, the scaffold needs to be perfused by an orthogonal continuous medium flow in a bioreactor environment.

METHODS: 4 or 8 bioreactors equipped with polycaprolactone (PCL) fibre scaffolds (up to 3 mm thick) were cultured in parallel. The cells were inoculated in the scaffold, cultured overnight in incubator and then transferred in the bioreactor where the medium recirculation was operated. The glucose consumption and lactate accumulation were monitored during the perfusion culture as an indicator of cell growth. At the end of the culture, the following analyses were performed: DNA quantification after papaine digestion, scaffold staining by calceine to analyze the distribution of living cells, immunocytochemistry and qPCR.

RESULTS: A new system including four small bioreactors was created to support the growth and differentiation of human stem cells in perfused 3D fibre scaffold, see Fig 1. This system was used in two different applications: the expansion of human skeletal muscle satellite cells (hSkMSC) and the expansion and differentiation of human embryonic stem cells (hESC). It enabled the orthogonal perfusion of scaffold of volumes in a range between 15 uL and 3 mL. pH and pO_2 were monitored by miniaturized single-use sensors.

Process optimization was carried out for the expansion of hSkMSC cultured in electrospun PCL fibre scaffolds in two bioreactor systems, i.e. eight bioreactors. Several factors were investigated: the scaffold surface treatment by plasma and/or coating, scaffold porosity, initial cell seeding density, and recirculation rate, see Fig 1. It was observed that the higher porosity significantly increased the cell in-depth penetration into the scaffolds. After a 7-day culture period, the highest cell fold increase, i.e. 25 fold, was obtained in the scaffolds seeded with the lowest density. It was showed that human skeletal muscle satellite cells cultured in this system were not carcinogenic in a mouse model. This system was then used for the culture of hESC. The effect of the recirculation rate, the porosity and the cell density were re-visited for these cells. It was showed that the cell pluripotency was maintained. A successful neural differentiation of 2 weeks expressed neural marker Pax6 at day 5 followed by beta-III tubulin expression at day 15.

DISCUSSION & CONCLUSIONS: To our knowledge, this is the first presentation of a bioreactor enabling the orthogonal perfusion of thick electrospun fibre scaffold. These bioreactors can be used as stand alone or as parallel scale-down of a larger bioreactor. This system is easy to operate and generates reliable data in parallel cultures allowing the direct comparison of the different parameter values. The cell cultures in this system have proven to be healthy and to maintain their markers.

ACKNOWLEDGEMENTS: This work was supported by FP7-Health, HESUB, #601700.

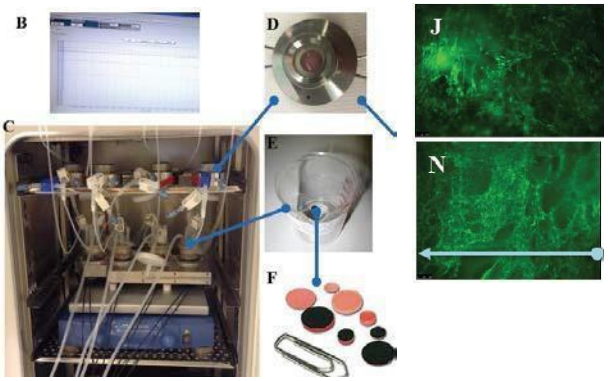

Fig 1: Bioreactor: (D) top; (C) overview; (B) sensors of pH and pO_2 ((F) detail); (E) reservoir; (J) top of the scaffold with SkMSC in 3 mm thick electrospun fiber PCL scaffold (calcein); (N) orthogonal view of the scaffold showing a uniform cell distribution – arrow head and tip pointing at the top and the bottom edge of the scaffold.

Interleukin-10 knockout mouse : In vivo validation model for a novel anti-inflammatory scaffold

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INTRODUCTION: The purpose of this study is to prove that Interleukin-10 knockout (IL-10 KO) mouse is appropriate model for evaluation of a novel anti-inflammatory scaffold through kidney regeneration.

METHODS: To elucidate the deficient level of IL-10 gene in kidney depending on age and gender, 4 weeks and 24 weeks-old male and female mice were analyzed. The $Mg(OH)_2$ incorporated scaffold (Mg-PLGA) was fabricated by a freeze drying method. Sixty mice were randomly divided into 3 groups; (1) Ctrl/IL-10KO,
(2) PLGA/IL-10KO and (3) Mg-PLGA/IL-10KO group. The scaffolds (size 5×5×2 mm^3) were implanted into injured kidney, and harvested at week 1, 4, 8, and 12 post-surgery. Histological, immunohistochemical (IHC) and gene expression for inflammation fibrosis and renal regeneration were analyzed.

RESULTS: In 4 weeks old mice, the *IL-10* mRNA level in *IL-10 KO* mice kidney was higher than that of C57BL/6, and at this age, gender was not related. However, 24 weeks old *IL-10 KO* female mice revealed significant low expression of IL-10.
In gross images, PLGA/IL-10KO mice showed kidney shrinking morphology. Mg-PLGA scaffold showed more swelling at 1 week after implantation and faster degrading than PLGA. From week 8, scaffold was disappeared, and minor scar remained at the scaffold implanted region.
In histological analysis, the implanted scaffolds were degraded and formed empty space at week 1. The kidney of PLGA/IL-10KO mice showed significantly enhanced inflammatory cells infiltration into adjacent area. The PLGA degraded acidic debris located at the adjacent region of scaffold, and it caused renal tubule injury and death. In the empty space, RBC and other types of cells were less identified. At week 4, empty space in PLGA/IL-10KO group was not completely filled and the scaffold debris was still remained. Mg-PLGA/IL-10KO group started to form glomerular precursors, renal tubule and blood vessel. At week 8, PLGA/IL-10KO group formed several glomerular, but the number and size were lower than that of Mg-PLGA/IL-10KO.

In detection of cytotoxic T-cell, monocytes and macrophages in PLGA/IL-10KO group, about 2 - 3 times higher positive cell frequency than that of Mg-PLGA/IL-10KO.

In real-time PCR, pro-inflammatory/-fibrosis related bioactive molecules were higher in PLGA/IL-10KO group than Mg-PLGA/IL-10KO, while, host stem cell recruitment and tissue regeneration related gene expression was lower.

DISCUSSION & CONCLUSIONS: IL-10 KO mouse is sensitive to immunogen causing inflammation and fibrosis by PLAG scaffold, thus, IL-10 KO mouse is the appropriate animal model to prove the anti-inflammatory and tissue regenerative effect of the $Mg(OH)_2$ incorporated PLGA scaffold.

ACKNOWLEDGEMENTS: This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (2014R1A1A3049460);
(NRF-2014M3A9D3033887); funded by the Korea government (MSIP) (NRF- 2015R1C1A1A01053509); funded by the Ministry of Education (2015R1D1A3A03020378); and supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare (HI14C1642).

Ultrasound fields non-invasively pattern microparticles in situ

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INTRODUCTION: Technologies that enable rapid patterning of cells and microparticles within three-dimensional (3D) hydrogels will accelerate fabrication of complex tissues. Ultrasound standing wave fields (USWFs) provide a non-invasive approach for patterning cells and/or microparticles rapidly. Furthermore, USWF-patterning of endothelial cells can accelerate microvessel network formation, and direct microvessel morphology within 3D hydrogel constructs [2,3]. Ultrasound propagates through tissue as a focused beam, and thus is ideal for translation in vivo. The goal of this study was to develop a dual-transducer USWF system to enable rapid, non-invasive, volumetric patterning of cells and microparticles within hydrogels in situ.

METHODS: A dual-transducer system comprised of two, 1-MHz ultrasound sources was developed to generate an USWF in the region of intersecting sound fields (Fig. 1A). USWFs are characterized by pressure nodes and anti-nodes. The distance between nodal planes, d, in this system is given by

$$d = \lambda / [2\sin(\theta/2)] \qquad (1)$$

where λ is the wavelength of the incident sound field and θ is the angle between propagation paths of the two sources [4]. The distance between nodal planes can be controlled by choice of θ and/or frequency. Solutions of Sephadex beads suspended in water were exposed to USWFs generated by interfering sound fields with θ of 60°, 120°, and 180°. Distances between planar bands of beads were quantified. The dual-transducer system was also tested for its ability to pattern microparticles in situ. C57BL/6J mice were anesthetized and the target region of one flank was positioned within the USWF. Sephadex beads, suspended in type I collagen solutions, were injected subcutaneously into both flanks. One flank was exposed to the USWF. The other served as a sham control. After exposure, USWF patterning was visualized using high-frequency (38-MHz) ultrasound imaging.

RESULTS: In vitro, USWFs generated by a dual-transducer system rapidly patterned Sephadex microparticles suspended in water into planar bands (Fig. 1B). Distances between planar bands were controlled by adjusting angle (θ), and measured distances (d) were consistent with theoretical predictions (Eq.1). The dual-transducer system patterned microparticles within collagen hydrogels injected under the skin of mice (Fig. 1C, 'USWF'). No patterning was evident in hydrogels in sham-exposed flanks (Fig. 1C, 'Sham').

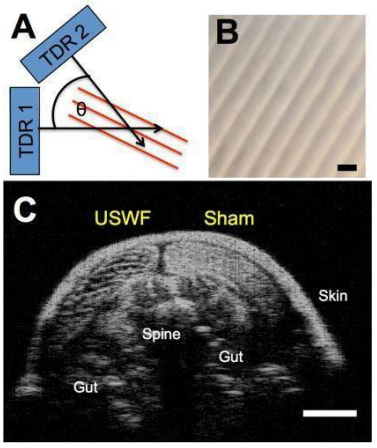

Fig. 1: A) Dual-transducer schematic. B) Ultrasound transducers angled at 120° produce planar bands of white microparticles spaced 840 ± 24 μm apart. Scale bar = 1 mm. C) High-frequency ultrasound imaging was used to visualize USWF-patterned microparticles in the flank of a mouse. Scale bar = 5 mm.

DISCUSSION & CONCLUSIONS: A dual-transducer USWF system was developed for rapid, non-invasive, 3D spatial patterning of microparticles within collagen hydrogels injected in situ. Patterning geometries were controlled by design of acoustic fields.

ACKNOWLEDGEMENTS: This work was supported by a grant from the NIH (R01 EB018210) and an AHA pre-doctoral fellowship (15PRE25300000).

Compact membranes of chitosan/hyaluronic acid polyelectrolyte complexes for cartilage repair

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INTRODUCTION: Cartilage exhibits limited ability to heal, due to its avascularity and consequent low access to nutrients or circulating cells. Its inability to reconstruct and the need to maintain the phenotype have inspired planar and three dimensional devices that promote chondrocyte seeding and phenotype maintenance. We present compact polyelectrolyte complexes (PECs) membranes for the regeneration of cartilage defects. These structures consist of chitosan (CHT) and chondroitin sulphate (CS) complexes, which are known to help in chondrogenesis [1]).

METHODS: CHT and CS were mixed vigorously at 0.5 mg/mL (pH 5.5, NaCl 0.5 M) to synthesize PECs, and poured into Petri dishes to dry at 37 ºC and yield membranes [2]. Morphology was assessed by SEM and AFM. Mechanical properties as a function of salt concentration (0–2.5 M) were measured by dynamic mechanical analysis (DMA), as well as water uptake. TGF-β3 doped membranes were seeded with ATDC5 pre-chondrocyte cells for 7, 14 and 21 days, after which the expression of collagen types I, II and X was assessed by immunocytochemistry. Pure CHT membranes and membranes without TGF-β3 were used as controls.

RESULTS: The synthesis of CHT/CS membranes is a Green Chemistry approach, free of cytotoxic reagents, which resulted in compact and rough membranes (Fig. 1A). Exposure to increasing salt concentrations triggered an increase of water absorption over 300% in NaCl 2.5 M. This response is accompanied by a decrease of the tensile storage modulus (Fig. 1B), revealing the plasticizing effect of salt ions over such structures. The membranes were doped with TGF-β3 (associated with the increased expression of cartilage markers) and retained 99% of the loaded mass during 14 days. The materials did not elicit a cytotoxic response in ATDC5 cells. Seeded cells rearranged as aggregates, typical of chondrogenesis, and expressed cartilage markers, namely collagen types I and II (Fig. 1C). The TGF-β3 loaded in the membranes elicited a decrease of collagen X expression, a marker of hypertrophic phenotype.

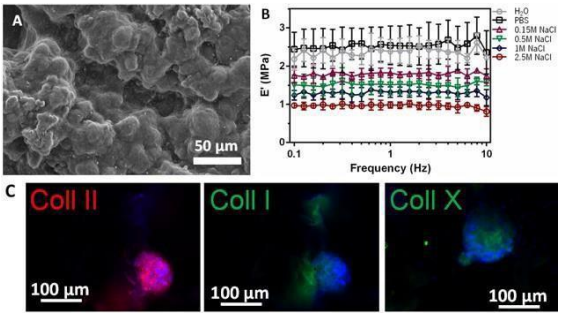

Fig. 1: (A) SEM top view of the CHI/CS membranes. (B) Storage modulus (E') of the membranes. (C) Fluorescence microscopy images of TGF-β3 impregnated CHT/CS membranes seeded with ATDC5 cells after 21 days in culture.

DISCUSSION & CONCLUSIONS: The rough surface of the membranes provides a substrate with anchoring points for cell adhesion, and exhibit mechanical properties similar to many soft tissues. Taking cartilage as a model soft tissue, the membranes promoted a chondrogenic phenotype in the pre-chondrocyte cell line ATDC5. Cell aggregates formed and expressed collagen type II. This is a cartilage-exclusive protein, which ratio relative to collagen type I increased for 21 days and is characteristic of hyaline cartilage formation. TGF-β3 loaded membranes induced decreased expression of hypertrophy markers (collagen X), contrary to pure CHT and membranes without TGF-β3. In the future, membranes with variable amounts of TGF-β3 may be stacked to establish a concentration gradient to trigger a 3D-regulation of the chondrogenic/hypertrophic cell phenotypes, thus recreating the cartilage/bone interface niche.

ACKNOWLEDGEMENTS: Fundação para a Ciência e Tecnologia (grants SFRH/BPD/95446/2013 and SFRH/BPD/111354/2015), "Fundo Social Europeu" and "Programa Operacional de Potencial Humano".

Development of an extracellular matrix hydrogel as a valuable and versatile tool in intestinal tissue engineering.

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INTRODUCTION: Decellularised intestinal replacements seeded with autologous stem cells have been highlighted by our group as a promising solution to intestinal failure (Totonelli et al. 2012). A hydrogel derived from these acellular scaffolds would represent another potentially valuable tool in intestinal tissue engineering. This non-immunogenic gel which possesses the extracellular matrix (ECM) information of the native tissue could hypothetically be used for; cell delivery of autologous cells to restore function of damaged tissues, the 3D-culture and expansion of organoid stem cells for the repopulation of decellularised scaffolds or as the ECM component of a hybrid engineered intestinal replacement. Here, we develop and derive such a hydrogel from decellularised intestine and characterise the decellularisation, ECM preservation, gelation, rheology and cytocompatibility, investigating its potential in intestinal tissue engineering.

METHODS: A non-immunogenic piglet intestinal scaffold was obtained via decellularisation. The appropriate number of cycles of the detergent enzymatic treatment was determined by quantifying the DNA and extracellular matrix (ECM) components with results supported by histological staining. To create a gel, the acellular scaffold was lyophilised, milled, digested for 72 hours and brought to a physiological pH and temperature. The resulting gel was characterised with turbidimetric tests, oscillation rheology, conductivity measurements and scanning electron microscopy. Preliminary cell culture experiments were performed using intestinal organoids to investigate the toxicity of the gel and investigate its potential as a 3D support for cell culture for tissue engineering applications.

RESULTS: The piglet intestine required just one cycle of the DET protocol to significantly reduce the DNA in the scaffold and immunofluorescence staining confirmed all nuclei were removed. The decellularisation protocol was shown to preserve the extracellular matix components with collagen, GAG and elastin not significantly reduced. Turbidimetric tests validated the efficacy of the gelation protocol with normalised absorbances forming sigmoidal curves and osscillation rheology supported these results showing an average gelation time of 20minutes. Cell experiments proved that the gel was non-toxic and non-immunogenic facilitating cell viability with phenotypic mophology.

DISCUSSION & CONCLUSIONS:

A ECM hydrogel derived from a decellularised intestine which is non-immunogenic, cytocompatible and stable represents a valuable tool for a plethora of applications within the field of intestinal tissue engineering.

3D cell cultures in porous scaffolds with oriented microtubules designed for dental regeneration

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INTRODUCTION: Tooth loss is a common result of a variety of oral diseases due to physiological causes, trauma, genetic disorders and aging, and can lead to physical and mental suffering that markedly lower the individual's quality of life [1-3]. Tooth is a complex organ that is composed of mineralized tissues and soft connective tissues. Dentin is the most voluminous tissue of the tooth and its formation (dentinogenesis) is a highly regulated process displaying several similarities with osteogenesis.

METHODS: In this study a low-cost scaffold made by gelatin biomineralized with magnesium-doped hydroxyapatite and blended with chitosan, was developed for hard tissue engineering. We synthetized a dentin-like scaffolds using a controlled freeze-drying process permitting the formation of microscopic channels comparable to dentin tubules and appropriate for cell penetration and matrix deposition.

Mesenchymal stem cells (MSCs) and dental pulp steam cells (DPSCs) were seeded in direct contact with the scaffolds and cultured with medium supplemented with osteogenic factors. Cell viability and cell morphology were analysed up to 14 days of cultured. Moreover gene and protein quantification were investigated.

RESULTS: The scaffolds show an aligned porosity suitable for the colonization of its inner part by the seeded cells (Fig. 1). The scaffold had no cytotoxicity, cells morphology was accordant to a non-stress cell condition and a good adhesion to the scaffold. The SEM observations showed the scaffold's aligned porosity and rugosity, cells were found through almost the entire height of the scaffold.

DISCUSSION & CONCLUSIONS: 3D cell culture with MSCs and DPSCs showed the promising properties of the new scaffolds for tooth regeneration. In detail, the chemical composition of the biomineralized gelatin facilitate the cell adhesion, the aligned porosity is suitable for cell colonization and fine cell/material interactions together with mineral component permit the cells differentiation and matrix deposition.

This preliminary study indicate the potential of a low-cost biomineralized gelatin scaffold as a novel tool for 3D cell culture in dental regeneration.

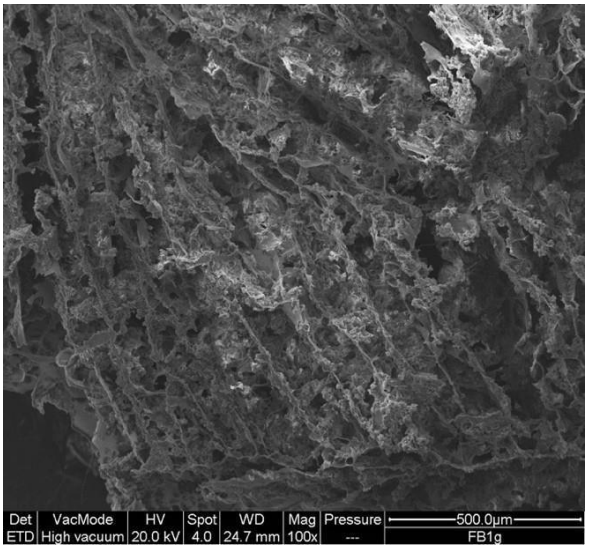

Fig.1: SEM image of biomineralized gelatin-based scaffold with a aligned channel-like porosity.

ACKNOWLEDGEMENTS: The authors would like to thank the European Project SMILEY (NMP4-SL-2012-310637) for providing financial support to this project.

The effect of the trehalose on the cell properties of the dental pulp derived mesenchymal stem cells

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INTRODUCTION: Trehalose is a non-reducing disaccharide composed of two glucose units. It has high water retention property, and its capacity to be used as preservant was recognized previously [1]. The effect of trehalose as a cryopreservation supplement on stem cells was analyzed, and the loss of stemness factors was not observed [2]. Recently, it was found that trehalose induces autophagy via an mTOR independent pathway important for a therapeutic approach against viral diseases [3]. The effect of trehalose on the mesenchymal stem cell culture is still required for the investigation.

METHODS: Dental pulp derived mesenchymal stem cells were previously isolated and characterized [4]. The cells were cultured in the DMEM medium supplemented with 10% fetal bovine serum and 1% pen/strep on collagen (type 1) coated culture flasks. Trehalose was supplemented in the medium at concentration of 100 mM. The adhesion assay was determined by measuring the remaining attached cells on the collagen coated surface after serial washing with PBS. The cell proliferation was measured by WST-1 reagent during the period of 3 days. The cytoskeleton structure was analyzed by Phalloidin staining. The mesenchymal stem cells were differentiated into osteogenic cell lines. After the alizarin red S staining, the differentiation efficiency was compared.

RESULTS: The cells appeared in slightly spherical cell morphology in the culture with trehalose supplemented medium compared to the non-trehalose supplemented control group. The F-actin structure of the cells was not disrupted (Fig.1), but the cell adhesion was decreased 20% and the cell proliferation was accelerated in the presence of the trehalose. After 72 h of culture, the cell duplication time was shorten from 68 h to 45 h. The osteogenic differentiation was detected to be more efficient in the culture medium without the trehalose supplementation compared to the cells in the trehalose medium. The disaccharide affected the cells to form cell clusters in which the mineral deposits were formed in the subsequent culture.

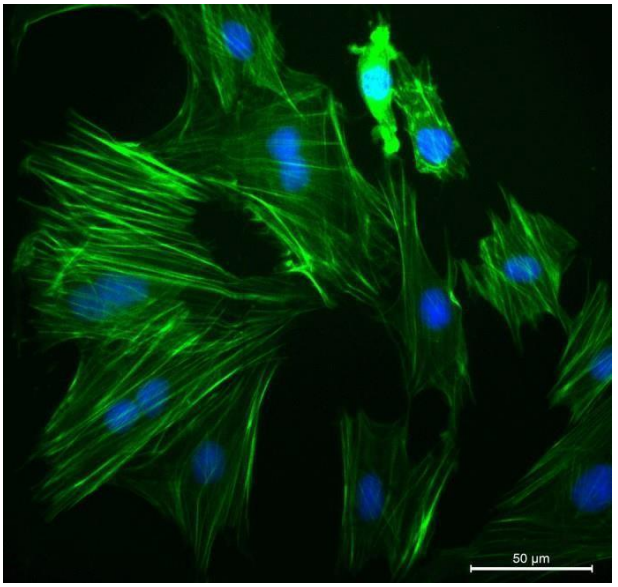

Fig. 1:.F-actin structures of the dental pulp derived mesenchymal stem cells was preserved in the trehalose containing culture media.

DISCUSSION & CONCLUSIONS: The trehalose in the culture medium slightly changed the mesenchymal stem cells with respect to the cell adhesion, proliferation and differentiation. The cells were loosely attached, which affected the osteogenic differentiation. The late response of the proliferation rate to trehalose might indicate to an indirect effect.

ACKNOWLEDGEMENTS: For her kind help and instructions, I would like to thank Cansu Subasi, and Yusufhan Yazir for his support in this study.

Modification of c.p Mg pure by MAO technique for biomedical applications

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INTRODUCTION: Magnesium (Mg) is an attractive material for biomedical applications in which degradable implants are required, because it is non(cyto)toxic nor are its degradation products[1]. Yet, bare Mg corrodes quickly with coinciding release of hydrogen gas, which may form pockets that may cause tissue damage. Therefore, tuning of the degradation rate is warranted, e.g. by adding of surface coating. Micro anodizing oxidation (MAO) is suitable to generate a coating in which an electrical potential between a tuneable electrolyte solution and specified current and voltage generate an oxidized surface coating on Mg. The morphology, roughness and composition can be controlled[2]. The production of a coating not only improves the corrosion resistance of the material but also influences cell-material interactions including adhesion, proliferation, differentiation and function. We set out to investigate the influence of coating types on cellularbehaviour.

METHODS: Samples of c.p Mg (99.98% pure Mg) of 1cm^2 and 0.3mm thickness were polished and cleaned. Next, samples were anodized in a basic solution of NaOH and $Na_2SiO_3.9H_2O$ at 370V by using a DC power supply for 600s. Characterization of the samples were performed by scanning electron microscope (SEM) and Energy dispersive spectroscopy EDS. Surface information such as wettability (contact angle) and roughness was measured. Biological assays comprised of hemolysis and cytotoxicity test, while short term adhesion (24h) of PK84 fibroblasts was assessed by fluorescence stain of cytoskeleton with FITC-phalloidin. All measurements were compared to untreated c.p Mg.

RESULTS: Using MAO a homogenous porous coating on c.p Mg was generated. The coating had a topography in the order of micrometres. The composition was basically an oxide-hydroxide magnesium ceramic. Coated c.p Mg was hemacompatible and non-cytotoxic in contrast to its non-coated control. Fibroblasts efficiently adhered and spread on coated c.p Mg surfaces, while on c.p Mg adhesion occurred but was associated with increased apoptosis. The contact angle of the coated c.p Mg was 27,80 ± 3,36 compared to 80,89 ± 6,54 for bare c.p Mg, which explains the differences in adhesion and survival.

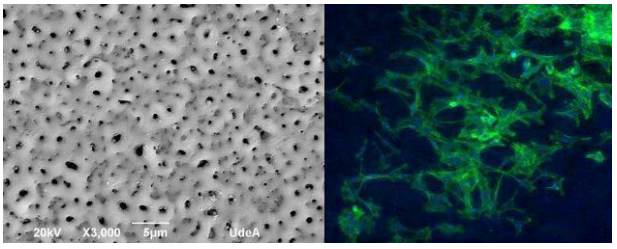

Fig. 1: SEM micrographs of c.p Mg modified by MAO (left), fluorescence micrograph (right) of adhered PK84 fibroblasts on coated c.p Mg (FITC-phalloidin stained).

DISCUSSION & CONCLUSIONS: *MAO is a technique that allow the control of the structure on the layer produced in Mg by the modification of some variables such as electrolyte solution, voltage, current and time modify the structure of layer of Mg. The coating obtained have a porous configuration which is favourable for attachment initially of the extracellular adherence protein promotes and subsequently for the cells.*

ACKNOWLEDGEMENTS: The authors acknowledge the financial assistance of the (COLCIENCIAS) through the project 111556933337 and Estrategia de Sostenibilidad 2014-2015 of UdeA. M.E:R is supported by PhD grant (Colciencias 567) and from the Eurica Program (Erasmus Mundus)

Bioactive, elastic and biodegradable double – layered emulsion electrospun DegraPol tube delivering PDGF-BB for tendon rupture repair

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INTRODUCTION: Healing of tendon ruptures represents a major challenge in musculoskeletal injuries. Approaches of repair by conventional suture may lead to re-rupture. A new, highly elastic polyester urethane (DegraPol® (DP))[1,2] was explored as a delivery device for platelet derived growth factor – BB (PDGF-BB) to promote tendon healing. Morphological, mechanical and delivery properties of the bioactive DP scaffolds were studied. Assessment of double-layered emulsion electrospun DP was performed *in vivo*, in a rabbit Achilles tendon rupture model.

METHODS: Water-in-oil emulsions were produced by dropwise addition of aqueous phase with biomolecules to the polymer solution, stirred, ultrasonicated and electrospun using in-house built electrospinning device. Changes in fiber morphology and diameter as a function of different electrospinning parameters were determined from SEM images. Mechanical properties of bioactive DP scaffolds were determined from stress/strain curves and compared to pure DP scaffolds. Release kinetics of fluorescein and FITC-BSA were studied as model molecules. The delivery of rhPDGF-BB was analysed by ELISA. Bioactivity of the released PDGF was tested on rabbit tenocytes *in vitro* by analysing cell proliferation (EdU assay). Performance of bioactive scaffolds in a form of double-layered tube, with inner layer delivering PDGF-BB at the site of tendon rupture (Figure 1A) was assessed in an *in vivo* rabbit model where biomechanical and histomorphological analysis of the regenerating tendons was performed 3 weeks post – surgery. One-way ANOVA was used to test differences between groups (*$p < 0.05$, **$p < 0.01$, *** $p < 0.001$).

RESULTS: Emulsion electrospinning resulted in significantly smaller fiber diameters, compared to single electrospinning. PDGF-BB was released in a sustained manner within a period of 30 days (Figure 1B). No differences were observed in Young's modulus, but bioactive scaffolds had decreased strain at break [%], however still high enough for proper surgeon handling. Tenocyte proliferation was higher on them and significantly different from pure DP scaffolds (Figure 1C). *In vivo* results revealed the positive impact of PDGF-BB by significant higher ultimate load and stress of extracted tendons compared to conditions with only pure DP electrospun tubes.

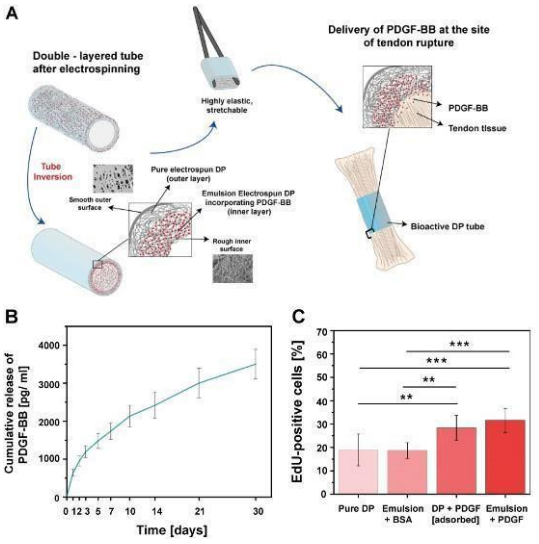

Fig. 1: A) Scheme of double-layered DP tube. B) Release kinetics of PDGF-BB. C) Bioactivity of incorporated PDGF-BB in DP scaffolds.

DISCUSSION & CONCLUSIONS: The data suggests that emulsion electrospun DP tubes deliver bioactive PDGF-BB, thus increasing tenocyte proliferation and leading to enhanced biomechanical properties of regenerating tendons *in vivo* 3 weeks post-operation, presenting a promising application in aiding tendon regeneration after rupture.

ACKNOWLEDGEMENTS: This [2] work was supported by the EMDO foundation and fellowship by *ab medica*, Lainate, Milan, Italy for O.E.

In vitro model of the tumor draining lymph node to study immune escape

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INTRODUCTION: In cancer patients, the downstream tumor-draining lymph node (TDLN) is constantly bathed in cytokines and antigens derived from the tumor, and as a result develop immune suppressive microenvironments that enable metastatic growth[1]. Tumor cells are thought to spread to the TDLN via local lymphatic vessels, where metastasis is a primary prognostic indicator[2]. While the conditions that favor versus reject tumor metastasis in the TDLN remain unclear, there are also no existing in vitro tools to study these effects. To better understand the role of the TDLN microenvironment in promoting tumor metastasis, we developed an in vitro lymph node model. We used this model to test how the presence of tumor-draining factors, such as TGFβ and IL-10, inhibit $CD8^+$ effector T cell function and enable tumor cell invasion.

METHODS: Fibroblastic reticular cells, lymphatic endothelial cells, and dendritic cells (DCs) harvested from LNs and bone marrow of C57BL/6 mice were mixed in type I collagen gel and loaded into a PDMS microdevice for 3D culture under physiologically relevant flow conditions (Fig 1).

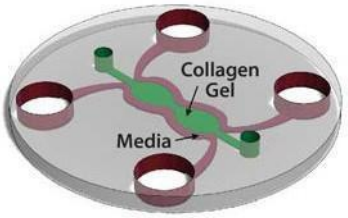

Fig. 1: In vitro lymph node model to study the TDLN microenvironment

In addition, CD8+ OT-1 cells, which specifically recognize the model antigen ovalbumin (OVA), were added to the device. The LNs were primed with either OVA peptide only to drive T cell activation, or OVA peptide plus immuno-suppressive cytokines TGFβ (2 ng/ml), IL-10 (10 ng/ml), and tumor conditioned media (CM) from B16-VEGFC-OVA melanoma cells to promote immune suppression. After 2 days, B16-VEGF-OVA-GFP cells were added to the LN inlet, and their migration was monitored by live-cell imaging for 3 days. Analysis of T cell activation (CD44, CD62L, PD1) was measured by FACS.

RESULTS: After 3 days of co-culture, B16 tumor cells proliferate into TDLNs conditioned with immunosuppressive cytokines, while they do not survive in the activated LNs (Fig 2).

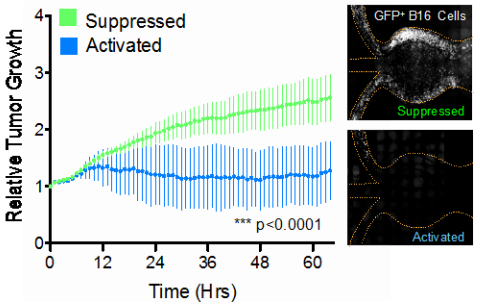

Fig. 2: B16-VEGF-OVA cells (white) survive and proliferate in the TDLN under suppressed conditions (peptide, TGFβ, IL-10) but are killed under activated conditions (peptide only) n= 5

Under immunosuppressive conditions, $CD8^+$ T cells maintain a $CD44^+CD62L^-$ effector phenotype, but increase PD1, a molecule expressed on non-functional, exhausted T cells. In contrast, activated $CD8^+$ T cells differentiate into $CD44^+CD62L^+$ memory cells after tumor cell killing and clearance of antigen, and have a $PD1^{lo}$ phenotype.

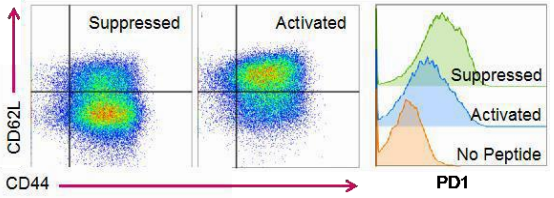

Fig. 3: $CD8^+$ T cells are $CD44^{hi}CD62L^{lo}$ $PD1^{hi}$ in suppressive TDLN conditions, suggesting tumor cell infiltration is due to dysfunctional phenotype.

DISCUSSION & CONCLUSIONS: The development of an in vitro TDLN can help us better understand how tumors prime draining lymph nodes to enable immune escape. In addition, such a tool can provide important and complimentary insights to animal models for the design and trial of immunoengineered therapies and anti-tumor vaccines.

Artificial membranes tuning for lymphatic wall repair

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INTRODUCTION: Chylothorax is an uncommon form of pleural effusion, which generally occurs after cardiac surgery and almost any surgical operation in the chest. The aim of this project is to develop a bioresorbable vascular patch for lymphatic wall repair [1]. Here, we project to develop new materials (i.e. membranes) having two different levels of porosity [2]. First short tests made with Polycaprolactone (PCL) membranes and PCL was blended with different biocompatible, bioresorbable membranes. It shows that human dermal lymphatic endothelial (HDLEC) cells can bind and spread on certain membrane and not on others suggesting that the chemical structure and the morphology of the membranes is important.

METHODS: The membranes were prepared using a modified phase inversion technique [2]. A polymer solution was prepared by dissolving PCL in N-methyl-2-pyrrolidone (NMP) at 60^0C during 24h. The concentration of PCL was set to 15% (wt). The polymer solution, at 20^0C, was casted with a thickness about 250 µm covered by the polyester track etched membrane (Sterlitech,USA). Two types of porosity were prepared in the same condition. The part of the final membrane in direct contact with water presented a homogeneous microporous structure and the other part, which was covered by the track etched membrane, formed large macropores due to the localized arrival of non-solvent.

RESULTS: *Fig 1 (left)* the asymmetrical structure of double porosity membrane presented macropores which were largely open towards the active surface. The macrochambers displayed on a length of about 70-100 µm for a diameter in the order of 30–40 µm. The macrochamber wall possessed a highly porous, sponge-like structure with interconnected micropores [2] of a diameter in the range of 1-8 µm.

In *Fig 1 (right)* HDLEC were cultivated on the PCL membranes with double porosity.

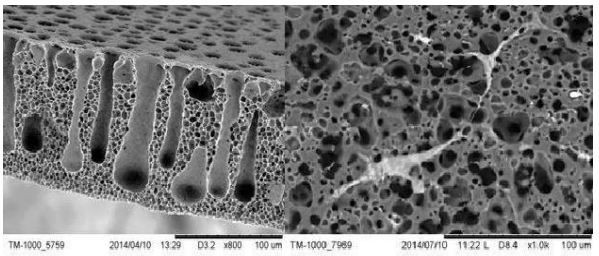

Fig. 1: ESEM micrographs of membranes, Right; membrane with double porosity, Left; HDLEC colonization after 24h of incubation.

And mesenchymal stem cells were cultivated inside the macropores to provide in situ growth factor for the cellular distribution, morphology and physiology over 9 days. The viability was measured by live/dead cell staining assay and lactate dehydrogenase (LDH) assay.

DISCUSSION & CONCLUSIONS: A well organized monolayer of polygonal HDLEC cells on PCL double porosity membrane obtained [1,2]; macro-porosities, opened at one membrane surface, allowing the colonization of 3D material by cells and micro-pores present in the walls of the macro-pores to ensure the transfer of compounds required for the development of cells or of compounds released by cells. The encouraging biological data obtained with flat sheet membranes with double porosity level can be integrated in vascular patch loaded with lymphatic cell to repair chylothorax.

ACKNOWLEDGEMENTS: This project is funded by Erasmus Mundus Doctorate in Membrane Engineering (5^{th} Ed.)- EACEA.

Biodegradable controlled release microparticles as artificial antigen presenting cells (aAPC) for control of T cell behaviour

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INTRODUCTION: The immune system is emerging as an important clinical target in tissue engineering and regenerative medicine. Recent advances in immunoengineering have identified the *ex vivo* expansion and activation, and long term control of T cell behaviour, as key to ensuring successful engraftment of transplanted materials. Control of T cell behaviour is usually mediated by the interaction of a T cell with an Antigen Presenting Cell (APC). Whilst the primary engagement molecules involved in this interaction have been identified, there are currently limited material interfaces able to deliver these signals to immune cells in order to direct their behaviour. Previously, Delcassian *et al* described 2D interfaces with controlled ligand-receptor nanopattern spacing as artificial APCs for the control of T cell activation [1]. Here, we present our continued development of aAPCs to include new materials capable of the delivery of simultaneous soluble factors to immune cells through biodegradeable and controlled release systems for long term control of T cell behaviour.

METHODS: PLGA was homogenised using a water-in-oil-in-water double emulsion method to synthesise microparticles between 20-200µm. Microparticles were functionalised to encapsulate T cell specific human soluble proteins and small molecules, including CD3 and CD28. Microparticles were analysed by SEM, encapsulation and release profiles were analysed using ELISA protein quantification kits, and were sized using a laser diffraction method. Primary human blood mononuclear cells were extracted from leukocyte cones using density centrifugation, and negatively depleted to isolate CD4+ cells. Human cells were incubated with immunomodulatory materials, and the response to directed activation measured by flow cytometry analysis.

RESULTS: PLGA microparticles containing various immunomodulatory proteins were fabricated. Typical microparticles can be seen in Figure 1, where microparticles show relatively narrow size distribution across a range of compositions. Additionally, these microparticles present a porous PLGA matrix, likely due to the inclusion of PBS in the fabrication step, which helps to stabilise human proteins [2].

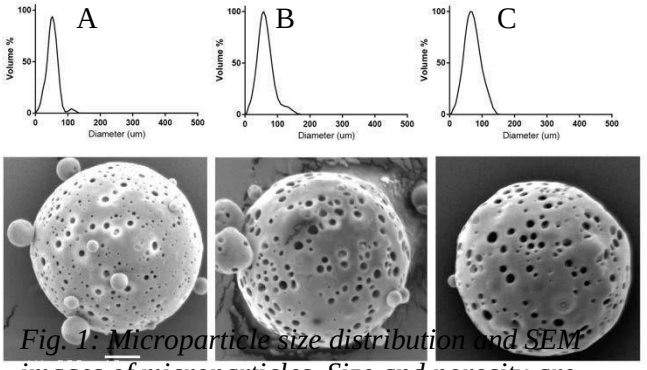

Fig. 1: Microparticle size distribution and SEM images of microparticles. Size and porosity are conserved across 3 different samples. A) blank control particles B) particles containing Human serum albumin protein C) particles containing HSA protein and immunomodulators.

DISCUSSION & CONCLUSIONS: These microparticle materials present a development on current aAPCs, as they allow immune cell engagement through surface receptors and the controlled release of soluble factors over time. The analysis of cellular responses to these materials has helped us to identify biodegradeable material aAPCs that may offer an attractive solution for the long–term control and directed differentiation of specific immune cell function.

1

ACKNOWLEDGEMENTS: This research was supported by an EPSRC E-TERM Landscape Fellowship and funding from the UKRMP Acellular Hub.

Resveratrol alleviates plasma cytokine production in chronic psychological stress: relationship with cardivascular disease

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INTRODUCTION: Converging lines of evidence from both experimental and clinical studies indicate that psychological stress may account for the cardiovascular disease via inflammation. Proinflammatory cytokines, such as TNF-α and IL-1β, impair endothelium-dependent vasorelaxation in vascular beds, decreases the release of nitric oxide, suppresses endothelial NO synthase expression. Previously, we have shown protective effects of TNF-α inhibitors on unpredictable chronic mild stress (UCMS)-induced vascular dysfunction and depression in rats [1,2]. Resveratrol (trans-3,5,4-trihydroxystilbene) has anti-inflammatory and cardioprotective properties. Recently, we have reported beneficial effects of resveratrol on vascular dysfunction in psychological stress[4]. In this background, we examined the effect of resveratrol on proinflammatory and inflammatory cytokine production in UCMS rats.

METHODS: Animals were divided into the following study groups (n = 10 per group): Control animals not exposed to UCMS, animals exposed UCMS, resveratrol treated group (20 mg/kg/day, i.p., during 12 weeks) while exposed to UCMS. UCMS groups with or without treatment were subjected to different types of stressors: restraint for 4 h, cage tilting for 24 h, wet bedding for 24 h, swimming in 4 ^{o}C cold water for 5 min, swimming in 45 ^{o}C hot water for 5 min, pairing with another stressed animal for 48 h, level shaking for 10 min, pairing with another stressed animal for 48 h, level shaking for 10 min, nip tail for 1 min and inversion of the light/dark cycle for 24 h. Rats received one of these stressors per day and the same stressor was not applied for 2 days so that the animals could not predict the occurrence of stimulation. The control groups receiving no stress had free access to food and water. At the end of the 12-week period of UCMS, blood was collected and the aortas of the rats were removed. To assess the effects of UCMS on circulating inflammatory markers, we measured the serum concentrations of tumor necrosis factor-α (TNF-α), IL-1β, IL-6, C-reactive protein (CRP), monocyte chemoattractant protein 1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1) and corticosterone levels were determined by ELISA. These measurements were performed with an ELISA kit (Invitrogen/GIBCO) using a microtiter ELISA reader. The measurements were performed according to the manufacturer's protocol.

All data were expressed as a mean value± standard error of the mean. Significance was tested by one-way analyses of variance (ANOVA) with a posthoc Tukey's test and Kruskal–Wallis test. Probabilities of less than 5% ($P<0.05$) were considered significant.

RESULTS: The TNF–α, IL-1β, IL-6, CRP, MCP-1, ICAM-1 and also corticosterone levels in the supernatants were determined with an ELISA reader. TNF–α, IL-1β, IL-6, CRP, MCP-1, ICAM-1 and also corticosterone levels increased significantly in UCMS where chronic resveratrol treatment decreased the cytokine levels. The levels of TNF-α were measured also in the brain and determined that resveratrol treatment decreased the TNF- α levels ($P<0,05$) (*Fig. 1*).

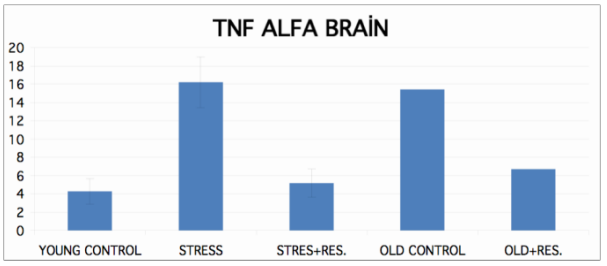

Fig. 1. TNF-alpha levels of brain tissue determined by ELISA analysis.

DISCUSSION & CONCLUSIONS: UCMS increased proinflammatory and inflammatory cytokine levels in plasma and TNF-α in brain tissue where this changes can be markedly improved by chronic resveratrol administration. Therefore, resveratrol has potential as a therapeutic intervention for preserving vascular function during stress.

Various wavelengths of light-emitting diode light regulate the proliferation of human outer root sheath cells via wnt/β-catenin and the ERK pathways

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INTRODUCTION: Light emitting diode (LED) has been demonstrated to promote hair growth in clinical trials. However, the underlying mechanisms are not fully understood. The aim of this study was to determine the effect of LED irradiation on human outer root sheath cells (hORSCs).

METHODS: The effect of the LED irradiation on cell proliferative effect of hORSCs was examined by MTT assay. hORSCs were irradiated using four ranges of LED wavelengths from 415nm to 830nm with 1, 5 and 10 J/cm2 The effect of LED irradiation on the expression of Wnt/β-catenin signaling pathway and ERK pathway in hORSCs was examined by real-time PCR and Western blot assay. For statistical analysis, we performed ANOVA. All tests were one-sided, and a *p*-value of < 0.05 was considered statisticallysignificant.

RESULTS: LED irradiation at 660 nm and 830nm significantly increased on hORSCs proliferation and induced β-catenin, Wnt5a, Axin2, Lef1 and Sox9 mRNA expression and β-catenin protein expression. Bcl2/bax mRNA ratio increased after various wavelength of LED irradiation. Phosphorylation of ERK, c-Jun and p38 in hORSCs was observed after 660nm light irradiation and PD98059 treatment (20uM) before irradiation reduced the phosphorylation of ERK and c-Jun. Various wavelength of LED-treated hORSCs showed substantial upregulation of IL-6, IL-8, IL-16, TNF-a, IGF-1, TGF-b1, TGF-b2 and VEGF mRNA expression. 660 and 830nm light accelerated the migration of hORSCs in vitro.

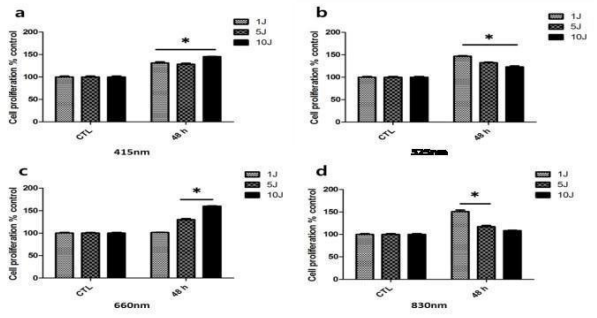

Fig. 1: The effects of LED lights on the hORSC viability. Cell viability was enhanced by LED light exposure energy dependently, especially at 660nm and 830nm.

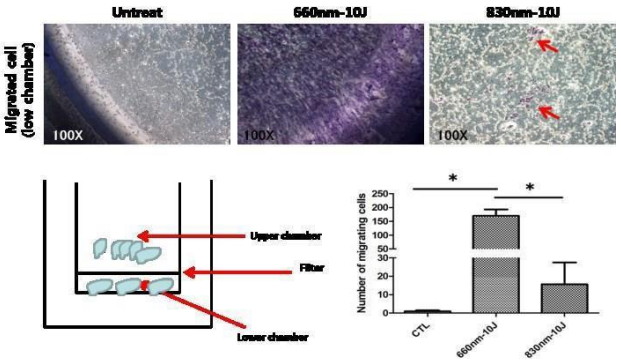

Fig. 2: LED significantly increased the migration of hORSCs at 660nm and 830nm of/10J.

DISCUSSION & CONCLUSIONS: These results demonstrate that Various wavelength of LED irradiation induced hORSC proliferation and migration and inhibit apoptosis in vitro. The growth promoting effect of LED irradiation in hORSC appears to be associated with the direct stimulation of the Wnt5a/β-catenin signaling pathway. Red Light also promoted cell proliferation by activating ERK signaling in hORSCs, which is independent on Wnt5a/β-catenin signaling pathway. ERK signaling activation seems to induce expression of many growth factors and some inflammatory cytokines with growth-promoting activity.

ACKNOWLEDGEMENTS: This study was supported by a grant from Clinical Research Laboratory of the Catholic University of Korea, St. Paul's Hospital (2014).

Repeated microneedle stimulation induces enhanced hair growth through the wnt/β-catenin pathway in a murine model

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INTRODUCTION: Microneedle treatment is a method that creates transdermal microchannels across the stratum corneum barrier layer of skin. Wound-induced hair follicle regeneration via the Wnt/β-catenin signaling pathway following wounding in adult mice was demonstrated. No previous study showed a therapeutic effect of microneedle on hair growth by wounding. The aim of this study is to investigate the effect of repeated microwound formed by microneedle treatment on hair growth and hair-growth-related genes in a murine model.

METHODS: A disk-type microneedle roller was applied to each group of mice five times a week for three weeks. First, to identify the optimal length and cycle, microneedles of lengths of 0.15, 0.25, 0.5, and 1 mm and cycles of 3, 6, 10, and 13 cycles were applied. Second, the effect of hair growth and hair-growth-related genes such as Wnt3a, β-catenin, VEGF, and Wnt10b was observed using optimized microneedle. Outcomes were observed using visual inspection, real-time polymerase chain reaction, and immunohisto-chemistry. We performed ANOVAs followed by Kruskal–Wallis tests.

RESULTS: We found that the optimal length and cycle of microneedle treatment on hair growth was 0.25 mm/10 cycle and 0.5mm/10 cycle. Repeated microneedle stimulation promoted hair growth, and it also induced the enhanced expression of Wnt3a, β-catenin, VEGF, and Wnt10b.

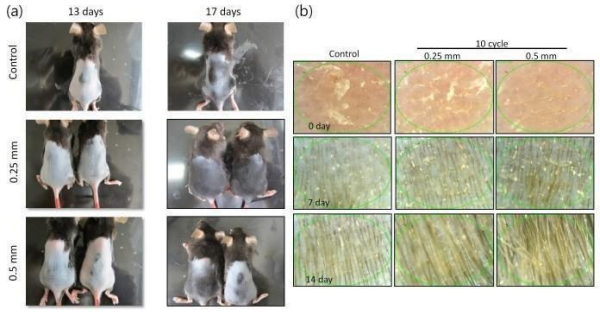

Fig. 1: (a) Visual inspection 13 and 17 days after the first microneedle stimulation showed more prominent hair growth than that in the control group in both the 0.25 mm/10 cycles and 0.5 mm/10 cycles groups. (b) 50-fold magnified photographs taken 7 days and 14 days after the first microneedle stimulation showed uniform hair growth and no visible structural abnormalities in both the 0.25 mm/10 cycles and 0.5 mm/10 cycles groups.

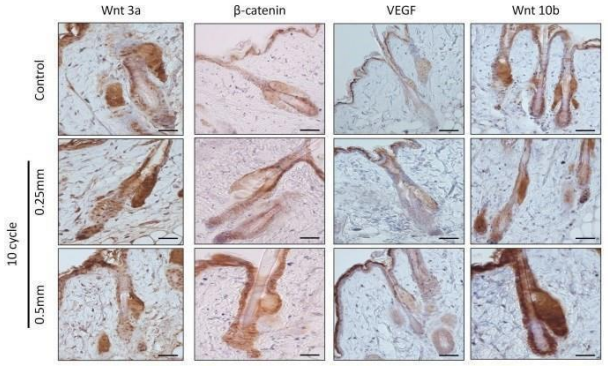

Fig. 2: (a) Immunohistochemistry revealed that expression of Wnt3a, β-catenin, VEGF, and Wnt10b all increased in both the 0.25 mm/10 cycles and 0.5 mm/10 cycles groups compared with the controls (×200, scale bar: 25 μm)

DISCUSSION & CONCLUSIONS: We showed that repeated microneedle stimulation induces hair growth and increases Wnt3a, β-catenin, VEGF, Wnt10b mRNA and protein expression in a murine model. We also demonstrated that 0.25 mm/10 cycles and 0.5 mm/10 cycles showed the best results. Our study provides evidence that microneedle stimulation can induce hair growth via activation of a wound healing process that includes the Wnt/β-catenin pathway and VEGF.

ACKNOWLEDGEMENTS: This study was supported by a National Research Foundation of Korea grant funded by the Korean government (2011-0008687)

Development of an immunocompetent tissue engineered mucosal model to study oral disease

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INTRODUCTION: The role of macrophages in the innate immune response towards pathogens at the oral mucosal surface or in oral cancer progression is widely acknowledged but under investigated. This is because 2D monolayer cultures do not replicate the tissue complexity seen in diseased tissue and so it is often difficult to delineate molecular mechanisms. Tissue-engineered human oral mucosal models have been used but to date these do not contain such innate immune cells as macrophages and so the role of immune cells is often downplayed. Here we develop and characterise an *in vitro* tissue engineered oral mucosal model that contains macrophages to study oral disease processes.

METHODS: Human blood-derived macrophages (Mϕ) or monocyte cell line (THP-1) were cultured with normal oral fibroblasts (NOF) within a type 1 collagen hydrogel. Either immortalized normal oral keratinocytes (iNOK) or oral squamous cell carcinoma (OSCC) cells (H357) were seeded on top of the hydrogel and cultured at an air-to-liquid interface for up to 14 days. The presence, viability and expression of Mϕ markers was analysed by histology and flow cytometry when retrieved from the hydrogel. Mϕ function was measured by cytokine release upon stimulation with LPS. Tumour cell invasion into the collagen matrix in the presence or absence of Mϕ/THP-1 cells was measured by histology and immunohistochemistry.

RESULTS: CD68-positive Mϕ/THP-1 cells were observed in the collagen matrix of both normal and OSCC mucosal models. These cells were >95% viable and expressed known differentiation markers (CD14, CD45, CD80, CD81, CD163, CD206) when analysed by flow cytometry.

Upon stimulation with LPS, Mϕ-containing models secreted significantly more IL-6 than those containing iNOK & NOF or Mϕ alone, showing that the Mϕ contained within the mucosal models are responsive to inflammatory stimuli. Moreover, this response is amplified in multi-cell models suggesting cross talk between Mϕ, keratinocytes and fibroblasts (Fig. 1A).

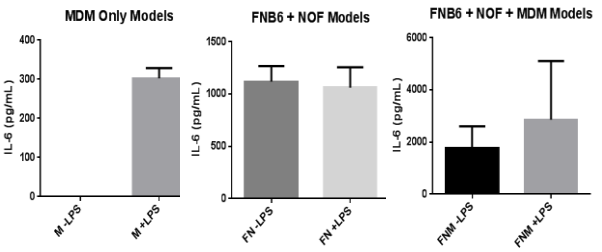

Fig. 1 Release of IL-6 from Mϕ-containing mucosal models.

To investigate the role of Mϕ in OSCC, Mϕ or THP-1 cells were added to the collagen matrix and their effect on normal epithelium or OSCC invasion analysed. Addition of Mϕ derived from healthy volunteers had no effect on either iNOK or OSCC models. In contrast, THP-1 cells caused extensive invasion in both iNOK and OSCC models (Fig. 2) and increased OSCC proliferation. These data suggest that an altered Mϕ phenotype significantly affects epithelial cell biology where as normal Mϕ do not.

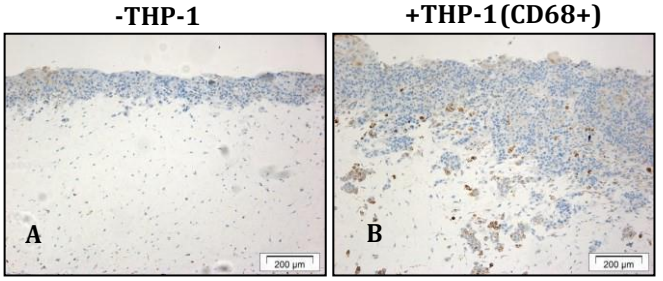

Fig. 2: Histology images showing (A) OSCC model without THP-1 cells and (B) increased tumour cell invasion in the presence of THP-1 cells (CD68+, Brown).

DISCUSSION & CONCLUSIONS: We have developed and characterised an immuno-responsive, tissue-engineered oral mucosal model containing Mϕ that can be used to investigate microbial infection or determine the effects of the immune system in oral cancer biology. This represents a significant advancement in 3D tissue models and paves the way towards the generation of a fully immunocompetent system containing lymphocytes and dendritic cells.

The effects of hydroxyapatite nanoparticle shape on the acute inflammatory response

(*in vitro and in vivo*)

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INTRODUCTION: Wear debris generated from biomaterial implants can activate inflammatory cells, stimulate reactive oxygen species (ROS) release and stimulate bone resorption and aseptic loosening [1]. Few studies have investigated how particle morphology can affect inflammation. In the present study hydroxyapatite (HA) nanoparticles were fabricated with different morphologies (long rods, short rods, sheets, fibers). The production of ROS from primary human monocytes (MNCs) and neutrophils (PMNCs), as well as caspase 3/7 activity, was evaluated following exposure to HANPs.

METHODS: Cell Isolation: Human polymorphonuclear cells (PMNC) and human monocytes (MNCs) were isolated from the buffy coats of anonymous blood donors.

Luminol amplified chemiluminescence: The amount of reactive oxygen species (ROS) released by PMNC and MNC was quantified by luminol amplified chemiluminescence. MNCs and PMNCs were seeded with 100ug/ml of nanoparticle solution at 37°C. MNCs were activated using 0.5μM phorbol myristate acetate (PMA) while PMNCs were activated by 0.25 μM PMA. Measurements were taken every 2 minutes, for up to 2 hours, using a plate reader (Tecan) in the luminescence mode. Total ROS was quantified by integrating the area under the chemiluminescence kinetic curve (AUC) using Origin Software. All measurements were performed in medium containing a 4:1 ratio of PBS to RPMI-1640, 100 μM of glucose, 50 mM luminol, 0.1M NaOH, and 2 μg/ml horse radish peroxidase (HRP).

RESULTS: MNCs produced greater amounts of ROS when exposed to the fiber particles, as compared to the sheet and long rod particles. PMNCs also followed the same trend as the MNCs, although this difference was not found to be significant. The fiber particles also caused increased levels of caspase 3/7, a known marker for apoptosis, indicating that the fiber particles cause increased death via the apoptosis pathway. Preliminary in vivo results further confirm that the fibers induce increased acute inflammation.

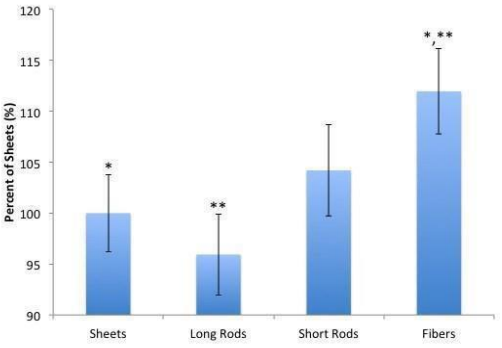

Fig. 1: ROS produced by primary human monocyte/macropahges.

DISCUSSION & CONCLUSIONS: Fiber particles stimulated greater ROS production in vitro, and in vivo, compared to sheets and long rods. The particle shape has been reported to affect the rate of particle internalization and translocation in phagocytic cells [2,3]. Fiber shaped particles may more easily disrupt the cell membrane, similar to the effects of asbestos fibers in connective tissues.

ACKNOWLEDGEMENTS:VINNOVA (VINNMER project 2010-01907).

Cell response to gelatin based hydrogels in the presence of encapsulated macrophages: An *in vitro* model of incoming cell/implanted cell interactions

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INTRODUCTION: In order to introduce a biomaterial with a cellular component, wounding of the target area is inevitable. The implantation or injection of the material result in the mobilization of immune cells. The success of the tissue engineering strategy is strongly related to the inflammatory response, mainly through the activity of macrophages that are key cells in immune response. They can be polarized either into M1 or M2 phenotype, depending on the activation signals. M1 macrophages are considered to be pro-inflammatory, while M2 to promote tissue regeneration[1]. However, it is still not clear how these cells can interact with other incoming cells following implantation. The main objective of this study is to develop an *in-vitro* model of interaction between encapsulated macrophages and incoming cells. Naïve THP-1 monocytes (autogenic cells), HUVEC (allogenic vascular endothelial cells), and 3T3 cells (xenogenic fibroblasts) were selected as incoming cells for this study.

METHODS: THP-1 cells were encapsulated in enzymatically crosslinked gelatin and cultured for 3 days in different conditions (RPMI-1640 with or without 10 ng/ml of IL-4). On the 3rd day, the cell culture medium was removed and incoming cells (HUVEC, THP-1 or 3T3) were added to all the samples (Fig. 1). Cells were cultured for a further 3 days or 6 days. The metabolic activity of those cells was measured by Alamar Blue assay, and cytokine analysis in supernatant was performed with ELISA kit. The cell behavior was analyzed by fluorescence microscopy and scanning electron microscopy (SEM).

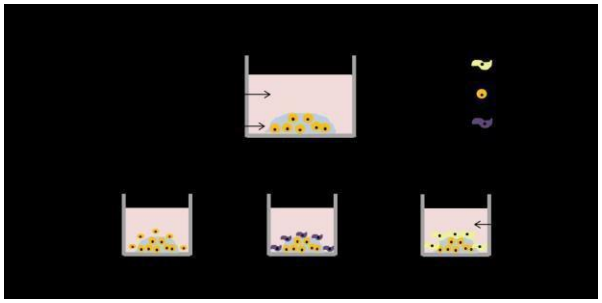

Fig.1: Experimental design of the Hydrogel-based co-culture system.

RESULTS: Addition of cytokine (IL-4) I in the culture medium did not affect encapsulated THP-1 cell viability on day 3. Fluorescence microscopy analysis showed that after 6 days THP-1-laden hydrogels attracted less 3T3 cells and more HUVEC cells on the gel surface, cells were pre-labelled to differentiate between different cell types. Furthermore, after 9 days of culture IL-4-stimulated THP-1 cells attracted more HUVEC cells, whereas more 3T3 cells were attracted in the absence of IL-4. This was confirmed by SEM analysis.

Cytokine analyses of supernatants from THP-1/THP-1 co-culture showed less M1 macrophage phenotype (TNF-α release) before the addition of incoming cells culture when IL-4 was added to the culture medium. However, for the THP-1/HUVEC and THP-1/3T3 co-culture experiments, there was no difference in TNF-α release as a function of the IL-4 on the medium. After 6 days, for all the co-culture condition, M2 macrophage phenotype was observed even though the absence of IL-4 on the medium (significant CCL18, IL-10, IL-1RA release).

DISCUSSION & CONCLUSIONS: The design and maturation of an engineered tissue is important for its integration within the host. However, the initial reaction of the incoming cells also has a determining effect on the final outcome. Here we demonstrated that the encapsulated macrophages, mimicking resident macrophages, have an impact on the behavior of incoming cells in a cell-type specific manner. Such a model can be used to better understand possible immunoengineering strategies using macrophages and also in studies of immune response to xenogenic and allogenic cells in a controlled manner.

ACKNOWLEDGEMENTS: "EU FP7 grant agreement no. 602694" (IMMODGEL).

Biomechanical cues in in situ cardiovascular tissue engineering – the role of shear stress

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INTRODUCTION: New synthetic, bioresorbable and off-the-shelf available synthetic heart valve replacements are being developed that aim to restore heart valve functionality for a lifetime. These heart valve substitutes should be able to take over heart valve functionality while providing a temporary roadmap for endogenous cells to create new, living and functional heart valve tissue *in situ*. Following this rationale, we have developed such acellular synthetic heart valves, manufactured from porous electrospun scaffolds. Upon implantation, these valves evoked a regenerative response *in situ*, ignited by the host' inflammatory response. Interestingly, these valves showed a strongly increased tissue formation on the pulmonary side of the heart valve leaflet compared to the ventricular side, which is likely to be caused by the divergent flow patterns on either scaffold surfaces. Divergent flow patterns cause different wall shear stresses (WSS), which may affect cell infiltration, activation and subsequent tissue formation [1,2].

Therefore, it is the aim of this study to investigate *in vitro* the influence of physiologically relevant shear stress levels on the early inflammatory responses (cell infiltration, activation and cytokine secretion) after scaffold implantation.

METHODS: 3D electrospun polycaprolactone (PCL) scaffolds were placed in a custom-made flow chamber that was connected to a mesofluidics-based test platform, which mimics the hemodynamic environment [3]. The flow chambers were adapted to locally vary and control the WSS on the scaffold surface. The scaffolds were exposed to a circulating cell suspension of human peripheral blood mononuclear cells in pulsatile flow with low (0.7 Pa) or high (7 Pa) peak WSS (mimicking the WSS on the pulmonary and the ventricular side of the heart valve leaflet, respectively) for 24 hrs. Samples of the circulating cell population were analysed over time with flow cytometry to assess cell depletion, as an indirect measure of cell infiltration into the scaffold. After 24 hrs the scaffolds were sacrificed and further processed for 1) immunostainings, 2) tissue assays and 3) gene expression analysis.

RESULTS: The low peak WSS on the scaffold surface resulted in a more pronounced decrease in the number of circulating monocytes up to 3 hours of flow compared to the high WSS (figure 1), while cell viability remained unaffected. This suggests an accelerated infiltration of monocytes into the scaffold at low WSS. No difference was seen between the types of monocyte subsets that infiltrated in both groups [4].

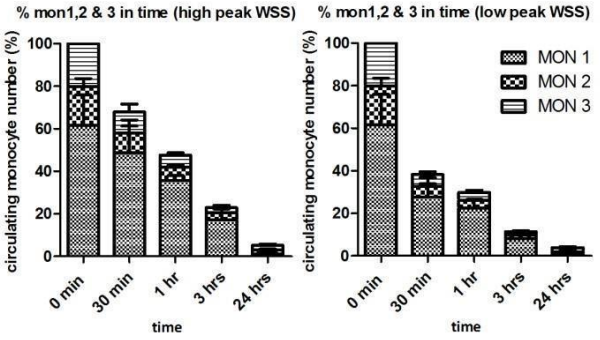

Fig. 1: Average percentages (±SD) of the circulating monocyte subsets per WSS group in time. Cell counts were corrected for the circulating cell number and normalized on the initial monocyte count of the starting PBMC population (n=3 per group).

DISCUSSION & CONCLUSIONS: These results show that the local WSS may have an effect on the infiltrating monocyte number. Future analysis will focus on the gene expression patterns between the different conditions to investigate the effect of a divergent WSS on cell activation (pro/anti-inflammatory) and eventually tissue regeneration.

ACKNOWLEDGEMENTS: We acknowledge the support from the Netherlands Cardio Vascular Research Initiative: The Dutch Heart Foundation, Dutch Federation of University Medical Centres, the Netherlands Organisation for Health Research and Development and the Royal Netherlands Academy of Sciences.

Three-dimensional interpenetrating networks to modulate the differentiation of mesenchymal stem cells

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INTRODUCTION: Mechanical cues such as substrate stiffness can direct different biological processes [1]. How cells sense and react to such mechanical stimulation in 3D is still poorly understood. In this work we analysed how stem cell shape and matrix stiffness regulate chondrogenesis in a physiologically relevant 3D environment. To this end, we developed an interpenetrating network (IPN) hydrogel consisting of type I collagen and alginate, which allowed us to tailor both the stiffness of the gel as well as the shape a MSC would adopt in this environment. The overall objective of this study was to investigate the interplay between cell shape and substrate stiffness in regulating the differentiation of MSC, and to explore specific molecular mechanisms of mechanotransduction.

METHODS: IPNs are made by final concentrations of 1.5% low molecular weight alginate (Pronova) and 1.3 mg/ml of rat-tail collagen type-I (Corning). This blend was mixed with $5x10^6$ cells/ml p2 porcine MSCs and transferred to a custom-made mold to obtain gels of cylindrical shape. Variation of the incubation time before the cross-linking of alginate, led to a modulation of cell morphology, while varying the length of exposure to $CaCl_2$ or sodium citrate produced gels whit different stiffness. Constructs were mechanically tested and maintained in a chemically defined media supplemented with 10ng/ml TGF-β3 in a low oxygen environment and assessed at day 0, 7 and 21 in terms of cell viability, biochemical content, qRT-PCR and histologically.

RESULTS: The stiffness of the IPN hydrogels decreased from approximately 18 kPa to 4 kPa by decreasing $CaCl_2$ mediated cross-linking of the alginate network. The Young's Modulus of the constructs was constant over 21 days of culture. Cell morphology was successfully modulated (Fig. 1), obtaining a round or spread shape group in both soft and stiff gels. MSC remained viable in the 3D IPNs, depositing a matrix rich in glycosaminoglycans (GAG) and collagens.

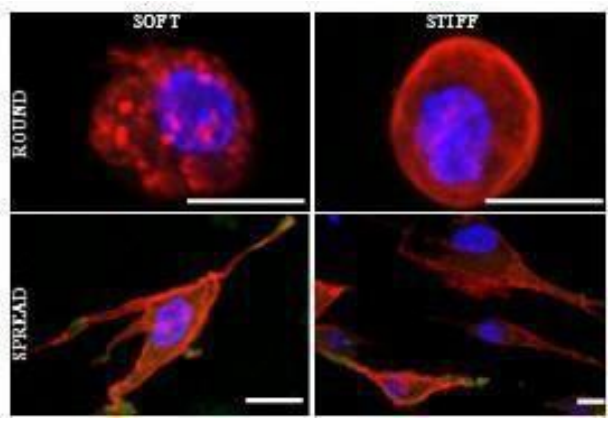

Fig. 1: Immunofluorescence for actin (red) vinculin (green) and nuclei (blue). Morphological validation of MSC embedded in 3D-IPNs gels.

Total matrix depended more on cell shape than on the stiffness of the local environment, with greater chondrogenesis with round MSC. These results were confirmed by gene expression analysis. Moreover, localization of molecules involved in the adhesion dynamics and mechanotransduction pathways of MSCs, such as vinculin and Yap, were analysed in these 3D-IPNs. YAP was found to be predominantly cytoplasmic, even in stiff gels.

DISCUSSION & CONCLUSIONS: Mimicking the complexity of ECM adhesion elements is required to reproduce the biological 3D microenvironment. IPNs can satisfy this requirement and furthermore can be used to modulate the morphology and contractility of MSCs. In particular, our study demonstrates that cell morphology has a higher impact on chondrogenesis then local substrate stiffness. All together these results gave us a better understanding of the MSC mechanobiology in a 3D environment, which has important implications for the development of novel cartilage tissue engineering strategies.

ACKNOWLEDGEMENTS: This study was supported by Science Foundation Ireland (12/IA/1554; 12/US/I2489). We wish to confirm that there are no known conflicts of interest

Chondrogenic differentiation and collagen synthesis of human chondrocytes in the absence of ascorbic acid

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INTRODUCTION: The introduction of automation in the manufacturing of engineered cartilage will be crucial to standardize production methods and to maximize prospective scale-up and cost-effectiveness in the long-term. However, the limited stability of certain bioactive supplements currently used in cartilage engineering requires specific components to be stored at different temperatures and poses significant challenges to the automation of liquid handling.
Ascorbic acid (AA) is generally considered to be a critical supplement for cartilage tissue engineering. Due to its instability, AA is typically added from frozen aliquots at each medium change, preventing straightforward automation. The aim of this study was to investigate its effect on the chondrogenesis of human nasal chondrocytes (hNC) in order to define appropriate requirements for automation strategies.

METHODS: hNC were re-differentiated as micromass pellets for 2 weeks in chondrogenic medium with or without ascorbic acid-2-phosphate (AA2P), a more stable derivative of AA. Cell viability was assessed by TUNEL assay. Safranin-O staining and biochemical analyses verified glycosaminoglycans (GAG) deposition. Collagen type I and II were visualized via immunohistochemistry and quantified by ELISA. mRNA expression of collagen types I & II, Sox-9, aggrecan, and versican was quantified by RT-PCR. The structure and organization of collagen fibrils were observed with scanning electron microscopy.

RESULTS: The omission of AA2P did not affect cell viability. hNC expression of all genes analysed was similar with or without AA2P. Pellets cultured with or without AA2P had similar Safranin-O staining, consistent with quantification of GAG/DNA. Immunohistochemistry of pellets showed that hNC cultured without AA2P had synthesized abundant type II collagen. While the amount of type II collagen was 35% less in pellets without AA2P, the hydroxylation of total collagen was not significantly different. SEM analysis showed a similar collagen fibrils in pellets cultured with or without AA2P.

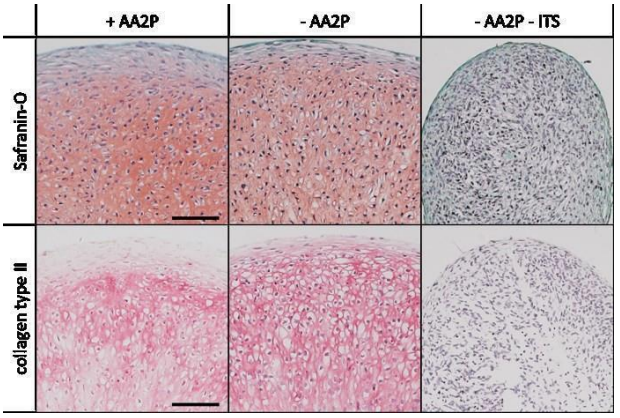

Fig. 1: Safranin-O and collagen type II staining of pellets cultured in presence or absence of AA2P. Scale bar 200 µm.

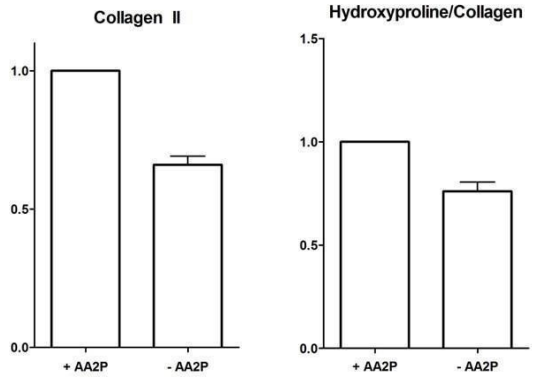

Fig. 2: Quantification of collagen type II and hydroxyproline (normalized to +AA2P).

DISCUSSION & CONCLUSIONS: Taken together, our results demonstrate that the addition of AA2P to hNC cultures had little influence on chondrogenic differentiation and the extracellular matrix accumulation and structural organization. Therefore, AA2P potentially may not be required for a cartilage tissue engineering process. Although the amount of collagen type II was slightly reduced in the absence of AA2P, the impact on the quality/potency of the engineered cartilage will need to be investigated to address the question "how good is good enough?" and to ultimately define relevant quality release criteria.

ACKNOWLEDGEMENTS: Funding has been received under the EU FP7 Programme ("BIO-COMET"; No. 278807).

Tissue formation by hMSCs undergoing dynamic compression in a hybrid scaffold

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INTRODUCTION: Mechanical stimuli and substrate stiffness are known to play a key role on tissue formation and mineralization capabilities of cells [1]. Whereas soft scaffolds are better to mimic the extracellular environment, they are not able to support sufficient load for physiological loading. Thus, we propose a 3D hybrid scaffold embedding collagen gel as support for dynamic mechanical stimulation of mesenchymal stem cells. The objective of this study consisted in assessing the role of short bursts of compression on tissue formation and mineralization in a hybrid construct closely mimicking bone architecture.

METHODS: ***Collagen/hMSCs injection.*** 3D Insert® PCL (3D Biotek, USA) underwent air plasma treatment (5 min, 30W, 1 mBar). hMSCs ($20*10^{\wedge 3}$ cells/sample) were suspended in 2 mg/ml bovine type 1 collagen and statically seeded on samples. ***Mechanical stimulation.*** From day 6, samples were compressed applying a 5% ramp followed by peak-to-peak 1% sinewaves at 1 Hz for 15 min. Three different conditions were tested considering unloaded (U), loaded from day 6 to day 10 (L1) and loaded as L1 and from day 16 to day 20 (L2) samples. ***Assays and MicroCT.*** At day 1, 3, 7, 14, 21 and 28, samples were collected for testing cell viability, DNA and osteocalcin content. Samples were stained with 1% osmium tetroxide, scanned by MicroCT and reconstructed by ScanIP (Simpleware, UK).

RESULTS: Tissue content (Fig.1 top) experienced an increase at day 21 for all conditions although L1 gave the highest amount. The growth of tissue was enhanced on the surface (OUT) of scaffolds, while the internal content (IN) amounted to 40±12%, 45±7% and 47±8% respectively for U, L1 and L2 samples. Cells in L2 samples did not show any significant proliferation but enhanced deposition of ECM. Mineral (Fig.1 bottom) in U was detected at day 14 amounting to 2 mm^3 and 0.5 mm^3 respectively OUT and IN. On the contrary, cells in L1 samples produced mineral from day 7 but the mineral phase at day 28 was only 25% of the amount detected in U. Conversely, mineral content in L2 was 30% higher at day 28 compared to U ($p<0.05$). Moreover, the mineralization IN for L2 samples was 3-fold higher than the other tested conditions from day 21.

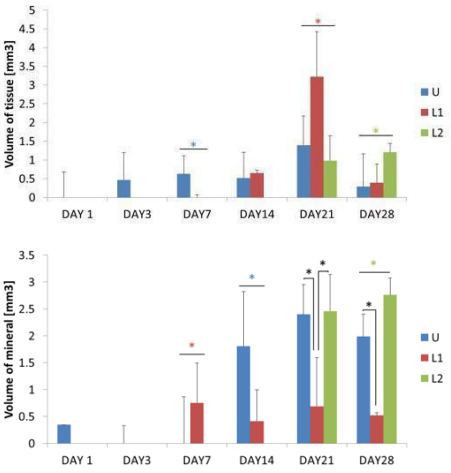

*Fig. 1: Volume of tissue (top) and mineral (bottom) quantified by MicroCT (*p<0.05).*

DISCUSSION & CONCLUSIONS: Tissue growth involved volumes either inside or outside the ROI at day 21 for L1, suggesting cyclic stimulation as a trigger for increased proliferation when applied at early stage of culture. Cyclic load is also playing a role in the mineralization process preventing crystal deposition when applied at early stage of culture. Conversely, applying cyclic load at late stage of culture on pre-compressed samples induced mineral formation. This study shows that short bursts of compression are effective in modifying tissue formation and mineralization potential of hMSCs over time, opening the frontiers to a new approach aiming to use mechanical stimulation for the development of engineered in vitro tissue as replacement for large bone fractures.

ACKNOWLEDGEMENTS: Funding from the European Research Council (FP7-258321) is acknowledged.

DISCLOSURE: The authors have nothing to disclose.

A dual environment 3D printed bioreactor for kidney tissue engineering

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INTRODUCTION: The instances of end stage renal failure are increasing year on year at huge economic and social cost. Tissue engineering has great potential for finding a renal replacement therapy with research taking many streams including; decellularised tissue, 2D cell culture, 3D printing and bioartificial kidneys[1,2]. Although some progress has been made in developing a bioartificial kidney, tissue engineering of the kidney is still in its infancy.

Current strategies aim to create embryonic stage kidneys which could later be transplanted for maturation, although such constructs have shown a limited survival in cell culture conditions. This may in part be due to culture in a single static environment. The immature kidney consists of the ureteric bud and metanephric mesenchyme, shown to have different expression which is key to the kidney development[3]. It is hypothesised that due to the single environment in current culture systems cells are confronted with conflicting signals. The aim of our device is to mimic *in vivo* conditions using a 3D dual environment to examine the influence on kidney cell organisation and growth.

METHODS: The device was designed using CAD software (SolidEdge) for minimal handling when constructed. Tubing connectors were integrated into the design, with a screw thread for easy assembly and a nitrile O-ring is to seal the device.

The viscosity of cell culture media was modified using xanthan gum to better represent blood flow. Rheology testing was performed at varying shear rates with the results used as parameters for computational fluid dynamics (CFD). Artificial urine was used as the second flow condition.

The flow channel design was analysed using CFD (ANSYS) to ensure a maximum area of the scaffold is exposed to 0.1 Pa of shear stress[4].

Devices were 3D printed in VeroClear, and scaffolds electrospun from 14 wt% PCL in Chloroform methanol (5:1). After sterilization the bioreactor was seeded with 100,000 RC-124 human kidney epithelia cells and conditioned for 24 hours.

Devices were validated by cell viability assay (CellTitre-Blue), DNA quantification assay (PicoGreen), RT-qPCR (GAPDH, AQP-2, KRT-8, ANPEP, E-CAD) and fluorescence microscopy.

RESULTS: Viscosity investigation showed that 0.5 g/L xanthan gum produced a viscosity parameters comparable to that of blood. CFD analysis indicated evenly distributed shear at 1.67 ml/min. The experimental set-up can be seen below with viable cells after 24 hours of conditioning, within the dual environment (*Fig.1)*.

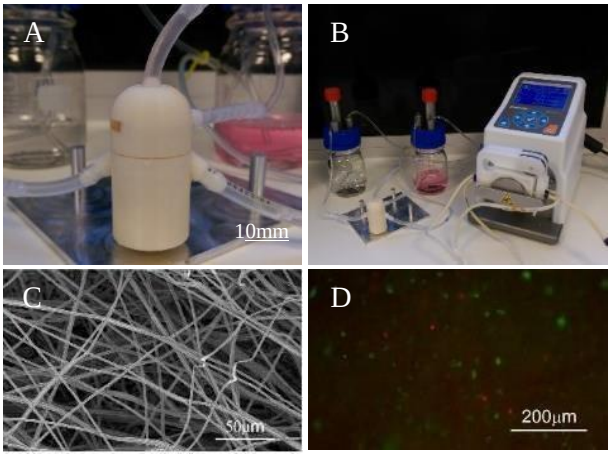

Fig. 1: The experimental set-up (A, B), SEM of the electrospun scaffolds (C) and a Live/Dead image after 24 hours in the dual environment (D) (Live-Green, Dead-Red).

DISCUSSION & CONCLUSIONS: This study displays the potential of cell conditioning within a dual-environment. The results highlight the promise of a complex 3D printed bioreactor environment to aid kidney cell organisation and proliferation. Further work with iPSC or embryonic cells is needed to assess the devices effects on self-organisation.

ACKNOWLEDGEMENTS: This research is funded by an Engineering and Physical Sciences Research Council (EPSRC) Doctoral Training Partnership (DTP) Studentship.

Rotational dual-chamber bioreactor to generate 3D tissue interfaces

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INTRODUCTION: Advances in Tissue Engineering (TE) are changing the paradigm of cell culture, by altering it from 2D to 3D. This is enabling the ability to improve the complexity of in vitro created tissues[1]. Along with the 3D structures, in vitro disease modelling better mimics the native environment, allowing the pre-clinical trials to be more efficient in the future. However, cell culture systems, as tissue culture well-plates and bioreactors, are not evolving at the same speed rate of the constructs being reported in the TE field. There are now some cell culture platforms for spheroids and small constructs. However there is a lack of technology adapted to recreate tissue interfaces or barriers which are essential for drug efficacy and regenerative medicine, as skin, pulmonary or intestinal membranes or even osteochondral and bone-tendon interfaces, for which we developed a cell culture system.

METHODS: A rotational dual-chamber bioreactor was fabricated able to rotate in vertical and horizontal directions. Dual-chambers were designed creating two different environments through two inlets and outlets. A cyclic compression drive was also created. As proof of concept assay was performed in a 3D low acyl gellan gum (2% w/v) sponge using a vertical turning delay of 30mins during 48h under a flow perfusion of 600µL/min, seeding 200'000 human adipose-derived stem cells.

RESULTS: A bioreactor comprising a multiposition stirred platform on which are connected dual chambers by magnetic attraction, was developed (figure 1). The dual chambers can have rotational movement, which is horizontal and/or vertical. The multi-chamber bioreactor is adapted for co-culturing different cell types allowing the generation of multi-cell type interfaced tissues. The chamber cap allows cyclic compression over the respective cell-scaffold construct, creating a physical stimulus. The bioreactor was tested for the vertical turning movement and is now under optimization for each variable parameter during cell culture. In figure 2 it is possible to observe the effect of this movement in metabolic activity, which can be correlated to cell number, and in cell viability and distribution.

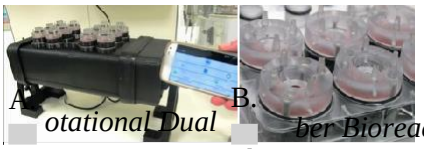

Fig. 1: A. Rotational Dual-chamber Bioreactor, B. Dual-chambers adaptable to 6-well culture plates. [2]

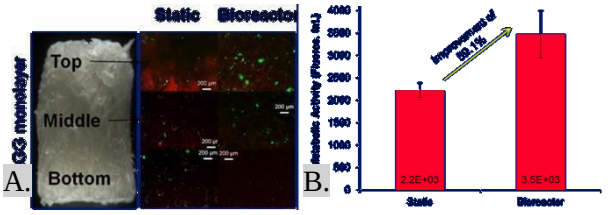

Fig. 2: A. Live/dead assay, B. Metabolic activity

DISCUSSION & CONCLUSIONS: In figure 2 is possible to see the effect of the turning movement during cell adhesion in cell distribution and metabolic activity. The size of the scaffold is dependent on the ability of the medium to enter and exit the interior of the 3D structure. By introducing the stirring movement, the diffusion potential in 3D structures increases. This allows the use of bigger scaffolds. The compression drive can be important for applications on osteochondral and bone-tendon TE. The described technology has the potential to be used in the development of different in vitro tissue model platforms, either 3D interfaces or 2D membranes. These can allow replacing or decreasing the animal models for scientific research and in the pharmaceutical field for drug screening and evaluation during pre-clinical trials.

ACKNOWLEDGEMENTS: This study received funding from Portuguese Foundation for Science and Technology through the project OsteoCart (PTDC/CTM-BPC/115977/2009) and the Raphaël Canadas' PhD grant (SFRH/BD/92565/2013).

Mechanical cues and cell shape regulate the myogenic versus chondrogenic fate decision of MSCs

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INTRODUCTION: Multipotent stromal cells (MSCs) can be isolated from numerous different tissues and induced to differentiate towards multiple lineages, making them an attractive cell type for musculoskeletal tissue engineering. Previous studies have demonstrated that the application of uniaxial cyclic tensile strain (CTS) can enhance myogenesis and suppress chondrogenesis of MSCs seeded on 2D substrates [1,2]. However, there is currently a paucity of studies investigating the influence of CTS on MSCs encapsulated in physiologically relevant 3D matrices. Here, we present a novel bioreactor for application of CTS to 3D cell laden hydrogels. This bioreactor is then used to investigate the influence of CTS on the chondrogenic vs. myogenic fate decision of MSCs and the dependency of this response on (i) substrate stiffness and (ii) cell morphology.

METHODS: A novel bioreactor system (Fig.1) was developed to facilitate application of precise uniaxial tensile strains to 3D hydrogels under controlled, sterile conditions. Bone marrow derived MSCs were encapsulated in collagen/alginate blended hydrogels and maintained in bioreactors in MSC differentiation medium. CTS was applied for 4 hours per day for 4 days at a strain magnitude of 10% and frequency of 0.5Hz. Substrate stiffness (soft vs. stiff) was controlled by varying alginate concentration. Cell morphology (round vs. spread, Fig. 2) was controlled by introducing a delay period of 12 hours prior to alginate crosslinking for spread groups. Cell lineage commitment was evaluated via relative gene expression analyses (RT-PCR) and confocal imaging of cytoskeletal components.

RESULTS: Cyclic tensile strain (CTS) was found to enhance the expression of myogenic markers (↑ACTA2, MYH1, CNN1) regardless of substrate stiffness, but inhibit chondrogenesis of MSCs encapsulated in stiffer matrices only (↓COL2A1, ACAN). Furthermore, CTS was shown to synergize with a spread cellular morphology to enhance markers of myogenesis.

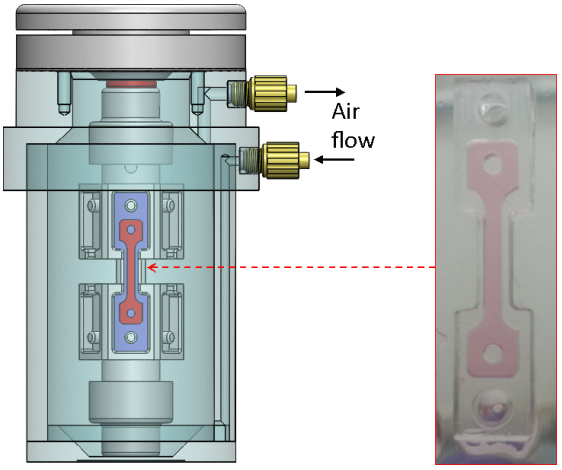

Figure 1: A novel bioreactor was developed to apply uniaxial tensile strain to 3D hydrogels

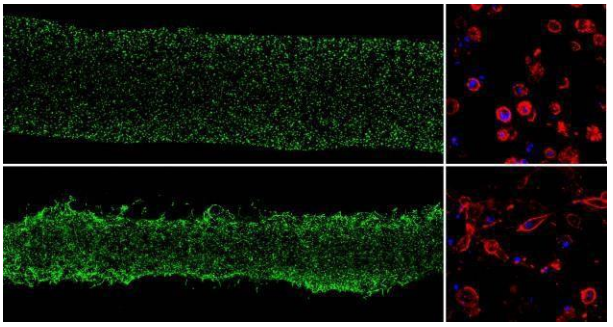

Figure 2: Live/Dead (left) and F-actin (right) staining of round (top) and spread (bottom) MSCs in 3D collagen/alginate hydrogels.

DISCUSSION & CONCLUSIONS: A key challenge when developing successful tissue engineering strategies is to elucidate the influence of biophysical cues on stem cell fate. The application of CTS was shown to direct MSC fate by suppressing chondrogenic markers and promoting a myogenic phenotype. However, these effects were found to be dependent on various environmental factors, suggesting that the local substrate must be optimized to synergize with mechanical stimuli to direct MSC differentiation.

Cyclic tensile strain enhances both intramembranous and endochondral ossification of MSCs encapsulated within fibrin hydrogels

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INTRODUCTION: Multipotent stromal cells (MSCs) can be isolated from numerous different tissues and induced to differentiate towards multiple lineages, making them an attractive cell type for bone tissue engineering. Bone development occurs via two distinct mechanisms: *intramembranous ossification* (direct differentiation of mesenchymal stem cells into osteoblasts) and *endochondral ossification* (the formation of a cartilage template and its replacement by bone) [1]. The latter is the process by which long bones are formed. It has long been suggested that ossification during limb development occurs in the regions of maximal tensile strains [2], while *in vitro* studies have demonstrated that the application of uniaxial cyclic tensile strain (CTS) to MSCs cultured on 2D substrates can induce osteogenesis [3]. However, few studies have investigated how CTS influences osteogenic differentiation of MSCs in more clinically relevant 3D grafts. Here we utilize a custom bioreactor system to investigate the influence of CTS on MSC lineage commitment in the absence of growth factors. Recognizing that long bones form via a cartilage intermediary, we then examined the influence of long term CTS on chondrogenesis and endochondral ossification of MSCs encapsulated in 3D hydrogels.

METHODS: A custom bioreactor system was utilized to apply precise uniaxial tensile strains to 3D hydrogels under controlled, sterile conditions. Bone marrow derived MSCs were encapsulated in fibrin hydrogels and maintained in these bioreactors (in medium ± TGF-β3). CTS (5% strain, 0.5 Hz; 10% strain 0.5 Hz; 10% strain, 1 Hz) was applied for 4 hours per day for up to 21 days. Cell lineage commitment was evaluated via biochemical assays, relative gene expression analyses (RT-PCR) and confocal imaging of cytoskeletal components. Gene expression data are present as fold-differences (± standard error) normalized to the mean of the free-swelling (non-loaded) control group and significance was accepted at a level of $P<0.05$.

RESULTS: CTS was found to promote intramembranous ossification and inhibit adipogenesis of fibrin encapsulated MSCs in the absence of chondrogenic growth factors (data not shown). In chondrogenic differentiation medium, CTS promoted proteoglycan and collagen content, cellular alignment (Fig.1) and endochondral ossification of MSCs in a magnitude and frequency dependent manner. Of the range examined, a regimen of 10% strain at 1Hz resulted in maximum expression of various endochondral markers (Fig. 2) in chondro-inductive medium.

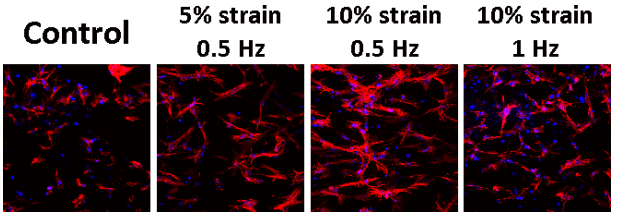

Figure 1: F-actin staining of MSCs encapsulated in 3D fibrin hydrogels after 3 weeks of cyclic tensile strain

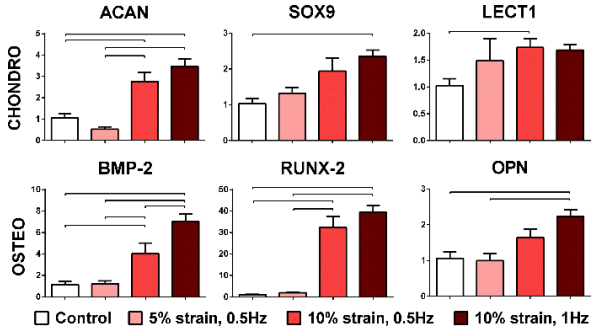

Figure 2: Relative endochondral gene expression of MSCs encapsulated in 3D fibrin hydrogels after 3 weeks of cyclic tensile strain.

DISCUSSION & CONCLUSIONS: A key challenge when developing successful bone tissue engineering strategies is to elucidate the influence of biophysical cues on stem cell fate. In this study, we demonstrate that mechanical strain can enhance both direct intramembranous and indirect endochondral ossification of MSCs in 3D fibrin gels. The results of this study point to the importance of *in vivo* mechanical cues for determining the terminal phenotype of MSCs

Dynamic bioreactor culture in hypoxic conditions facilitates the development of large cartilage tissues using mesenchymal stem cells

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INTRODUCTION: Mesenchymal stem cells (MSCs) cultured in three dimensional (3D) hydrogels are capable of producing robust cartilage tissue. However nutritional diffusion gradients arise during 3D cell culture resulting in cartilage tissues with significant spatial distributions in matrix components [1]. This problem becomes exacerbated when attempting to engineer larger cartilage tissues using MSCs [2]. Previous attempts at employing dynamic bioreactor culture regimes to overcome these nutritional limitations have reported suppression of MSC chondrogenesis compared to static culture conditions [3]. We hypothesize that this suppression is due to the enhanced oxygen availability that arises under dynamic culture, as higher oxygen tensions are known to suppress chondrogenesis of MSCs [4]. The objective of this study was therefore to develop a dynamic bioreactor system capable of engineering large cartilage tissues with a homogenous spatial distribution of matrix components by controlling the levels of oxygen and nutrient delivery throughout MSC laden alginate hydrogels.

METHODS: A dynamic culture regime was developed using a custom made bioreactor. Briefly a fixation platform containing MSC laden alginate hydrogels was attached to a linear actuator and translated through a bath of media at a speed of 0.05mm/s and an amplitude of 6mm (Fig 1a). This culture regime was directly compared to static free swelling conditions where the actuator was not translated. Small (5mmØ X 3mm) and large (10mmØ X 6mm) channeled (Ø = 1mm) MSC laden alginate hydrogels were cultured in either dynamic or static conditions for 28 days in chondrogenic media. The influence of the external oxygen tension on the response to the dynamic culture regime was also explored by performing the experiment in both normoxic (20% $p0_2$) and hypoxic (3% $p0_2$) conditions.

RESULTS: Significantly higher sGAG accumulation was found with dynamic culture compared to static conditions in larger constructs (10mmØ X 6mm) cultured at 3% $p0_2$ (Fig 1b). The response to such dynamic culture was dependent on the external oxygen tension, as this culture regime significantly suppressed sGAG production at 20% $p0_2$ for all sized constructs (Fig 1b). Histological analysis revealed that the core regions of large statically cultured samples stained weakly for sGAG (Fig 1c). However dynamic culture at 3% $p0_2$ resulted in the development of a tissue with a homogenous distribution of sGAG (Fig 1c). Weak sGAG staining were present in constructs cultured at 20$p0_2$% in dynamic conditions (Fig 1c).

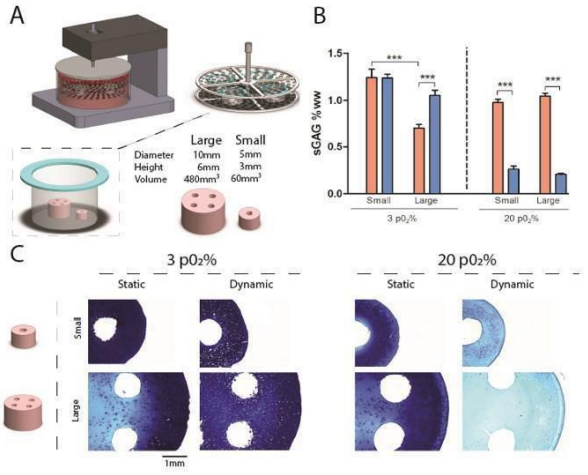

Fig. 3: (a) Bioreactor Design (b) Biochemical sGAG content normalized (%ww) (ANOVA, P<0.01) & (c) Histological staining for sGAG after 28 days of culture.

DISCUSSION & CONCLUSIONS: Taken together these results demonstrate that dynamic culture regimes that provide adequate nutrient and an appropriate oxygen environment can be employed to engineer cartilage tissues of scale with homogenous distributions of matrix components. Such dynamic culture regimes will be required to engineer larger cartilage tissues for treatment of large chondral defects and whole osteoarthritic surfaces.

Modyflow – an open-source bioreactor and testing device

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INTRODUCTION:

The raising demands on testing devices for biomedical research require more and more complex systems. The increasing complexity and specialization of these devices render comparisons across different research groups nearly impossible. Moreover, the financial aspect of the development of these devices tends to grow a limiting factor. The idea of this project was to develop a new standardized testing device for cardiovascular research topics and to publically provide it as open source project.

METHODS:

To allow a broad application and acceptance of the device, it was designed in a highly modular way. This way, adaptions to individual requirements are rendered possible. Furthermore, unique features were added to provide an incentive to use the device and to facilitate the working process.

RESULTS:

A highly complex stainless steel bioreactor was developed with four main modules with exchangeable connectors. This way, different testing environments can be created easily (fig. 1). The device can provide physiologic flow conditions, due to an integrated compliance and resistance. A specialized insertion sluice is integrated, allowing the placement of TAVI-implants during operation.

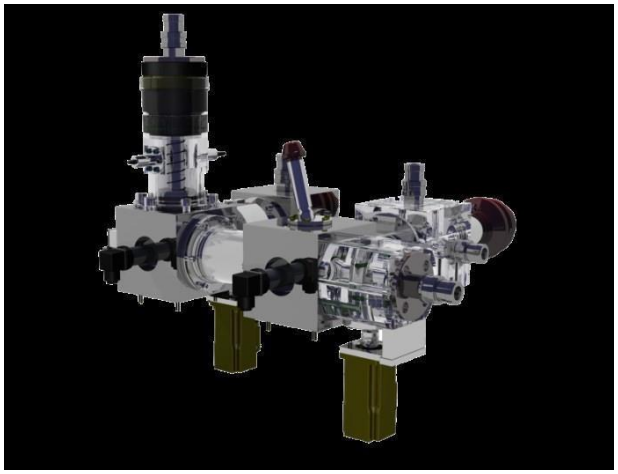

Fig. 1: Modular Bioreactor Modyflow

Multiple sensors (temperature, pH, O2, flow and pressure) and actuators are integrated to control all necessary environmental conditions. Visual observation is possible via a high-speed camera. The device is controlled via a programmable logic control (PLC) which allows industrial standard real-time surveillance of the device. The PLC allows a simple adaption of sensors, actuators and software which further supports the modularity of the device. All necessary data are bundled in the PLC and presented to the user in a clear and structured application.

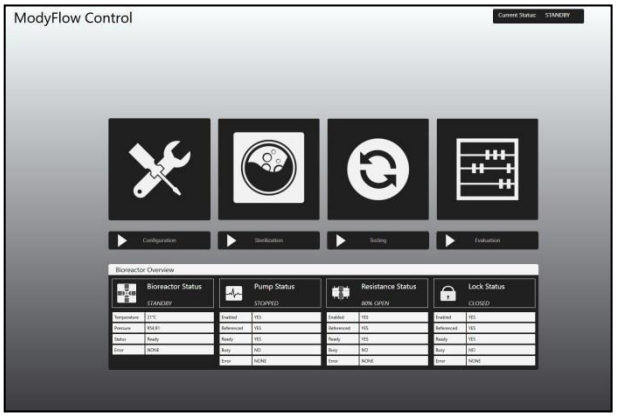

Fig. 2: User Interface of Control Application

Finally, the device offers unique features, such as an integrated sterilization procedure and means for wireless and remote control. To promote the use of the device, all necessary data is available online (www.modyflow.com), with the aim to create a new standard in cardiovascular research. Technical drawings, PLC configuration and source code are freely available.

DISCUSSION & CONCLUSION:

The introduced device provides the means to support multiple different research topics. Its modular design and highly adaptable software provides flexibility needed in modern biomedical research. The open-source availability allows researchers access to an advanced testing device applicable in many fields of cardiovascular research.

DISCLOSURE STATEMENT:

The authors have nothing to disclose.

Translation of vibration loading system from 2D into 3D setting for osteoinduction of human adipose stem cells

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INTRODUCTION: Certain types of mechanical strains are known to stimulate bone formation. This can be seen in athletes *e.g.* tennis players have higher bone mineral density in their dominant playing arm [1]. Mechanical stimulus can also be sensed by mesenchymal stem cells. We showed previously that high magnitude, high frequency (HMHF) vibration loading can be used to enhance osteogenic differentiation of human adipose stem cells (hASCs) [2]. In contrast, adipogenic differentiation of hASCs was inhibited by HMHF vibration loading. The aim of this work was to translate our vibration loading system from 2D into 3D setting. Four different hydrogels were evaluated for the 3D experiment. Finally, osteogenic differentiation of hASCs was evaluated after two weeks of vibrationstimulation.

METHODS: *Vibration loading:* In-house developed vibration loading device was used to stimulate cells by vertical HMHF (100 Hz, 2.5 G_{peak}) vibration. Cells were plated on hydrogels at 48-well plates, which were stacked firmly into well plate holders on top of the subwoofer-based (Lab12, Eminence speaker) device. Vibration parameters were controlled and monitored by LabVIEW-based graphical user interface.

Selection of suitable hydrogel: Bovine collagen type I (Gibco) at 2.5-3.0 mg/ml, rat tail collagen type I at 2.5 mg/ml (Gibco), gellan gum 5 mg/ml (Sigma) mixed with 10% collagen type I (GG-Col), and hydrazone crosslinked hyaluronic acid (HA-HA) (synthesized at Tampere University of Technology) hydrogels were compared in cell culture for hASC morphology and viability. Gel volume was 200 µl on 48-well plate (1.8 mm thickness) and different cell densities from 250 000 to 100 000 c/200 µl were tested.

Analyses: The effects on cell viability (Live/Dead, Invitrogen), cytoskeleton (phalloidin-TRITC, Sigma), cell number (CyQuant, Invitrogen), alkaline phosphatase activity (qALP, Sigma), and gene expression (qRT-PCR, Applied Biosystems) were analysed after 14 days of culture in osteogenic medium [2].

RESULTS: *Selection of suitable hydrogel:* Cells cultured in GG-Col and HA-HA presented rounded morphology, whereas in both collagen gels hASCs were able to attach, spread and proliferate. The HA-HA hydrogels were difficult to handle due to fast formation time (seconds). GG-Col detached from the wells during vibration loading. Rat and bovine collagen I were able to withstand the vibration loading and were selected for further experiments.

Vibration stimulation in 3D: The first experiment was conducted with 3.0 mg/ml bovine collagen I with 250 000 c/200 µl gel. Cell morphology and viability (Live/Dead and phalloidin stainings) were similar between static control and vibrated cells after 14 days. There was no difference in cell number or qALP between static and vibrated cells. However, gene expression of osteogenic markers such as DLX5, Runx2A and ALP was higher in vibrated cells. At 21 days the gels started to shrink and detached from the edges of the wells. Rat tail collagen I showed less shrinkage than bovine collagen I. Therefore, rat tail collagen I with lower cell density (100 000 c/200 µl) was selected for further vibration experiments, which are still under way.

DISCUSSION & CONCLUSIONS: Bovine collagen type I was selected as the 3D matrix and hASCs seeded in the hydrogels were able to withstand HMHF vibration loading with no adverse effect on viability or cell number. A trend of higher expression of osteogenic genes was detected in vibrated cells versus static control. Further repeats of the experiments are conducted with rat tail collagen I.

ACKNOWLEDGEMENTS: Thanks to Prof. Minna Kellomäki and MSc Jennika Karvinen (Tampere University of Technology) for HA-HA hydrogels.

Bioreactor for small diameter vascular grafts

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INTRODUCTION: Designing of vascular grafts (VG) suggests study of endothelial/progenitor cell (EC) interaction with inner VG surface and their functioning in a conditions mimicking blood flow. Different kinds of bioreactors (e.g. BOSE Inc or custom designed [1]) were used for testing of cell growth and shearing stress was shown to be the main factor influencing on EC [2]. Here we offer simple bioreactor for testing of EC interaction with inner surface of VG (diameter 1-3 mm, 50-60 mm long) and preparation of cell loaded VG for subsequent *in vivo* study.

METHODS: Bioreactor consists of autoclavable module (AM) installed in 100×100×16 Petry dish (Sarstedt), block of electromagnets (BE) setted in CO_2-incubator and outer controller (fig 1). VG is installed in AM filled with culture medium. Controller provides 2 testing regimes (rotation and pumping) and 3 operating regimes (cycles of rotation and pumping). The inductors have inductivity 11,8 mH and actuates membrane pump or rotation unit. BE is fully protected from corrosion. Endothelial cells from human umbilical vein (HUVEC) were cultivated in IM DMEM supplied with 10% FBS and antibiotics at the surface of VG produced by electrospinning (1,7 mm inner diameter, 150±20 um wall thickness, 50 mm long) from polycaprolactone/gelatin solution in 1,1,1,3,3,3-hexafluoro-2-propanol and supplied with blood cell impermeable inner layer [3]. To test bioreactor VG was installed in sterile AM, AM was filled with 35 ml of culture medium and incubated for 24 h in CO_2-incubator. Then 1×10^5cells (100 ul) were injected into VG closed with a cap and incubated 24 h in a seeding mode (rotation) and then 48 h in a flow mode (rotation and pumping).

RESULTS: In a seeding mode rotation vary from 90° once per 30 s for the first hour to 90° once per 5 min for next 24 hours. In a flow mode VG was rotated once per 3 min with subsequent 3 pulses of the pump (50 µl pulse per 300 ms with 700 ms interval). The conditions listed do not cause heating of electromagnets and culture medium. Cell distribution and orientation studied under SteReo Discovery V12 in a fluorescent mode (cells were stained with SIBR Green I) demonstrate uniform cell distribution, good seeding efficacy and orientation of the cells across (just after seeding) or along (after cultivation in a flow mode) VG.

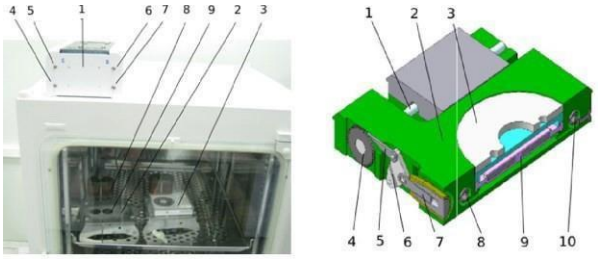

Fig. 1: (A) Bioreactor consist of 1 controller, 2 block of electromagnets and 3 autoclavable module. 4,6 Selector of testing regimes; 5,7 Selector of operating regimes; 8 Solenoid actuator of membrane pump; 9 Solenoid actuator of rotation unit. (B) Autoclavable module. 1 vascular graft; 2 basic AM unit; 3 membrane pump; 4 Rotation unit with cogwheel; 5 pusher; 6 rocker, 7 steel core covered with silicon rubber; 8 output valve; 9 silicon membrane with a steel core; 10 input valve

DISCUSSION & CONCLUSIONS: Simple, inexpensive and easy to use bioreactor is presented. The bioreactor was successfully used for HUVEC cultivation onto inner walls of VG and preparation of cell-loaded VG for subsequent transplantation and *in vivo* experiments.

ACKNOWLEDGEMENTS: This work was supported by a Russian Science Foundation grant (№ 14-15-00493).

Quantitative analysis of cell-matrix interaction

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INTRODUCTION: Extracellular matrix provides a dynamic environment acting as a critical regulator of cell behaviour. Two general mechanosensing pathways have been so far described to integrate the mechanical forces arising from the cell milieu: the focal adhesion (FA) complexes that link integrin signalling and actin cytoskeleton, and the Hippo Pathway through its main effector YAP1. Although it has been already evidenced that FA control the Hippo pathway through a FAK–Src–PI3K cascade [1], a bidirectional crosstalk has not been established yet. Here we develop an *in vitro* analysis model allowing us to quantitatively evaluate the interplay between the two pathways at a single cell level, thus providing a quantitative measure of cell-matrix interaction.

METHODS: Immortalized adipose tissue-derived mesenchymal stem cells were cultured on fibronectin (FN)-coated micropatterned surfaces with different adhesion area by controlling the availability of binding sites. Cells were fixed and stained for VCL as a focal adhesion marker and for YAP1. Single-plane images were obtained by confocal microscopy under identical scan settings. YAP1 nucleus/cytoplasm ratio was calculated using an Image J plug-in modified from Horzum *et al*. [2]. FA number, size and total area were determined using a customized Image J plug-in [3]. The method was tested also in gain and loss of function experiments for both YAP and VCL and in different coating conditions (FN, Poly-L-Lys).

RESULTS: The image analysis reveals a nuclear YAP accumulation logarithmic dependence on the spreading area. The YAP1 nucleus/cytoplasm ratio was not significantly decreased in cells with reduced adhesion area when compared with the one with the same spreading surface, showing that YAP nuclear function is controlled by cell area rather than by the availability of adhesion sites. Similarly, we also found that FA number and total area are increasing in a linear way with cell spreading area, and there were no significant changes between cells with different adhesion area but the same spreading surface. When YAP is impaired, a significant reduction in FA spike formation could be noticed. In cells missing FAs a similar alteration in the ratio between the nuclear and cytoplasmic forms of YAP1 was detected, thus evidencing an interplay between the two systems.

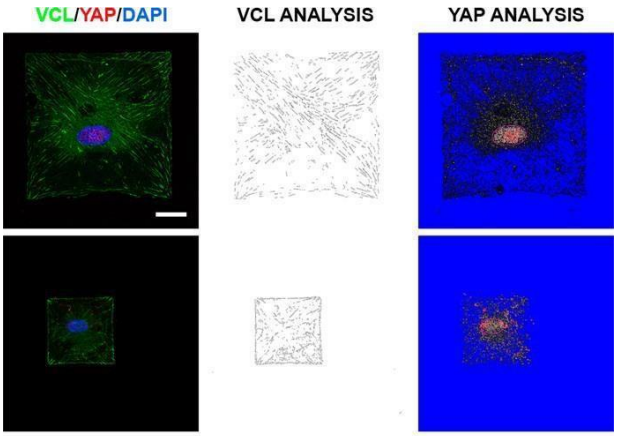

Fig. 1: Representative image of the input (left) and output (center, righ) of the analysis for cells with an area of 10000 µm^2 (up) and 2025 µm^2 (down). Scale bar: 20 µm.

DISCUSSION & CONCLUSIONS: Our system allows for a sensitive FA and YAP quantification at a single cell level, suited to detect slight alterations in both pathways. The first analysis indicates that both FAs and YAP are dependent on the spreading rather than on the availability of adhesion sites. Methods employed to disrupt either FAs or YAP1 expression alter the other pathway, suggesting a reciprocal interaction amongthem.

ACKNOWLEDGEMENTS: Supported by the project no. LQ1605 from the National Program of Sustainability II and FNUSA-ICRC (No. CZ.1.05/1.1.00/02.0123). MA Sáez-Soro for his technical assistance.

Low-frequency pulsed electromagnetic fields as treatment for bone healing

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INTRODUCTION: Electrical potential and ionic current exist naturally within cells. Any alteration of electrical potential and ionic current can lead to changes in cell signaling pathways and gene expression, resulting in differences in proliferation and differentiation. Leveraging on the potential of Pulse Electromagnetic Frequency (PEMF) to guide cells growth and differentiation, research efforts have been geared towards the use of PEMF to accelerate the bone regeneration process either in combination with growth factors [1] or cells [2]. In this study, we examined the osteogenic capabilities of hAMSCs when subjected to different selected PEMF configuration.

METHODS: In Study 1, hAMSC plated at a density of 1 x10^4 cells/cm^2 were treated with different PEMF configurations (CIT 4, 10, 16, 18, 20, 31, 64, 81, 114 or 124), once a day for 5 days per week (Monday to Friday). Cell proliferation, cell matrix mineralization and osteogenesis-related gene expression was examined. Cells cultured in osteogenic medium without PEMF treatment were used as control. In study 2, hAMSCs were plated with a density of 1 x 10^4 cells/cm^2 and cultured in osteogenic medium. Cells were subjected to CIT 20 treatment and left to rest for up to 6 hours. At pre-determined time points, samples were collected for protein array.

RESULTS: Across all groups, no significant different of cell proliferation and ALP activity was detected between control and hAMSCs treated with different PEMF configurations. In terms of gene expression, COL1A1 gene expression was significantly upregulated for CIT 20 and 124 treated cells. Notably, the ALP and osteocalcin gene expression of CIT 20 treated cells were significantly upregulated compared to control. Using a protein array, it was revealed that selected ERK1/2 and AKT signaling cascade were simulated for hAMSCs when subjected to CIT 20 treatment.

DISCUSSION & CONCLUSIONS: To date, there is still no consensus of the optimal PEMF configurations required to enhance osteogenic potential of cells. Although we did not observe any significant difference in terms of cell proliferation, ALP activity and the extent of matrix mineralisation between PEMF simulated and non-simulated hAMSCs (control), the osteoblasts maturation process can be characterised by the temporal expression of osteogenic-related genes [3]. In terms of gene expression, it was observed that hAMSCs treated with PEMF 20 exhibit a more prominent differentiation into osteogenic lineage compared to other treatment groups. Therefore, CIT 20 was chosen to further elucidate the underlying regulatory mechanism on osteogenic differentiation of hAMSCs when simulated by PEMF. Our results indicate that CIT 20 simultaneously activated the ERK1/2 and Akt pathways which are necessary to promote cell proliferation and protein translation [4]. We have shown that PEMF can be used to modulate cells osteogenic capabilities and has high potential to be used in combination with cell therapy to facilitate bone regeneration.

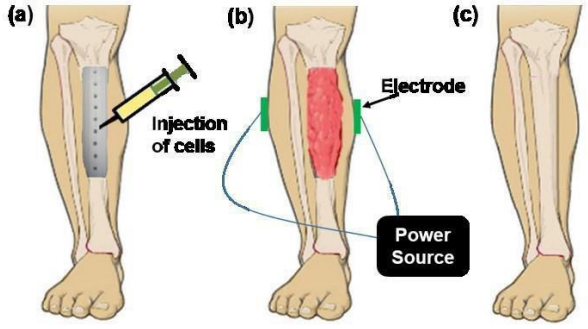

Figure 1 (a) Therapeutics cell source (e.g. hAMSCs) can be injected into the defect site whereby polymeric scaffold is used as a temporary supporting structure. (b) After which, PEMF device can be apply to drive hAMSCs towards the osteogenic lineage. (c) It is of ideal that the PEMF configuration is altered dependent on the stage of the bone regeneration process to accelerate the remodeling progression resulting in a fully regenerated bone.

[2] **ACKNOWLEDGEMENTS:** We'd like to thank Sachtleben GmbH for supporting the study.

On the role of scaffold pore geometry in stem cell mechanotransduction

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INTRODUCTION: A lot of effort has focused on the development of bone graft substitutes to accelerate bone repair. Silk fibroin scaffolds (SFSC) have shown to be highly suitable for bone tissue engineering (BTE) [1]. SFSC can be produced by several methods, such as salt leaching (SL) or inverse opal (IO). Compared to salt leached scaffolds (SLSC), IO scaffolds (IOSC) are homogeneous and structured scaffolds. IOSC have been shown to enhance extracellular matrix (ECM) mineralization compared to SLSC under static cell culture conditions, suggesting their potential for BTE. Differences in mineralized ECM were attributed to pore diameter distributions or pore geometry. BTE constructs aim to mimic the structural but also the mechanical characteristics of natural tissue [2]. It is well known that cells respond to mechanical stimuli. Incorporation of bioreactors in BTE eliminates nutrient transfer limitations of static cultures but also provide mechanical stimulation to the cells, mimicking the *in vivo* environment of bone. The aim of this study was to assess the potential of IOSC for BTE applications. In this study, we evaluated and compared the osteogenic effects of SFSC with two pore geometries SL and IO, respectively - under mechanical stimulation. Molecular biology and imaging techniques were combined to provide a better insight on how pore geometry influences how cells respond to mechanical stimuli. A better understanding on how cells sense and respond to mechanical stimulation depending on the scaffold pore geometry will help on the design scaffolds for BTE.

METHODS: SLSC were prepared as described previously [3]. IOSC were prepared by assembling monodisperse and spherical pore template microparticles into a crystalline lattice before infiltration with aqueous silk fibroin solution. Human mesenchymal stem cells were cultured on both scaffold types for up to 49 days with osteogenic supplementation in spinner flask bioreactors (70rpm). Cell distribution and collagen synthesis was assessed by Haematoxylin and Sirius red (SR) staining. Expression of bone related genes was quantified by real-time RT-PCR. Formation and spatial distribution of mineralized ECM was evaluated by micro-computed tomography.

RESULTS: Although spinner flask bioreactors supported homogeneous mineralized ECM formation in both IOSC and SLSC, the amount of mineralized ECM volume fraction (BV/TV) was nearly doubled for IOSC. On the contrary, SLSC enhanced collagen formation, as revealed by SR staining (especially at the edge of the scaffolds) and significantly upregulated of Collagen-I (Coll-I) ($p=0.016$) and Osteocalcin (OC) gene expression ($p=0.001$) compared to IOSC.

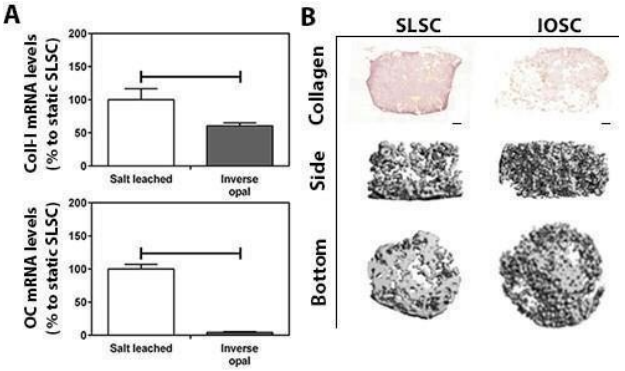

Fig. 1: (A) Coll-1 and OC relative mRNA levels, (B) collagen formation (scale bar 500µm) and 3D mineralized ECM.

DISCUSSION & CONCLUSIONS: Based on the results observed, it can be assumed that the differences in pore geometry of IOSC compared to SLSC have a direct effect on how cells sense and respond to mechanical stimulation. These results might suggest that homogeneous and structured IOSC enhanced cell differentiation and thus formation of a highly mineralized tissue compared to SLSC, however further research will be needed.

ACKNOWLEDGEMENTS: This project received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreements no. 329389, 262948 and 618603.

Effect of pH on nasal chondrocyte proliferation & re-differentiation

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INTRODUCTION: In conventional cell/tissue culture processes, culture medium is typically exchanged batch-wise at regular time intervals. This approach can lead to significant & uncontrolled fluctuations in pH. While pH has been shown to affect numerous cell types, there are currently no reports on the effect of pH on human nasal chondrocytes (NC). Thus, we aimed to assess: i) the effects of defined pH levels on NC proliferation, & ii) if the pH level during the expansion phase would affect the subsequent re-differentiation of NC.

METHODS: NC were expanded in media buffered to six different defined pH levels (6.5, 6.8, 7.2, 7.4, 7.6, 8.0). pH levels were monitored using PreSens sensor plates. Expanded NC were then re-differentiated in 3D pellet cultures in serum-free medium (pH approx. 7.4 for all pellets). To determine if pH differently affected the growth rates of subpopulations, NC were stained with fluorescent proliferation tracking dye carboxyfluorescein diacetate succinimidyl ester (CFSE), expanded at the six pH levels, analyzed by FACS, & data modeled with ModFit LT v4.

RESULTS: After five days of expansion, cell numbers at pH 6.8 & 7.2 were only 45% & 76% of those obtained at pH 7.4, respectively [Fig. 1]. We next determined that the pH during the expansion phase affected the subsequent re-differentiation capacity of NC. Pellets generated from NC expanded at pH 7.2 & 7.4 had the most uniform & intense Safranin-O staining [Fig. 2], the highest amount of GAG/DNA, & the highest ratio of collagen II:I mRNA (>20-fold higher). Analysis of CFSE stained NC showed heterogeneous growth rates. The mean & shape of the cell division distributions depended upon pH, with the highest mean division numbers for pH 7.2 & 7.4 [Fig. 3].

DISCUSSION & CONCLUSIONS: We have shown that rather small differences in pH can significantly influence the expansion of NC. Moreover, the pH level during expansion influences the subsequent re-differentiation of NC. The CFSE data further shows that not all cells within the pooled populations are affected equally, as the shape of the distribution for each pH level is different.

We now aim to implement the results of this study by defining pH based in-process controls in the bioreactor-based manufacturing of engineered cartilage for clinical applications in order to improve process standardization & product quality.

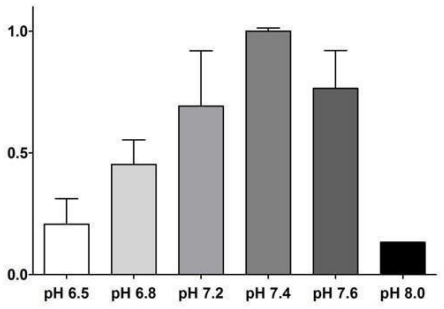

Fig. 1: Cell numbers after 5 days of expansion (values normalized to pH 7.4).

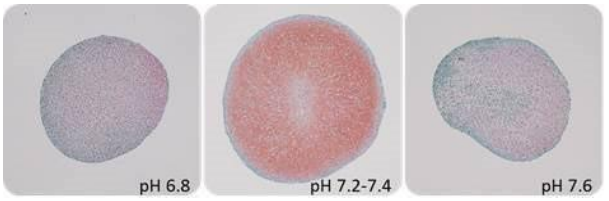

Fig. 2: Representative Safranin-O stained pellets generated from NC expanded at specific pH levels.

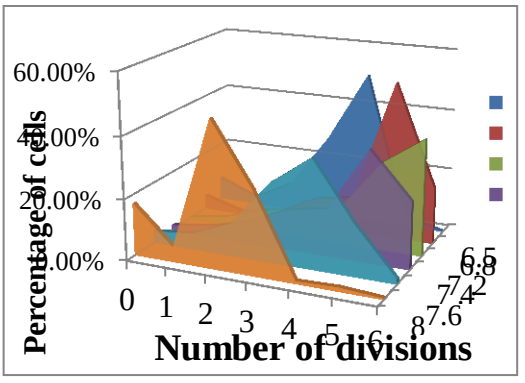

Fig. 3: FACS analysis of CFSE stained NC. At each pH level, % cells from the original cell population that divided during 5 days expansion.

ACKNOWLEDGEMENTS: Funding has been received under the EU FP7 Programme ("BIO-COMET"; No. 278807).

Poly (ε-caprolactone) scaffolds combined with extruded perfusion bioreactor prototypes as an integrated platform for articular cartilage tissue engineering

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INTRODUCTION: Articular cartilage tissue engineering combining cells, biomaterials and chemical/physical cues have appeared as an attractive alternative to the therapeutic strategies currently used, since they allow a better mimicking of the native cartilage microenvironment and hold the promise of achieving functional tissue [1,2]. Poly (ε-caprolactone) (PCL) is a biodegradable and biocompatible synthetic polymer, which can be easily processed into 3D scaffolds with desired geometry, porosity and high interconnectivity using additive manufacturing (AM) techniques, such as fused-deposition modelling (FDM) [3]. In this work, we hypothesized that the dynamic culture of PCL scaffolds seeded with bone marrow mesenchymal stem/stromal cells (BM MSC) in a custom-made perfusion bioreactor will improve the quality of the articular cartilage produced.

METHODS: PCL scaffolds were produced by FDM, characterized morphologically by SEM/μ-CT and in terms of their mechanical properties under compressive loading using a Instron® 5544 machine. Bioreactor prototypes were designed with the SolidWorks® software and produced in acrylonitrile butadiene styrene (ABS) using a MakerBot Replicator™ 2x. For cell culture studies, BM MSC were seeded on PCL scaffolds ($15x10^4$ cells/scaffold) and cultured under standard expansion conditions for 14 days. Afterwards, the constructs were placed in the bioreactor prototypes and cultured under perfusion (0.2mL/min) and in static conditions with chondrogenic media for 21 days. All the culture studies were performed under hypoxia (5% O_2) and equivalent cell numbers were obtained over time by AlamarBlue™ assay. The final tissue constructs were evaluated by Alcian Blue staining followed by the quantification of the sulfated glycosaminoglycans (GAGs) content. The expression of chondrogenic marker genes was also evaluated by qRT-PCR.

RESULTS: The cell-scaffold constructs cultured under perfusion conditions stained positively for GAGs formation (Fig.1 (A)) and presented a statistically significant higher content of sulfated GAGs when compared to the constructs cultured under static conditions (Fig.1 (B)). Moreover, perfusion conditions resulted in higher expressions of chondrogenic genes (COL II, Sox9, Aggrecan) and lower expressions of COL I and Runx2 (Fig.1 (C)). Chondrocyte hypertrophy was suggested by the higher expression of COL X, which highlights the need for culture conditions optimization, as well as the study of other materials/chemical cues to obtain native-like articular cartilagetissue.

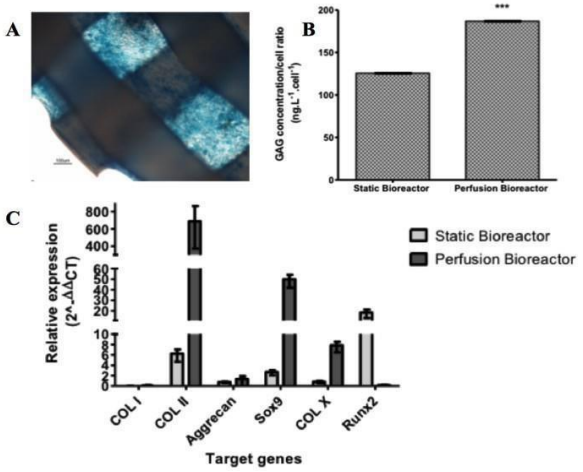

*Fig. 1: (A) Microscopic image of Alcian Blue staining protocol of a tissue construct cultured in perfusion conditions. (B) Quantification of sulfated GAG production per cell (***P < 0.001). (C) Gene expressions at the end of culture for perfusion and static conditions. Expressions are relative to GAPDH and normalized to day 0 of culture.*

DISCUSSION & CONCLUSIONS: Our findings demonstrated the suitability of our customized and versatile platform for articular cartilage tissue engineering and suggest a positive effect of shear stress stimuli in chondrogenesis of BM MSC seeded on PCL scaffolds.

ACKNOWLEDGEMENTS: Funding by FCT through the grants SFRH/BD/105771/2014 and UID/BIO/04565/2013 and by PORL 2020 (No 007317).

Automated perfusion-decellularization – Raising quality, reproducibility and safety in development of tissue engineered vascular grafts

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INTRODUCTION: Tissue-engineered vascular prostheses are a promising alternative for autologous and synthetic vessels in arterial bypass grafting. Therefore, the present study aimed at establishing a novel automated method for the acquisition of decellularized vascular grafts.

METHODS: Scaffolds were obtained from human saphenous vein segments (n=30). Native veins were bisected. One half was processed in a new decellularization (DC) bioreactor with a tailor-made protocol. The second half functioned as reference group and was treated according to a standard DC protocol. All steps were realized at 37°C.

The bioreactor allowed an automatic perfusion by a continuous pulsatile flow (60BPM; 10ml/min) with a 1% SD-SDS solution. Veins were incubated for 8, 16 or 24h (each n=10) respectively, followed by six subsequent perfusion washing cycles of 24h with PBS.

The reference group was treated by single detergent injection and immersion DC during incubation in a thermal rocker for the mentioned times. Afterwards immersion washing in PBS was performed in six cycles of 24h.

Samples were taken before and after DC. The efficiency of the DC process was evaluated with DAPI and HE staining, DNA quantification and subsequent gel electrophoresis. Histological evaluation was assessed by Movat's Pentachrome and Picrosirius-Red staining. Picrosirius staining further allowed the examination of elastin (green autofluorescence) and collagen (rhodamine channel) by fluorescence microscopy. Surface topography was evaluated by scanning electron microscopy (SEM).

RESULTS: DAPI and HE staining confirmed the DC efficiency of the perfusion-based setting after 16h and 24h (Fig. 1A): No residual cell nuclei were detected in automatically decellularized veins. Furthermore DNA quantification and gel electrophoresis substantiated these findings. However after 8h perfusion treatment single residual nuclei were detected. In contrast, the manually decellularized reference group showed an insufficient and inhomogeneous DC (Fig. 1B) at all times. The persistence of significant amounts of residual cell nuclei was revealed for all treatment times. DNA quantification methods validated these results.

Histological evaluation by Pentachrome and Picrosirius-Red staining demonstrated the preservation of the structural integrity of native extra cellular matrix (ECM). No signs of damage to the scaffolds could be discovered in all groups at all times.

SEM evaluation showed the removal of the superficial cell layer and a smooth and homogenous surface topography after perfusion DC. Furthermore, no structural damages combined with remaining fiber integrity proved the preservation of the ECM.

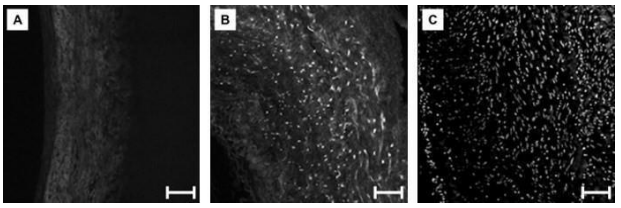

Fig. 1; DAPI staining: After 24h DC automated perfusion (A) no residual cell nuclei were detectable. Conventional treatment (B) revealed insufficient and inhomogeneous DC after 24 h. Native sample (C) showed a homogenous spread of cell nuclei in the wall of the vessel. Scale bars = 100µm

DISCUSSION & CONCLUSIONS: Our new DC bioreactor with tailor-made processing protocols provides the means to achieve complete and reproducible DC of vascular grafts. Additionally, the bioreactor aided procedure highly facilitates the DC process and leads to a superior DC efficiency compared to manual procedures while preserving ECM and simplifying handling.

Corneal endothelium differentiation capability of human MSC

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INTRODUCTION: The human cornea endothelium plays an important role in maintaining the hydration levels of the cornea stroma. These important functions are carried out by a very important Na/K ATPase allocated at the lateral area of the cells which are able to synthetize a specialized basement membrane called Descemet membrane consisting of collagens type IV and VIII, but it also contains fibronectin, vitronectin and various laminins [1]. Several works previously demonstrated that some stem cell markers such as Oct-3/4, Wnt-1, Pax-6 and Sox-2 could become upregulated in the posterior periphery of the endothelium during endothelial regeneration [2].
Due to its limited in vivo regeneration capability, cell therapy could be a therapeutic option for patients with endothelial dysfunction. However, human endothelial cells culture is still very challenging, and new alternative cell sources should be explored. In the present work, we evaluated the endothelial induction capability of four major sources of mesenchymal stem cells.

METHODS: Primary cell cultures of human MSC were established from 4 different tissue biopsies: adipose tissue (ADSC), bone marrow (BMSC), dental pulp (DPSC) and Wharton's jelly (HWJSC), using enzymatic digestion. Each type of MSC was cultured in DMEM medium with 10% foetal bovine serum following standard culture conditions. To evaluate the endothelial differentiation capability of each MSC type, cells were cultured in conditioning differentiation medium containing epithelial growth factor (EGF), adenine, hydrocortisone, insulin, triiodothyronine and cholera toxin for 28 days. Then, RNA corresponding to control and induced cells was isolated and the expression level of each human transcript was quantified by using Affymetrix Human Genome U133 plus 2.0 oligonucleotide microarrays. Probe-sets related to cornea endothelial differentiation showing upregulation upon induction were selected to determine which MSC type has higher differentiation potential for use in tissue engineering.

RESULTS: Out of the 159 probe-sets analysed here, 6 (FN1, COL4A3, COL8A1, LAMA3, SOX2, ATP1A2) became upregulated in all 4 MSC, whereas 26 probe-sets were upregulated by 3 MSC types, 57 in 2 and 53 in one type of MSC. 17 probe-sets were not upregulated by any of the cell types. The cell type showing higher number of probe-sets upregulated upon induction (99) was ADSC, followed by HWJSC (63), BMSC (59) and DPSC (48) (Figure 1).

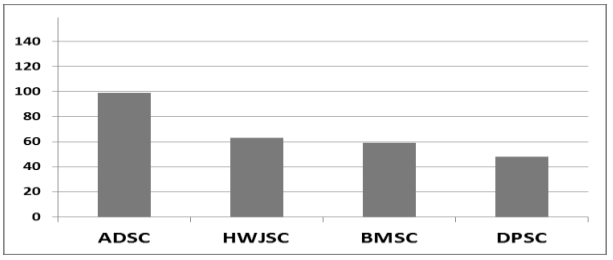

Figure 1. Number of probe-sets upregulated upon induction by the different MSC.

DISCUSSION & CONCLUSIONS: These results suggest that the four MSC types analysed here have potential for endothelial differentiation, although relevant differences exist among cell types. We would therefore recommend the use of ADSC as the most promising MSC type for endothelial cell induction using conditioning medium.

ACKNOWLEDGEMENTS: This study was supported by the Spanish Plan Estatal de Investigación Científica, Desarrollo e Innovación Tecnológica (I+D+I) 2013-2016 from the Spanish Ministry of Economy and Competitiveness (Instituto de Salud Carlos III), grant FIS PI14/0955 (co-financed by FEDER funds, European Union), and by Fundación Benéfica Anticáncer San Francisco Javier y Santa Cándida, Granada.

Ex vivo and *in vivo* evaluation of nanostructured bio-artificial nerve guide

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INTRODUCTION: Nerves are complex organs which have the capability to partially regenerate their components following structural damage. Short nerve gaps can be surgically repaired, but critical gaps require the use of nerve guides or grafting materials, with nerve autografts being the gold standard treatment. Unfortunately, there are several drawbacks and limitations associated to the autograft technique and nerve guides. Therefore, reconstruction and functional recovery of patients with critical nerve injuries are still important challenges in medicine and tissue engineering [1].

METHODS: In this study, autologous rat adipose stem cells (ASC) were isolated, expanded and used to generate fibrin-agarose hydrogels (FAH). These constructs were nanostructured for the elaboration of fasciculated nerve substitutes (Figure 1a,b) and then subjected to ex vivo histological and ultrastructural characterization. For in vivo analysis, 1 cm of the left sciatic nerve was removed from a group of rats and the nerve gap was repair by using autograft technique (n=5) or nanostructured bio-artificial nerve guides (n=5). After 12 weeks post repair, animals were subjected to electromyography, functional, gross anatomical and volumetric analyses andhistology.

RESULTS: Ex vivo analyses revealed that ASC were able to grow and proliferate into the nanostructured FAH over the time. Interestingly, ASC showed variations of PCNA and cortactin immunostaining with time and showed a progressive synthesis of extracellular matrix molecules.

In vivo analyses showed that nanostructured bio-artificial nerve guides were successfully used to bridge a critical nerve gap in the rat model. Time-course electromyography confirms the progressive re-innervation of the distal muscle in both groups. Time-course functional analyses showed a progressive recovery of the motor and sensory function in both groups, especially in autograft.

Histological analyses revealed the presence of nanostructured FAH 12 weeks after implantation. The immunohistochemical analysis clearly showed newly-formed axonal sprouts crossing the gap through the implanted substitute (Fig. 1c). In the case of autograft, the grafted nerve was completely remodeled and supports the peripheral nerve regeneration at the epineurial, perineurial and endoneurial levels (Fig. 1d).

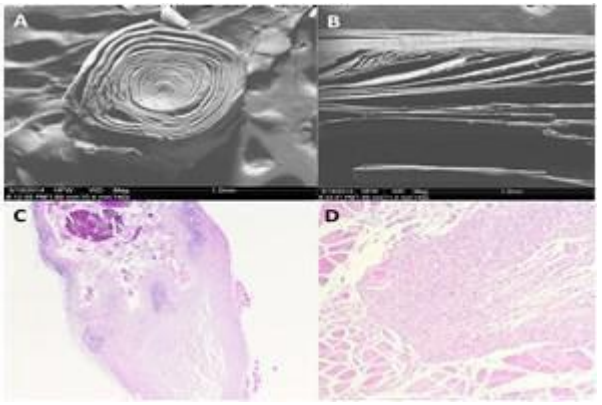

Fig. 1. Ex-vivo and in vivo representative images of nanostructured bio-artificial nerve guides.

DISCUSSION & CONCLUSIONS: We have generated a novel biodegradable and mechanically stable nerve guide based on the use of FAH and ASC. These guides were able to successfully bridge a critical nerve gap in a rat model, supporting a promising peripheral nerve regeneration profile from the functional and histological point of view. However, these results were not completely comparable to the autograft group and therefore, additional improvement of the model is still needed as an efficient and safe alternative to bridge critical nerve gaps.

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ACKNOWLEDGEMENTS: This study was supported by the Spanish Plan Estatal de Investigación Científica, Desarrollo e Innovación Tecnológica (I+D+I) 2013-2016 from the Spanish Ministry of Economy and Competitiveness (Instituto de Salud Carlos III), grant FIS PI14/1343 (co-financed by FEDER funds, European Union).

Can the application of hydrostatic pressure to 3D ESC-models enhance the maturation of engineered cartilage tissue?

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INTRODUCTION: Degeneration of cartilage resulting from injury or disease, combined with its notoriously poor capacity for repair, has created a high clinical demand for regenerative medicine constructs. Human embryonic stem cells (hESC) are pluripotent, thus offering an allogeneic cell source for this application. Recent advances in culture techniques have allowed for the production of xeno-free chondroprogenitors from hESC [1]. Previous work demonstrated that the application of these hESC-derived chondroprogenitors, when encapsulated in fibrin gels, enhanced cartilage repair in osteochondral defects of rat models [2]. Mechanical cues contribute to early cartilage development *in vivo.* The application of hydrostatic pressure an important physical stimulus has a positive effect *in vitro* [3]. This study aims to investigate the effects of hydrostatic pressure on *in vitro* 3D models using hESC-derived chondroprogenitors and human bone marrow derived mesenchymal stem cells (hMSC).

METHODS: hESC were differentiated into chondroprogenitors following a chemically defined protocol [1-2]. Chondroprogenitors and human bone marrow derived MSC were then either embedded into hydrogels or allowed to form cell spheroids. Hydrogels and cell spheroids underwent mechanical stimulation with intermittent hydrostatic pressure for 1 hour daily at 270 kPa and 1 Hz over 8 days. To assess the effect of hydrostatic pressure samples were compared with statically cultured controls. Biochemical assays, qPCR, histology and immunocytochemistry were utilised to investigate matrix production, expression levels of chondrogenic markers, cell viability and proliferation. Furthermore, chondroprogenitor-seeded fibrin gels were implanted into a cartilage defect at the epiphyses of *ex vivo* cultured chick femurs and stimulated for 5 days at 1Hz and 270 kPa for 1hour per day and then cultured statically for another 5 days.

RESULTS: Results suggest that mechanical stimulation of hMSC and chondroprogenitor hydrogels and spheroids using intermittent hydrostatic pressure resulted in increased production total protein and matrix proteins such as collagen and GAG. In addition, upregulation of chondrogenic markers was observed.

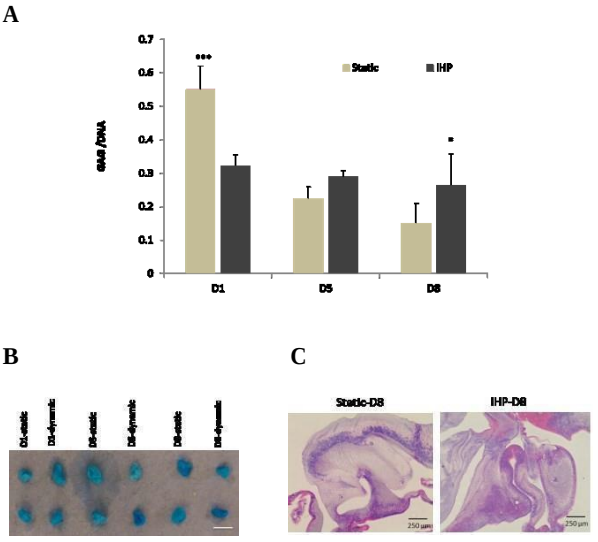

Fig. 1: Chondroprogenitor cells embedded in hydrogels and cultured for 8 days statically and dynamically. DMMB assay (A), Alcian Blue stain (B) and H&E stain (C) indicate increased matrix production. Scale bar: B= 2mm, C=250µm. Colour intensity in B represents amount of GAG produced.

DISCUSSION & CONCLUSIONS: hMSC and chondroprogenitor cells remain viable in static and dynamic culture. Upon mechanical stimulation in the hydrostatic force bioreactor, increased protein content and protein release was observed for cell spheroids and hydrogels. Future work will include the optimisation of stimulation regime for the ex vivo culture chick femurs and the investigation of alternative hydrogel materials.

ACKNOWLEDGEMENTS: The authors would like to thank the UKRMP Hub for funding. The authors would also like to acknowledge Mr Diogo Mosqueira-Alves-Moreira-Silva, Miss Jessica Bratt, Mr Luis Costa-Marques and Mr Shah Mijaan Ali.

The optimization of serum-free medium for co-culture NSCs and ECs in a three-dimensional microfluidic device

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INTRODUCTION: A key role of endothelial cells (ECs) in the CNS is to establish intimate interactions with neural stem cells (NSCs) by forming "neurovascular niches"[1]. Within the "niches", ECs produce instructive signals to preserve NSCs proliferative potential and multipotency. Microfluidics has proven to be a powerful technology to study cell-cell interactions in vitro due to spatially mimic the unique anatomical features of NSCs-ECs as well as lower cost[2].

METHODS: The microfluidic device consists of a NSCs culture chamber and side channel for ECs culture. Some micropillars were microfabricated as "windows" where NSCs and ECs can be spatially separated and also connected with each other (Fig.1).

The collagen-NSCs compound was driven into the NSCs culture chamber of microfluidic device, and expanded with the serum-free medium consisting of DMEM/F12/RPMI1640 supplemented with N2/EGF/bFGF. ECs was planted into one of the side channels when the average diameter of NSCs clusters was about 50-100μm. The initial growth factors in serum-free medium were replaced by different concentration of VEGF/bFGF/EGF/EPO. An orthogonal test with 4 factors and 3 levels was designed to screen the optimal serum-free medium formula for NSCs-ECs co-culture. The expression of the undifferentiated NSCs marker nestin were determined by qRT-PCR. The cell numbers of ECs were measured by semi-quantitative immunofluorescence assay.

RESULTS: The results of orthogonal test showed higher expression of the undifferentiated NSCs marker Nestin in the group 1、3、4、5 than other groups (Fig.2) ,especially group 4 has the highest expression. These results indicated that addition of certain quantities of VEGF and EPO in original serum-free medium is conductive to maintain NSCs undifferentiated characteristics. The statistic results of the number of ECs was showed in Fig.3. It appeared that all experimental groups were lower than the control group(with serum medium). Among them, the group 4 was the most close to the control group.

Fig. 1: Schematic of 3D microfluidics device for NSCs by co-culture with ECs.

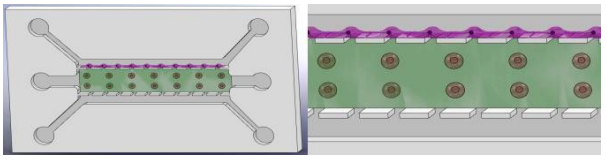
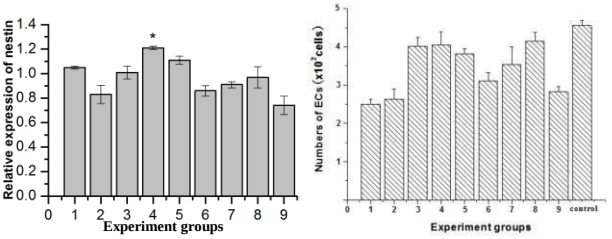


Fig.2: qRT-PCR revealed that the expression of Nestin genes in



*each orthogonal test group. (n=3; *P<0.05, Student's t test)*

Fig.3: Numbers of ECs in each orthogonal test group after 5 days culture

DISCUSSION & CONCLUSIONS: In this study, a 3D model of the NSCs-ECs "niche" based on microfluidics was fabricated. By orthogonal test, we obtained an optimal serum-free medium formula for NSCs by co-culture with ECs. That is the serum-free medium consisting of DMEM/F12/RPMI1640 (1:1:1) supplemented with VEGF(100ng/ml)/bFGF(10ng/ml)/EGF(20ng/ml)/ EPO(0.5U/ml).We also found that the serum-free serum was not conductive to ECs proliferation as compared with those in serum medium.

ACKNOWLEDGEMENTS: This work was supported by National Science Foundation of China (31300809).

A tissue-engineered dermal model rich in endogenous collagens recapitulates human native dermal tissue biomechanics better than classical monolayer or spheroid models

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INTRODUCTION: 3D cell models exploit more representative spatial, biological, biochemical and biophysical parameters that 2D flat monolayers to bridge the gap between in vitro and in vivo experiments. In this study, we propose to compare physical properties of a same cell type growing in configuration of increasing complexity, going from 2D monolayer to native tissue, passing by 3D models rich or poor in extracellular matrix.

METHODS: From a unique human skin biopsy, we extracted primary fibroblasts later seeded at precocious passage on monolayer (2D model), spheroid (3D model poor in extracellular matrix) [1] and tissue-engineered cell sheet (3D model rich in complex extracellular matrix) [2,3]. We also worked with native and fresh dermis tissue. In these cell models of increasing complexity, nanomechanical and viscoelastic properties at cell scale were measured by atomic force microscopy (AFM) [4] on living tissue models. Biphoton microscopy allowed collagens detection by second harmonic generation (SHG). Scanning electron microscopy (SEM) helped in model morphological characterization.

RESULTS: In all models, fibroblasts presented typical elongated cell shape, with cytoskeleton well-arranged along the long axis of the cell. Collagens fibers were only observed in cell sheet and native dermis models (Fig. 1). Interestingly, elastic moduli of cell sheet and native dermis tissue were similar, and significantly lower than those of monolayer and spheroid models (Fig. 2).

DISCUSSION & CONCLUSIONS: In this study, we proved the feasibility of carrying out AFM force measurements on fresh 3D models such as spheroids and cell sheets, as well as on living, fresh native human tissue. We observed that tissue-engineered dermal model recapitulates human native dermal tissue mechanical properties contrary to classical monolayer and spheroid models. Thus, we underlined the main role of environment and especially collagens on cell mechanical properties. In conclusion, from the mechanical point of view, we proved that the tissue-engineered cell sheet model rich in endogenous collagens is the closest model to human native dermis These results offer perspectives in wound healing, aging and cancer studies.

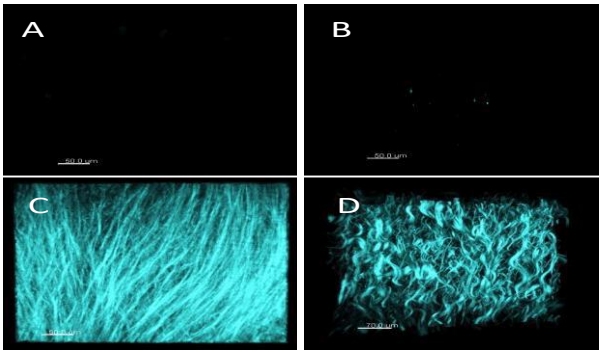

Fig. 1: Collagens observed by second harmonic generation (SHG) by biphoton microscopy. A. monolayer. B. spheroid. C. dermis substitute (tissue-engineered cell sheet). D. native dermis.

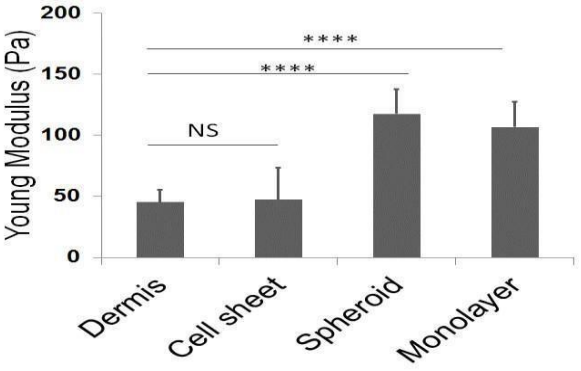

Fig. 2: Cell membrane viscoelastic properties measured by AFM.

ACKNOWLEDGEMENTS: This research was performed in the scope of the EBAM European Associated Laboratory (LEA) and is a result of networking efforts within COST TD1104.

Development of a tissue-engineered elastin-based heart valve prosthesis

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INTRODUCTION: The aim of this study was to develop a TEHV based on elastin-like recombinamers (ELRs). ELRs are bioengineered polymers based on the natural elastin, produced by recombinant techniques[1] and positioned midway between natural products and synthetic polymers, due to they keep the biological origin and nature in addition to the advantages of an engineered material[2].

METHODS: ELRs used in this work were obtained by using standard genetic engineering techniques and chemically modified by transformation of the α-amine group in the lateral lysine chain to bear cyclooctine and azide groups. Valves were formed by catalyst free click reactions between an azide group and an activated cyclooctine group[3]. Solutions of ELRs-cyclooctine and ELRs-azide (90 mg/mL) were prepared in PBS at the desired concentration and kept at 4°C for at least 24h. 30 million cells were suspended in ELRs solution previously prepared. 1.5 mL of each polymer solution, containing the cells were co-injected inside a 15 mm diameter aortic shape mould and kept 45 minutes at room temperature. Then the valves (n=3) were cultivated under static conditions for 7 days following dynamic conditioning for 14 days at 37 °C, 5% CO2 and 21% O2 in a custom-made bioreactor.

RESULTS: The valves were fully functional with an unobstructed opening (systolic phase) and complete closure (diastolic phase), as verified by frames extracted from high-speed movies and ultrasound. Immunohistochemistry revealed a pronounced deposition of collagens type I and III and alpha-SMA active cells aligned within the longitudinal direction of both wall and leaflets. The applied conditioning protocol resulted in an increase of the mechanical properties as determined by burst strength measurements. After conditioning, wall and leaflet tissues burst at 579.5 ± 145.8 mmHg and 642.8 ± 135.5 mmHg respectively. Collagen content of the walls and leaflets reached 22.6 ± 3.3 % and 30.7 ± 2.4 % respectively, relative to the native human aortic valve.

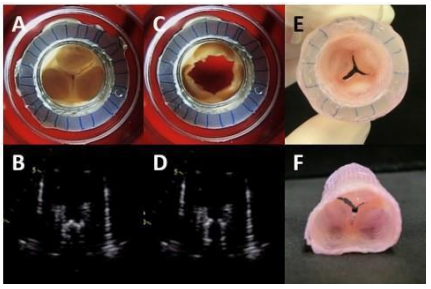

Fig. 1: Valve`s macroscopic appearance after 21 days of cultivation. Frames showing the closed (A) and open (B) positions of the valve, (B and D) ultrasound echography pictures of the EA-valves in the closed and open position respectively, and macroscopic appearance of the valve from the ventricular side (E) and vascular side including the wall (F).

DISCUSSION & CONCLUSIONS: The previous results show the first TEHV made exclusively of ELRs as scaffold. These valves exhibited excellent mechanical properties, good cell proliferation and development of new extracellular matrix, as immunochemistry and burst strength test reveals. These results highlight the potential of these functional TEHVs to be implanted in the aortic position.

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ACKNOWLEDGEMENTS: "The THEGRAIL project is funded by the European Union's 'Seventh Framework' Programme for research, technological development and demonstration under Grant Agreement n° HEALTH.2011.1.4-2- 278557 From 2012-01-01 to 2016-12-31″ Funding was also provided by the People Programme (Marie Curie Actions) of the European Union´s Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n°317512.

Manipulating the extracellular matrix for liver tissue engineering

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INTRODUCTION: Incidence of liver disease in the under-65s risen by 500% since 1970, with mortality increasing by 400% in the same time period[1]. A treatment is sought in the form of liver 'organoids'; lab grown devices which support the survival and function of hepatocytes. While scientists can maintain liver organoids in the lab, decades worth of research has failed to translate this into a fully functional organ[2]. This in part is due to the complexities of recapitulating a vital part of the in vivo environment; the extracellular matrix [ECM]. Tissue engineering novel scaffolds provides a strategy to tackle the problem of mimicking the ECM[3,4]. This study aims to demonstrate that manipulating cell derived ECM via chemical means improves scaffolds for liver tissue engineering.

METHODS: Poly-capro-lactone [PCL] scaffolds were created by electrospinning. Epithelials seeded onto the scaffold were subjected to drug treatment using histone deacetylase inhibitors to encourage protein production. These cells were stripped from the scaffolds to leave behind their ECM. The resulting hybrid ECM-PCL scaffolds were then seeded with HepG2s, and cultured for 3/5 days before being used for analysis. Scanning electron microscopy [SEM], mechanical and biochemical quantification, histology, and gene expression analyses were performed on the scaffolds.

RESULTS: SEM demonstrated markedly different scaffold topography [Fig1]. Additionally, gene expression was altered between conditions [Fig2].

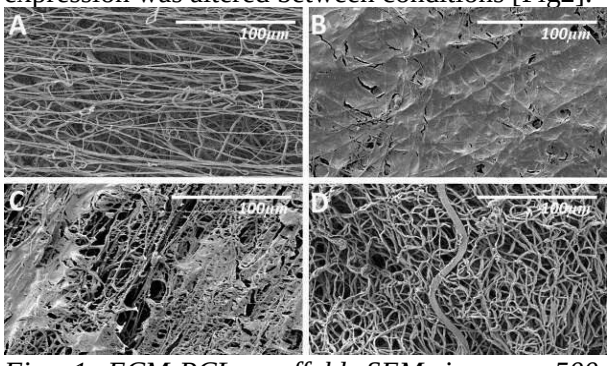

Fig. 1 ECM-PCL scaffold SEM images, 500x magnification. [A] Scaffold only, [B] Scaffold & untreated cells, [C] Scaffold & drug 1 treated cells and [D] Scaffold & drug 2 treated cells.

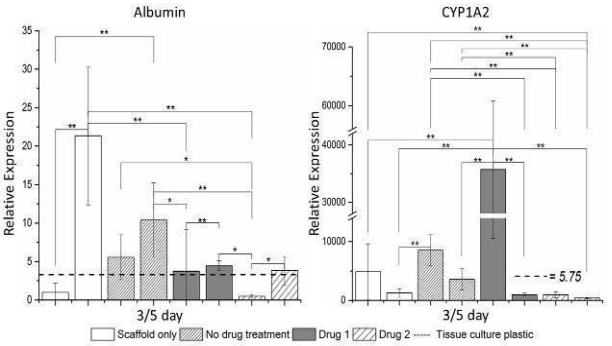

*Fig 2. HepG2 gene expression of liver associated genes. Normalised to GAPDH, relative to culture plastic HepG2s. N = 4, error bars represent SD. *p<0.05, **p<0.01; one way ANOVA with Tukey post hoc analysis.*

DISCUSSION & CONCLUSIONS: The production of extracellular matrix is significantly altered by drug treatment. Cytochrome P450 [CYP] genes are significantly upregulated, as is albumin. Exposure to the drug induced decellularized ECM influences the gene expression profile of the HepG2s [data not shown; Collagens I & IV, Fibronectin, Albumin and Cyp 1A1, 1A2 & 3A4]. These results demonstrate the great value of these drugs for the production of bespoke ECMs; and their potential for liver tissueengineering.

ACKNOWLEDGEMENTS: This work is funded by an Engineering & Physical Sciences Research Council [EPSRC] doctoral training partnership studentship and UK Regenerative Medicine Platform II [RMPII] grant MR/L022974/1.

Biocompatibility assessment of new fibrin-based interpenetrating polymer networks for human skin regenerative medicine

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INTRODUCTION: In skin regenerative medicine, the major challenge is still to produce a cost effective skin substitute, without any antigenic properties, easy to handle and giving satisfactory wound healing results [1]. To meet this need, new interpenetrating polymer networks (IPNs) associating a fibrin network with a co-network of polyethylene oxide (PEO) and Human Serum Albumin functionalized with methacrylate groups (HSAm) have been synthesized. These new biomaterials are entirely biodegradable and combine the mechanical properties of PEO with the healing properties of fibrin. In addition, fibrin precursors can be easily isolated from patients' blood which is an available autologous source making customized treatment possible. The aim of the present study is to evaluate the biocompatibility of these new biodegradable Fibrin/PEO-HSAm IPNs.

METHODS: The biocompatibility of the Fibrin/PEO-HSAm IPNs (0.5%/5%-5%, respectively) was assessed using organotypic culture. Pieces of IPNs were deposited on skin fragments isolated from 7 day-old chick embryos. After 7 days of culture, we measured the cell migration area from the explants and the cell adhesion by counting the number of detached cells under enzymatic dissociation. Tissue-culture coverslips were used as a control. Significance of the results was determined by student's test ($n \geq 30$, $*p<0.05$).

The biocompatibility was also evaluated with MTS cytotoxicity assay following the ISO-10993 guidelines (part 5). 10^4 L929 cells cultivated from passage 11 to 18 were seeded per well in 96-well plates and IPN samples were agitated in complete culture medium during 1 day at 37 °C. Then, the extracts were deposited on cells. 48 hours after cell seeding, a MTS cytotoxicity assay (Promega) was performed. Significance of the results was determined by one-way ANOVA ($n=6$, $p<0.001$).

RESULTS: Skin cells migrated and adhered significantly more on Fibrin/PEO-HSAm IPNs than on the control (see table 1). These results could be correlated with the biological properties of the fibrin network which is known for promoting cell migration and adhesion during wound healing in skin [2].

Table 1. Organotypic culture results.

	Control	IPNs
Migration (mm^2)	21.65 ± 2.35	32.07 ±1.20*
Adhesion (arbitrary units)	0.27 ± 0.01	0.42 ± 0.05*

MTS cytotoxicity test results indicated that the viability of L929 cells cultivated in contact with Fibrin/PEO-HSAm IPN extracts is greater than the 70%, threshold set in the ISO-10993 part 5 standard from which a material is considered as non-cytotoxic.

We demonstrated the biocompatibility *ex vivo* using organotypic culture and *in vitro* with the ISO-10993 MTS cytotoxicity assay. However, we need to validate further the biocompatibility of these new IPNs *in vivo* to test potential irritation and inflammation reactions.

DISCUSSION & CONCLUSIONS: Our results have shown that the new Fibrin/PEO-HSAm IPNs constitute promising biomaterials. The use of these new patient customized biomaterials may be extended to other tissue engineering applications such as soft tissue augmentation.

ACKNOWLEDGEMENTS: This work was supported by the ANR Tecsan Fibriderm and the Picardy region.

Effect of physicochemical features of biomimetic hydroxyapatite on endothelial cell behaviour

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INTRODUCTION: Calcium Phosphates (CaPs) such as biomimetic hydroxyapatite (bHA) have been extensively used as bone substitutes due to their similarity to the mineral phase of bone. However, the success of bone graft and subsequently bone regeneration depends mainly on postimplaiting neovascularization/ angiogenesis process [1]. Therefore, the knowledge of endothelial cell behavior in contact with CaPs is crucial for the further improvement of the materials. The aim of this study was to evaluate rat endothelial progenitor cells (rEPCs) behavior in contact with bHA. *In vitro* assays were performed varying the porosity, specific surface area (SSA) of bHA as well as its surface morphology in order to reveal their effect on rEPCs.

METHODS: bHA was obtained by mixing α-tricalcium phosphate (α- TCP) powder with a 2.5 wt.% Na_2HPO_4 aqueous solution. Two milling protocols were used to obtain fine (F) or coarse (C) particle sizes. Subsequently, samples with 0.35 $mL \cdot g^{-1}$ (35) or 0.65 $mL \cdot g^{-1}$ (65) liquid to powder ratio were prepared for both powders. The physicochemical characterization of the samples was described elsewhere [2]. For *in vitro* characterization, 15×10^3 rEPCs were seeded in EBM-2 medium supplemented with 5% FBS. Supernatants were collected and subsequently the content of Ca^{2+} and P_i was evaluated through colorimetric methods. Cell proliferation was quantified by LDH and cell morphology was visualized by SEM at 6 h, 3 and 7 days. The expression of genes involved in angiogenesis and vasculogenesis was analyzed by RT-qPCR at the same time points.

RESULTS: All samples released P_i to the cell culture medium, with higher amounts in C65 and F65 although it decreased over time (Fig. 1a.). In contrast, values of P_i were maintained constant in C35 and F35 during the study. Cells were able to proliferate on C35 and F35, whereas, in contrast, the number of cells decreased with time on C65 and F65, without significant differences between C and F for the same L/P ratio. SEM pictures revealed that rEPCs cultured on C35 formed capillary-like structures whereas on C65 they showed a flattened, regular morphology (Fig. 1b). Moreover, expression of angiogenic and vasculogenic genes was upregulated in low L/P ratio samples.

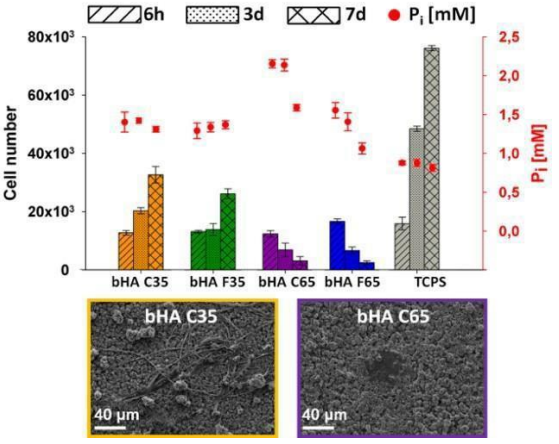

Fig.1 (a) Proliferation of rEPCs and P_i values from supernatants. (b) SEM images.

DISCUSSION & CONCLUSIONS: The modification of L/P ratio of samples affected differently the ion exchange with cell culture medium. The great release of P_i from samples with high L/P ratio samples might be associated to higher porosity (~55%) compared to low L/P ratio samples (~35%), which ensured higher accessibility of the fluid for ion exchange [2]. Interestingly, no correlation between SSA and ion reactivity was observed (SSA C35 and C65 ~20 m^2/g, F35 and F65 ~ 40 m^2/g). However, greater release of P_i from bHA C compared to bHA F with the same L/P ratio might be related to their higher proportion of larger pores as well as higher values of pore size. Consequently, the considerable release of P_i from bHA C65 and bHA F65 had great repercussion on cell viability over time confirming the negative effect of elevated concentrations of P_i on endothelial cell behaviour [3]. The capillary-like structures formed on low L/P ratio samples correlate with expression of pro-angiogenic genes, which indicates the potential capacity of these materials to induce neovascularization.

ACKNOWLEDGEMENTS: Spanish government (MAT2015-65601-R).

Development and characterization of a scaffold-free skin equivalents

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INTRODUCTION: Classically, the dermal part of full-thickness skin models are built on a scaffold often containing collagen in the form of a gel or of a porous scaffold with chemical or physical cross-linking improving their resistance. Since 10 years, scaffold-less full-thickness models were developed [1], based on the ability of dermal fibroblasts to form a dermal equivalent with several layers embedded in their own extracellular matrix. Our aim was to developed and characterize a skin equivalent model without scaffold support [2].

METHODS: We developed and evaluated a scaffold-free skin equivalents (SFSE), and characterized them by histology, transmission electron microscopy (TEM) and immunofluorescent analysis (IF). Then, we investigated their biophysical properties using innovative sensible imaging devices such as optical coherence tomography (OCT) and laser scanning microscopy. Their biomechanical properties were investigated by a suction device Cutometer.

RESULTS: The histological and immunofluorescence analyses showed a completely stratified epithelium close to native skin and the expression of a large number of protein typically found in normal human skin. Our data demonstrated the feasibility and sensitivity of the different biophysical and biomechanical devices even on the thin scaffold-free skin model. Cutometry results showed a viscoelastic behaviour of the SFSE similarly to the native skin with a progressive rise of deflection during the cycles. The mechanical properties of SFSE are attributed to the mature collagens fibers organized as bundles visible by TEM as well as elastic fibers demonstrated by IF where they are deposited in an organisation close to normal skin. OCT allowed the evaluation of the SFSE total thickness as well as its different compartments, perceptible by the contrast changes. The results showed a great inter-batch morphological reproducibility corresponding to those given by histology with a total thickness between 390 and 415 µm. However, the thickness evaluated by OCT directly on fresh samples is higher than the one evaluated by histology on post-fixed and dehydrated samples SFSE with notably a dermal equivalent much thicker.

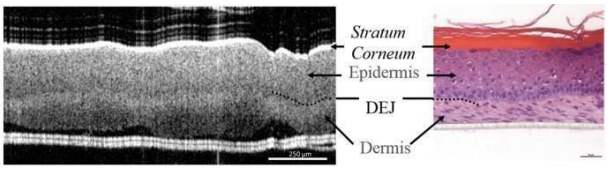

Fig. 1: Comparison between optical coherence tomography and histology

Laser scanning microscopy (LSM) has higher resolution than OCT allowing to evaluate the cells morphology and size and their homogeneity as well as the collagen fibers orientation and size. So result of production batches.

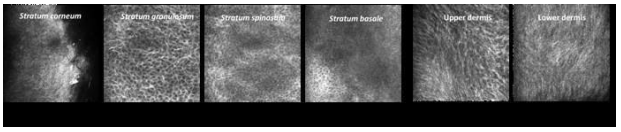

Fig. 2: Example of laser scanning microscopy images of the scaffold-less skin equivalent.

DISCUSSION & CONCLUSIONS: This work allowed to demonstrate that SFSE model is close to native skin and these tools have a great interest because they bring new non-invasive and real-time biophysical methods to evaluate morphological and functional properties of skin equivalent even without any scaffold in parallel with more classical analytical methods.

Regenerating the anterior cornea using recombinant human collagen nanoscaffolds and corneal epithelial stem cells

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INTRODUCTION: Limbal Epithelial Stem Cell (LESC) deficiency is an orphan disease leading to corneal blindness. Tissue engineering has become a treatment modality for this condition, as cultivated limbal epithelial transplantation (CLET) can successfully regenerate the anterior cornea [1]. In CLET, a limbal biopsy is cultivated and transplanted with the help of a carrier material, most commonly the human amniotic membrane (HAM). However, there are limitations associated with the use of HAM, including variable tissue quality, and limited transparency. The aim of this study is to investigate the application of the previously described recombinant human collagen type III - 2-methacryloyloxyethyl phosphorylcholine (RHCIII-MPC) hydrogels [1, 2] as a substitute carrier for CLET.

METHODS: RHCIII-MPC hydrogels [2] and HAM were tested for optical properties, microbial susceptibility, genotoxicity, and biocompatability. Composite grafts were generated by xeno-free cultivation of human LESCs.

RESULTS: RHCIII-MPC hydrogels had straylight values (s) 10 times lower compared to HAM (log(s): 0,44 vs. 1,37). Collagen scaffolds show no genotoxic effect, and appear to be 100-fold more resistant to microbial growth compared to HAM. No significant difference in cell viability and active proliferation rates were noted between both scaffolds *(p=0,07)*. Cultivated primary cells displayed an adherent progenitor phenotype (Fig. 1). Flow cytometry analysis shows that when compared to HAM, cells cultivated on RHCIII-MPC show similar expression of adhesion molecules (CD29/166), and fibroblast markers (CD90), but lower levels of stemness (CD73).

DISCUSSION & CONCLUSIONS: Primary LESCs can successfully be cultivated on RHCIII-MPC under standardized xeno-free conditions. The favourable optical characteristics, relative microbial resistance, successful composite graft generation, and previous successes in corneal regeneration [3] prove that RHCIII-MPC is a highly promising scaffold for CLET. Even though cells cultivated on collagen-MPC showed lower levels of stemness ($p<0,05$), more than 10% of cells expressed CD73. Fibronectin nano-lithography on RHCIII-MPC membranes is under investigation to potentially improve expression of stem cell markers [1]. Finally, a limbal stem cell deficiency rabbit experiment would be necessary to validate the use of RHCIII-MPC in CLET.

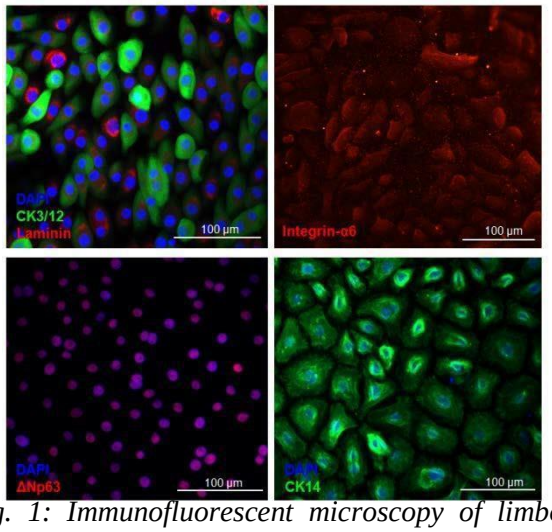

Fig. 1: Immunofluorescent microscopy of limbo-collagen composite graft (20x). Cultivated LESC's displayed a laminin (A), integrin-α6 (B), ΔNp63 (C), CK14 (D), and connexin 43. Differentiation marker CK3/12 (A) was also expressed.

ACKNOWLEDGEMENTS: This research was funded by "The Research Foundation-Flanders" (FWO) and EuroNanoMedII.

Regenerative medicine of *nucleus pulposus* niche through biomaterial-assisted transplantation of adipose stromal cell-derived nucleopulpocytes: preliminary experiments in sheep.

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INTRODUCTION: Intervertebral disc (IVD) degeneration is one of the major causes of low back pain (LBP). IVD degeneration is conceptualized as the consequence of the *nucleus pulposus* (NP) cell inability to maintain IVD extracellular matrix homeostasis. Interestingly, we recently demonstrated the ability of human adipose derived-stromal cells (ASC) to give rise to *nucleus pulposus* cells namely the nucleopulpocytes (NPCy)[1]. In addition, we also demonstrated the ability of ASC-derived NPCy to support the neo-formation of a NP-like tissue when transplanted with a Si-HPMC hydrogel in nude mice subcutis. To go further, the use of a clinically relevant animal model of IVD degeneration is required. Among the available animal models of IVD degeneration our data demonstrated that sheep exhibit a process of IVD degeneration quite superimposable to that of human. In addition, the size of sheep IVD allows the development of surgical protocols easily transferable to the human. In this context, and to further address whether sheep may be a relevant model for the development of IVD regenerative strategies, the aims of the present study were (i) to characterize the stemness properties of sheep ASC (sASC) including proliferation, clonogenicity and multipotency; (ii) to address the ability of sASC to give rise to NPCy in vitro; (iii) to demonstrate our ability to percutaneously inject Si-HPMC in sheep degenerated IVD.

METHODS: Proliferation and clonogenicity was assessed by cell counting and CFU-F. Their multipotency was demonstrated by measuring their ability to commit into the adipo-, osteo- and chondrogenic pathways by RT-qPCR and histological staining. Their ability to differentiate into NPCy was assessed by measuring the expression levels of NPCy specific markers including OVOS2, Pax1 and CA12 by RT-qPCR. Injection was done using a percutaneous fluoroscopy-guided approach, different volumes of Si-HPMC were injected using a Hamilton syringe.

RESULTS: Sheep ASC were found to exhibit a fibroblast-like morphology and have a doubling time of 2.38 days +/-1.12 (P 0 to P2). In addition, CFU-F assay indicated that 39.65% +/- 3.42 of ASCs were able to form colonies. Multipotency evaluation revealed adipogenic differentiation by an increase in leptin and PPARg transcript and Oil Red O positive stainings. Osteogenic differentiation was confirmed by an increase in collagen 1, osteocalcin and osteopontin transcript and Alizarin red positive staining. An increased expression of type II collagen and aggrecan transcripts and a positive alcian blue staining was observed in chondrogenic differentiation. Interestingly, the expression of transcripts coding for NPCy specific markers were increased upon nucleopulpogenic differentiation. Finally, our data also indicate that we were able to transplant about 400µl of Si-HPMC by percutaneous intradiscal injection in several lumbar IVD.

DISCUSSION & CONCLUSIONS: Our data have first confirmed the stemness properties of sASC by demonstrating their proliferation, clonogenicity and multipotency. In the context of IVD regenerative medicine, it was of particular interest to show that sASC were able to give rise to nucleopulpocytes when cultured in an inductive medium. Finally, our results also highlight our ability to inject a large volume of Si-HPMC in sheep IVD, compatible with future preclinical tests. In this context, sheep could be considered as a preclinically relevant animal model for comparing IVD regenerative strategies. Further studies dedicated to evaluate the regeneration of NP niche through biomaterial-assisted transplantation of sASC are now under investigation.

ACKNOWLEGDMENTS: ANR and AO foundation

DISCLOSURES: nothing to disclose.

Engineering of axially vascularized bone grafts towards the treatment of avascular bone necrosis

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INTRODUCTION: Avascular bone necrosis (AVN) is a degenerative disease caused by impaired vascularization and cellular death of bone, leading to challenging clinical scenarios.[1] The standard of care includes the use of pedicled or free autologous bone grafts, which are associated with morbidity and availability constraints.[2,3] Stromal vascular fraction (SVF) cells of human adipose tissue, known to contain vascular and skeletal progenitors, have previously been used to engineer osteogenic and vasculogenic grafts.[4] This study aims at combining such osteogenic/vasculogenic engineered grafts to a vascular bundle in order to manufacture pedicled bone graft substitutes, which were then tested in an ectopic model of AVN.

METHODS: SVF cells from human adipose tissue were seeded into hydroxyapatite cylinders (800 mm^3) and cultured for 5 days in a perfusion bioreactor system. Constructs were then inserted into hollow cylinders of devitalized bone (Tutobone®), mimicking necrotic bone in AVN and wrapped in a membrane (Biobrane®) to prevent any tissue ingrowth from the outside. In nude rats, a ligated arteriovenous bundle was created from the deep inferior epigastric vessels and introduced into the SVF-seeded constructs through a central drill hole. After up to 8 weeks subcutaneous implantation constructs were assessed by histology and microtomography with cell-free constructs as controls.

RESULTS: After 1 week in vivo, constructs with or without SVF cells were fully vascularized, but cell-based constructs displayed significantly higher (54% more) blood vessel density. At 8 weeks, bone tissue was formed only in cell-seeded constructs. Implanted SVF cells contributed both to bone and blood vessel formation in vivo, as documented by staining for human-specific ALU sequences. Rat-derived osteoclasts and tissue remodeling (M2) macrophages were found predominantly in SVF-seeded constructs. The surrounding necrotic bone was revitalized in both groups, as assessed by the ingrowth of blood vessels. [Fig.1]

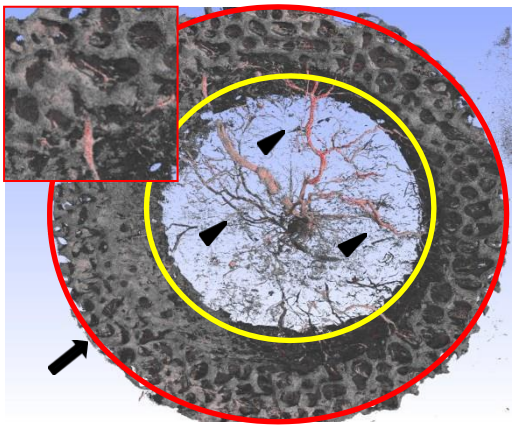

Fig. 1: Contrast – Microtomography with phosphotungstic acid shows a ramified vascular network (black arrowheads) after 8 weeks, arising primarily from the central vein of the AV bundle and reaching the outer shell of devitalized bone (red circle). Mineralized apatite in the inner core (inside yellow circle) was subtracted by image processing to facilitate view of formed blood vessels.

DISCUSSION & CONCLUSIONS: We successfully engineered pedicled bone graft substitutes by combining a vascular bundle with SVF seeding. In the ectopic AVN model, the vessels arising from the central pedicle efficiently revitalized the necrotic outer shell, while the SVF cells were essential to attract/polarize M2 macrophages and to induce bone and vessel formation in the inner core. Longer implantation times are now required to investigate the dynamics of osteoconduction into the outer layer of the construct, which would bear relevance for the treatment of AVN.

The endothelial barrier on-chip

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INTRODUCTION: A functional micro-vasculature is critical for the homeostasis of vascularised tissues. In addition, the identification of mesenchymal stem cells at perivascular sites of the endothelial barrier, suggests that pericytes have a role as multipotent progenitors involved in tissue repair [1]. Microfluidic technologies have shown the potential to closely mimic the vascular microenvironment and represent an alternative to animal models [2]. The aim of this project was to develop a microfluidic system comprised of a 3D microvascular network embedded in a hydrogel enabling the investigation of perivascular cells in a physiologically relevant context.

METHODS: The microfluidic mold was fabricated out of polycarbonate by using a computerized numerical control machine and comprises 3 different layers creating an empty chamber upon assembly. A removable capillary with an outer diameter of 150 µm placed within the chamber enables creation of a microchannel within the gel (see Fig.1).

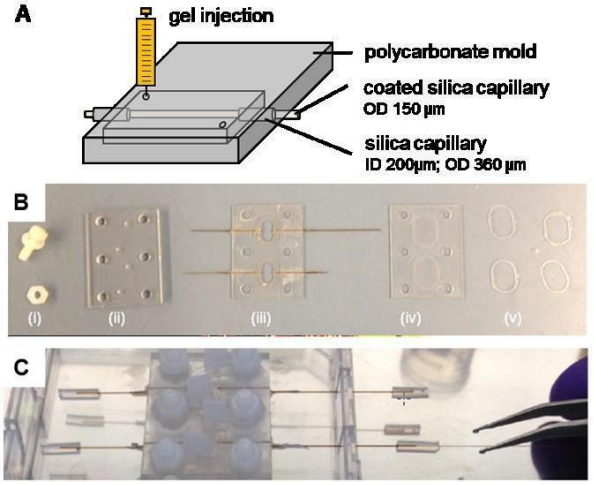

Fig. 1: A. *Schematic representation of the microfluidic chamber (only one gel chamber shown).* ***B.*** *Single parts of the mold: (i) polyacrylate screws with 4 mm diameter (ii, iii, iv) top, middle and bottom parts of the chip (v) PDMS rubber seal.* ***C.*** *Retraction of silica capillary from assembled chip after gel polymerization. ID: inner diameter; OD: outer diameter*

Collagen type I (2 mg/ml) gel was injected into the chamber and polymerized at 37°C for 60min. Microchannels were created by careful retraction of the capillary. The chip was connected to a reservoir of endothelial growth medium (EGM-2) and perfused using a piezo micro pump. GFP-tacked human umbilical vein endothelial cells (HUVECs) were injected into the microchannels and allowed to adhere for 2 hours. Cell-seeded microchannels were perfused with EGM-2 and observed by time-lapse microscopy for 48 hours.

RESULTS: Microchannels were successfully created within the collagen gel. Channels were regular with a diameter of 150 µm (Fig. 2A). Time lapse microscopy revealed efficient cell attachment and complete coverage of the surface of the microchannel (Fig. 2B, C). Good viability of HUVECs was observed over the full duration of the experiment and vessel sprouting occurred 28 h after initiation of perfusion (Fig. 2D, E).

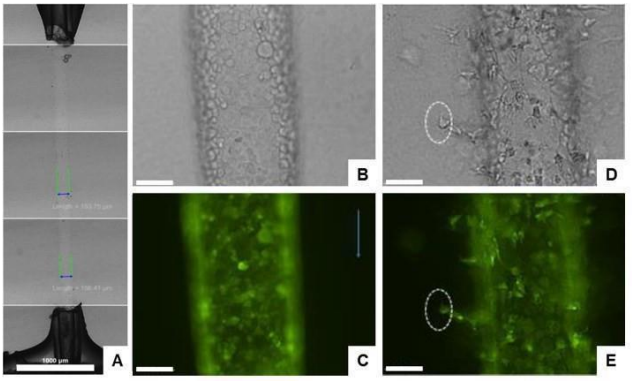

Fig. 2: A. *Microchannel within collagen gel.* ***B-E.*** *High magnification images of the microchannel seeded with GFP-HUVECs after 1 h (B, C) or 28 h (D, E) of perfusion. Circles indicate areas of sprouting. Scale bars 1000 µm (A); 100 µm (B-E).*

DISCUSSION & CONCLUSIONS: We have successfully developed a perfused microvascular model. The embedding of 3D microchannels within a hydrogel matrix will enable study of (i) interactions between perivascular cells (seeded in the hydrogel) and endothelial cells as well as (ii) perivascular and transendothelial cell migration.

ACKNOWLEDGEMENTS: This work is supported by the AO Foundation and the 3R Research Foundation Switzerland (#139-14).

Therapeutic effects of ultrasound on dermal wound healing in diabetic mice

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INTRODUCTION: Chronic, non-healing wounds, including diabetic, leg and pressure ulcers impose a significant healthcare burden worldwide [1]. Diabetes and obesity are major underlying causes of chronic wounds [1]. Currently, wound therapy is primarily supportive, aimed at reducing tissue ischemia and preventing bacterial infection. Some evidence supports the potential application of ultrasound for soft tissue wound repair [2, 3]. Here, the ability of ultrasound to enhance soft tissue wound repair was investigated in a murine model of chronic diabetic wounds.

METHODS: An ultrasound exposure system was developed to provide daily ultrasound exposures to full-thickness, 6-mm punch biopsy wounds made on genetically diabetic (*db+/db+)* mice. Acoustic fields were generated with a 1 MHz transducer and calibrated with a membrane hydrophone. Wounds were covered with acoustically transparent dressing and exposed to 1-MHz pulsed ultrasound (2 ms pulse, 100 Hz PRF) for 8 min/day for 10 days over a 2-week period. Pressure amplitudes of 0 (sham), 0.1, 0.2, and 0.4 MPa were investigated, corresponding to I_{SPTA} values of 0, 0.05, 0.2, and 0.7 W/cm^2, respectively.

Wound images were obtained immediately after injury and on treatment days. Wound closure was measured by tracing the leading edge of epithelium within the wound over time. Fourteen days after wounding, animals were sacrificed and the original wound area was excised and processed for histology. Five-micron sections were cut sequentially to capture the entire wound space. Sections nearest the wound center were stained with either hematoxylin and eosin (H&E) or Masson's Trichrome. Granulation tissue thickness was measured at the center of the wound.

RESULTS: No significant differences in the rate of re-epithelialization were observed in response to ultrasound exposure compared to sham-exposed controls. Two weeks after the initial injury, a statistically significant increase in granulation tissue thickness at the wound center was observed in mice exposed to 0.4 MPa (389 $\pm$ 85 μm; n=12) compared to sham exposures (105 $\pm$ 50 μm; n=10)(Fig. 1A, B). Increased collagen deposition (Fig. 1C), extracellular matrix remodeling (not shown), and revascularization (Fig. 1D, arrows) occurred in wounds exposed to 0.4 MPa ultrasound compared to shams. Exposures at 0.4 MPa increased skin temperature by 2.6±0.3°C to 29°C.

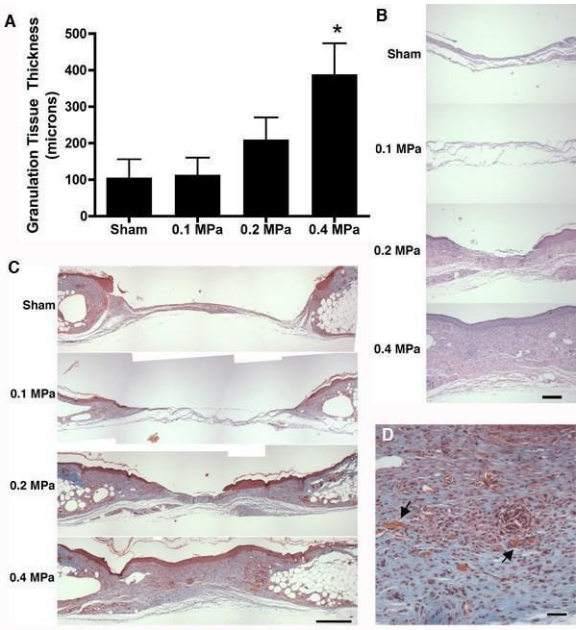

*Fig. 1: Effects of ultrasound on dermal healing. (A) Mean granulation tissue thickness at the center of each wound $\pm$ SEM. *Different from sham, $p<0.05$ (ANOVA). Sections from wound centers were stained with (B) H&E or (C,D) Masson's Trichrome. (D) Image of granulation tissue from a wound treated with 0.4 MPa ultrasound showing perfused blood vessels. Scale bars: 0.1 mm (B), 0.5 mm (C) and 50 μm (D).*

DISCUSSION & CONCLUSIONS: Daily exposure to ultrasound (1 MHz, 2 ms pulse, 100 Hz PRF, 0.4 MPa) can accelerate the repair of full-thickness wounds in diabetic mice by promoting granulation tissue deposition.

ACKNOWLEDGEMENTS: This study was supported in part by NIH grant R01 EB018210.

Enhanced human bone marrow mesenchymal stem cell chondrogenic differentiation in concave microwells with PDMS

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INTRODUCTION: Pellet culture or micromass culture is an in vitro technique that has been used widely to study in vitro chondrogenesis [1]. While pellet cultures are usually established in a polypropylene centrifuge tubes, it can be more cumbersome, time consuming and takes a great amount of culture media when multiple samples. We have recently developed a culture plate that has 25 concave microwells to substitute culture tube. In this study, we compared the in vitro chondrogenesis from bone marrow-derived mesenchymal stem cells (BMMSCs) using concave microwell plates with that obtained using culture tubes.

METHODS: Concave microwells (MicroFIT, Seongnam-si, Korea) with 3 mm diameters and 3 mm depth were fabricated using soft lithography techniques. Each culture dish contains 25 concave wells. hBMMSCs were dissociated to a single cell suspension by trypsinizing and then diluting to a final concentration of 1.25 x 10^7 cells/ml. 20 µl cell suspension (2.5 x 10^5 cells/20 µl) was directly seeded into each concave well. After 30 min, 3 ml of chondrogenic medium was gently added to the surface of the microwell plate. The pellets were cultured in 5% CO_2 atmosphere for 21 days. The medium was replaced every other day. Pellets were taken for analysis at 7, 14 and 21 days after culture.

RESULTS: Pellets cultured in concave microwell plate were larger than those cultured in tube, significantly on day 21. Pellets cultured in concave microwell plate had significantly higher level of GAG per DNA content and greater proteoglycan content that those cultured in tube at days 7 and 14 (Fig. 1). Three chondrogenic markers, SOX-9, COL2A1 and aggrecan, showed significantly higher expression from pellets cultured in concave microwell plate than those cultured in tubes at day 7 and 14. At day 21, there was not a significant difference in the expression of threes markers. COL10A1, the typical hypertrophy marker, was significantly lower in concave microwell plate during the whole culture period. Runx-2, a marker of both hypertrophy and osteogenesis, was significantly lower at day 7 in pellets cultured in concave microwell plate than those cultured in tube.

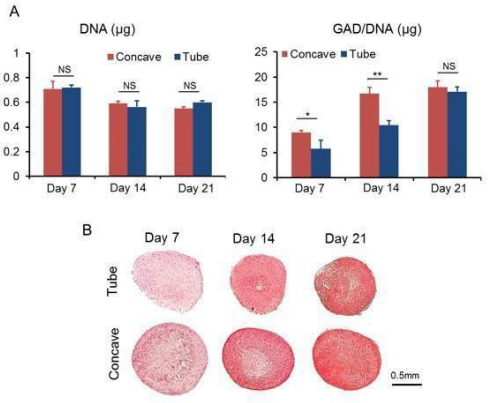

Fig. 1: Morphological and biochemical characterization of pellet cultured in concave microwell plate versus 5ml round culture tube after 7, 14 and 21 days.

DISCUSSION & CONCLUSIONS: In conclusion, early and enhanced chondrogenesis was obtained with the use of PDMS concave microwell plate compared with the use of culture tube. This concave microwell plate provides a convenient and effective tool for the study of in vitro chondrogenesis and may replace the use of propylene culture tube.

ACKNOWLEDGEMENTS: This study was supported by the National Research Foundation (NRF) funded by the Korean government (2015R1A2A1A09002793 and 2013R1A1A2062961).

Exracellular vesicles of the corneal epithelium: A functional regenerative response to corneal implants

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INTRODUCTION: The paracrine functions of extracellular vesicles (EV's) in carrying out normal physiological functions and modulations under aberrant conditions of stress, infection and wound healing are widely reported [1]. Exploiting the function of EV's as carriers and signalling molecules, recent works aim to enhance tissue regeneration as well as treatment of advanced diseases as cancer and immune diseases[1]. In this study, we report for the first time the release of EVs by the corneal epithelial cells among the pigs implanted with hydrogels made from PEG-maleimide conjugated collagen-like peptide (CLP-PEG) hydrogels following deep lamellar keratoplasty.

METHODS:

Animals: Gottingen mini-pigs (Ellegaard, Denmark) used were implanted with either RHCIII or CMP based implants with local ethical permission following OECD guidelines. All animals were monitored over a period of 12 months and the corneal sections were collected after one year.

Immunohistochemistry: Frozen corneal sections (7µm) and corneal epithelial cells cultured on the implants were fixed in 4% paraformaldehyde and stained with respective primary (mouse anti-CD9 antibody (EMD Millipore) and mouse anti-Rab7 antibody (Abcam), and secondary antibodies (anti-mouse and anti-rabbit) conjugated with Alexa-Fluor 488 and 594 florochromes as described in our previous study [2].

RESULTS:

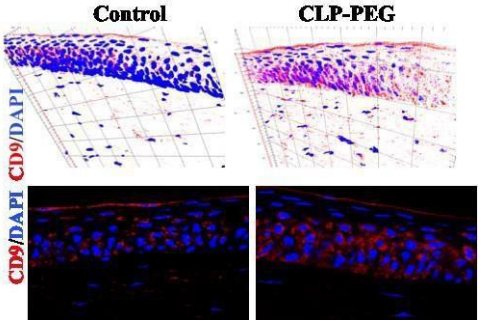

Fig. 1: Immuno-staining of pig corneal sections for EV marker CD9 shows an increased expression in the epithelium and stroma among the pigs with CMP implants

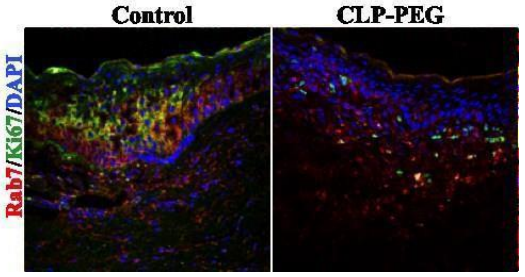

Fig. 2: Limbal region of cornea where epithelial stem cells are located, is stained with Rab7 (red) marker for endosomes and cell proliferating marker Ki67 (Green). There is an increase in proliferation of progenitor cells in the basal epithelium of CLP-PEG implanted corneas with staining for Rab7 only observed in stroma.

DISCUSSION & CONCLUSIONS: Our previous animal and pilot clinical studies using collagen based materials show neo-corneal regeneration even though mechanism is still elusive [3,4]. Here we show that the EV's, triggered by the implants, are released from the basal epithelium and play a crucial role in promoting regeneration through the activation of limbal stem cells. Our further studies employing in vitro methods show that the human corneal epithelial cells show a distinct interconnected network of EV's when grown on collagen implants further supporting our in vivo observations. Further studies to characterize the constituents of EV's triggering stem cell activity are underway.

ACKNOWLEDGEMENTS: Funding from a DBT-Vinnova project grant to MG (dnr 2013-04645), IGEN (Linköping University and Region Östergötland). We thank M Islam, J Phopase, M Ljunggren, P Fagerholm for their assistance in this project.

Decellularized human fetal intestine as a bioscaffold for bladder augmentation in rabbit model

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INTRODUCTION: Biological scaffolds produced from fetal tissues exhibit advantageous properties for tissue engineering. The extracellular matrix (ECM) in these scaffolds contains proteins that influence cell attachment, gene expression and cell differentiation and have a higher capability for tissue regeneration compared to mature tissues. Currently, gastrointestinal segments are often employed for bladder augmentation, but complications like stone formation, metabolic abnormalities and secondary malignancies commonly complicate this procedure [1, 2]. In this study we report a method to create a natural acellular matrix scaffold from human fetal small intestine and grafting to the bladder seromuscular layers as an experimental model of augmentation cystoplasty.

METHODS: The ethical committee of TUMS approved this study. Human fetal intestines were decellularized by immersion in a hypotonic solution containing 0.5% (w/v) SDS. The success of this protocol was evaluated by histological analysis, scanning electron microscopy and measurement of collagen content and sulfated glycosaminoglycan (sGAG) of the acellular construct. Eight adult rabbits were selected and underwent seromuscular dissection. The acellular scaffolds were then implanted on the exposed urothelium. Urodynamic studies and cystography were performed six months after the surgery. At 14, 120 and 180 days animals were sacrificed, the augmented bladders were resected and paraffin-embedded. Immunohistochemistry was performed to evaluate different types of cell seeding.

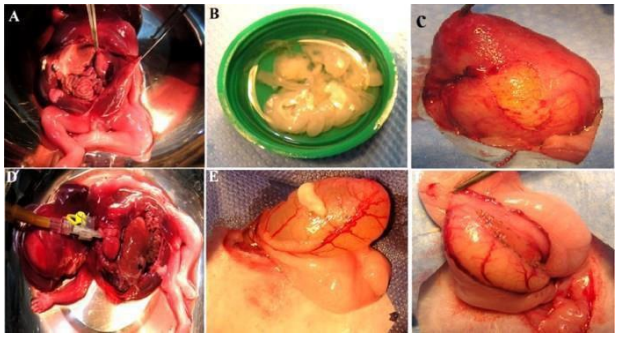

Fig. 1: fetal acellular intestine implantation

RESULTS: In acellular matrices, no nuclear remnants were detected and ECM architecture was also well preserved using this decellularization protocol. Histological analysis of the excised grafts revealed the formation of muscular layer and blood vessels in the implanted scaffolds similar to normal bladder. These findings demonstrate the effective seeding of this unique scaffold by host bladder cells. The tissue architecture of re-cellularized scaffold was very similar to the native bladder.

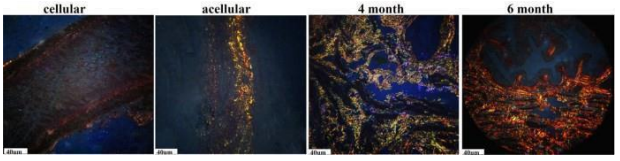

Fig.2: Picro-sirius red staining confirmed increased collagen staining during the implantation.

DISCUSSION & CONCLUSIONS: These findings demonstrate the effective seeding of this unique biological scaffold produced from human foetal intestinal tissue similar to the host bladder cells. The tissue architecture of re-cellularized scaffold was very similar to the native bladder. The foetal intestine acellular matrix could be an exceptional tissue for bladder augmentation in future experimental studies and may pave the road for clinical application.

DISCLOSURES: Authors declare no conflict of interest.

Application of omentum as a in vivo bioreactor for regeneration of decellularized human internal mammary artery

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INTRODUCTION: Coronary artery bypass grafting (CABG) is the mainstay treatment in coronary artery diseases, the leading cause of mortality around the globe. Among various conduits used for CABG, internal mammary artery (IMA) appears to be the preferable graft. However, increased prevalence of CADs, an increase in number of elderly patients with indication for CABG as well as expanded indication for repetition of this procedure has restricted availability of suitable autologous grafts for CABG, necessitating development of alternative conduits [1,2]. This study investigates the extent of cell seeding as well as lumen patency in acellular IMA scaffolds that have been re-cellularized by omentum as a natural bioreactor.

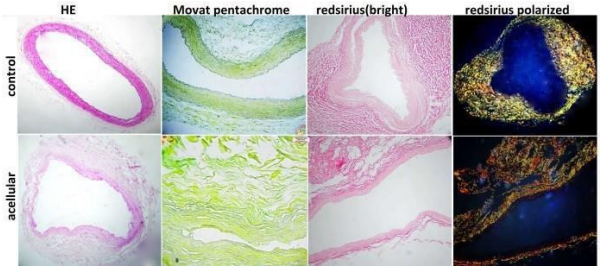

Fig.1. Hematoxylin, Movat's pentachrome and Sirius red staining of native and acellular human IMA.

METHODS: Sixteen Internal mammary arteries derived from human cadavers were decellularized by a protocol using Triton and SDS. Sixteen Virgin female Wistar rats were selected for implantation of acellular scaffold in their omentum. Following laparotomy omentum was retracted to the outside of the abdomen and the more vascularized portion of it was selected for the experiment. Initially the end of the omentum was ligated with a 3.0 USP Nylon thread and passed through the lumen of acellular vascular graft. A double-J catheter was also placed in the lumen to preserve its patency. Then scaffold was wrapped by omentum and the wrap which contained the graft was placed between two layers of rectus muscles that have been previously dissected. Samples were taken from scaffolds at 8 weeks and 12 weeks. Samples retrieved from implanted scaffolds as well as samples from both native and acellular scaffolds were investigated by histological studies including H&E, Sirius red and Movat's Pentachrome staining as well as Immunohistochemical studies.

RESULTS: All the grafts explanted at two and 3 month and the lumen were completely patent compared to the native scaffold. The histology of implanted IMA after 2 and 3 month showed progressive recellularization especially by smooth muscles over the media layer. CD 34 staining was positive surrounding the grafts which showed well angiogenesis to the grafts. Trichrome and Movat's pentachrome staining showed normal collagen formation in the inner media layer.

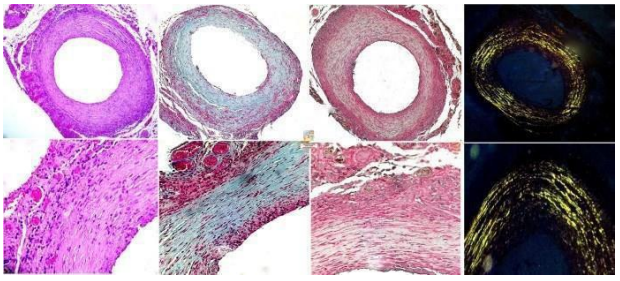

Fig.2. Hematoxylin, Masson trichrome, Movat's pentachrome and red Sirius staining of implanted acellular human IMA after 2 month.

DISCUSSION & CONCLUSIONS: Promising results obtained from the introduced protocol for in-vivo recellularization consisting of patent lumen and proper cell seeding encourages application of above mentioned technique for experimental vascular graft application. However, further investigation in larger size animals graft patency studies is needed prior to application of this technique in humans.

DISCLOSURES: no onflict of interest declared

Evaluating the role of autologous mesenchymal stem cell seeded on decellularized pericardium in the treatment of myocardial infarction: an animal study

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INTRODUCTION: Inappropriate left ventricular remodeling following myocardial infarction (MI) can result in subsequent severe cardiac dysfunction. Fibrotic remodeling, scar formation, and collagen disposition are key participants for repair of infarcted myocardium [1]. Here, we tested the hypothesis that decellularized pericardium (DP) or seeded pericardial patch with autologous adipose-derived mesenchymal stem cells (ADMSCs) can be safely used in a MI scar and improve heart function.

METHODS: Sixteen rabbits were randomly divided into four equal groups. The control group included rabbits without MI induction (G1). Four weeks after MI induction by ligation of the left anterior descending artery in other 12 rabbits (3 equal groups), animals of G2 received DP patch with labeled ADMSCs. DP patch was implanted in animals of G3. Rabbits in G4 remained without any intervention after MI induction. Serial examinations including echocardiography, scanning electron microscopy, histology and immunohistochemistry (IHC) were performed to evaluate the efficacy of the implanted scaffolds on recovery of the infracted myocardium.

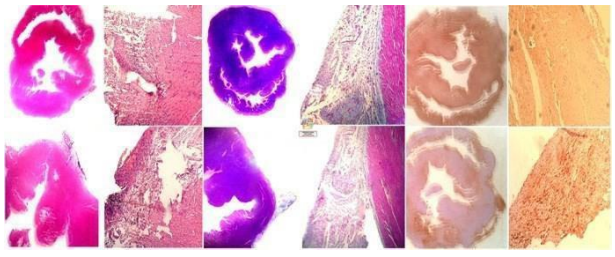

Fig.1. Hematoxylin & eosin, trichrome staining and immunohistochemistry of decellularized patch implantation after 1 month post-operation.

RESULTS: The results demonstrated that left ventricular contractile function and myocardial pathological changes were significantly improved in rabbits implanted with either DP or ADMSC-seeded pericardium. However, the seeded

Pericardium was more effective in scar repairing. IHC staining with Desmin and CD34 after 2 months of operation and positive immunofluorescence staining verified the differentiation of ADMSCs to functional cardiomyocytes.

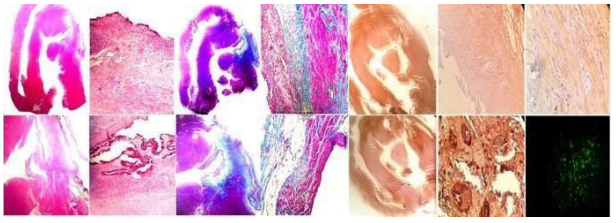

Fig.2. Hematoxylin & eosin, *trichrome staining, immunohistochemistry and immunofluorescent of decellularized patch seeded with adipose derived mesenchymal stem cell after 1 month post-operation.*

DISCUSSION & CONCLUSIONS: The ability of DP seeded with autologous ADMSC to repair MI remains controversial. Taken together, we can conclude that the application of pericardial patch seeded with autologous ADMSC can play an active role in treatment of MI and may prove a desirable approach to enhance cell engraftment. This novel method may gain vigorous momentum over other presented techniques for repair of MI. Based on the results of this study; DP seeded with autologous ADMSC may advance via animal trials into a clinical setting addressing patients withMI.

DISCLOSURES: Author declare no conflict of interest

A novel technique for perfusion based decellularization of pancreas: arterial versus pancreatic duct cannulation

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INTRODUCTION: Type 1 diabetes is the most common disease which is due to the lack of β cells function [1]. Regenerative medicine approaches such as a bioartificial pancreas has been proposed as potential therapeutic solutions .Increasing pancreatic islet survival and function is a starting point for obtaining a valuable bioartificial pancreas. Here we present two novel decellularization techniques in order to preserve pancreatic extracellular matrix architecture (ECM) in addition to complete decellularization of the organ.

METHODS: Forty rats were selected and divided into two groups. Twenty pancreas were harvested by cannulation of common bile duct. The second group was catheterized by aortic patch near the origin of superior mesenteric and celiac artery bifurcations. All the resected pancreases were washed by normal saline. Then different percentages of SDS-Triton based solution at different times points were applied. The decellularized samples by both method of cannulation were examined by histopathological evaluation, DNA quantification and collagen content.

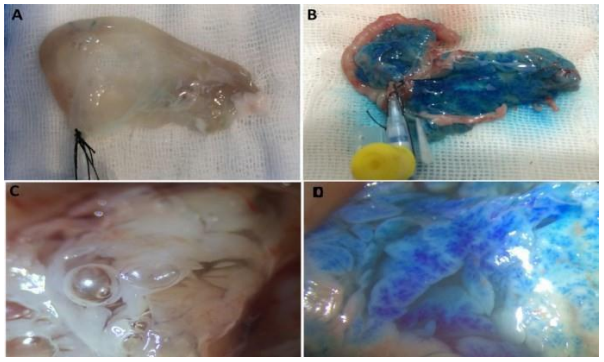

Fig. 1: *Decellularization of rat pancreas: A) after and B) before decellularization C) A microscopy image of pancreas after decellularization and D) before decellularization*

RESULTS: The Histopathological evaluation showed that perfusion base-decellularization of whole pancreas by pancreatic artery and ductal perfusion effectively removes cellular and nuclear material but can retaining innate molecular and spatial framework and stiffness with perfusable vasculature and appropriate ECM component to mimic pancreatic cell composition.

DISCUSSION & CONCLUSIONS: The novel strategy to produce a decellularized pancreas by ductal and arterial access may play a major role for pancreas tissue engineering. Furthermore, both methods could provide a 3D-engineered pancreas comparable to the native pancreas.

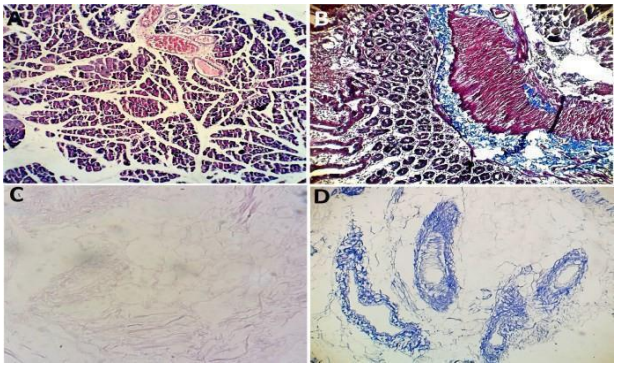

Fig.2: A) Hematoxylin & eosin staining of normal pancreas, C) Decellular pancreas, B), Trichrome staining of normal pancreas D) Decellular pancreas

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DISCLOSURES: The authors declare that there is no conflict of interest.

The ability of a bone morphogenic protein-2 releasing hydrogel to rapidly regenerate a bone defect cultured within a vascularised chorioallantoic membrane

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INTRODUCTION: With the rise in the aged population and the subsequent increase in orthopaedic related fractures, alternative approaches to bone regeneration and repair are urgently required. Current models employ *in vivo* techniques to study the potential efficacy of new bone regenerative strategies. To examine the numerous novel strategies/therapies to repair bone using these in vivo techniques is costly and time consuming. Hence alternative organ culture/*ex vivo* models can, in part, facilitate and streamline the testing of new therapies prior to transition to larger *in vivo* models. Using a 3D *ex vivo* segmental bone defect model implanted *in ovo* on the chorioallantoic membrane (CAM) we investigated the potential of a novel cell/biomaterial construct loaded with Bone Morphogenic Protein-2 (BMP-2) to repair a bone defect using an 8 day culture period.

METHODS: Hydrogels comprising of alginate / bovine extracellular matrix[1] combined with poly(DL-lactic-co-glycolic[2] p acid) (PLGA) copolymer) microparticles [DL using BMP-2 with release

human serum albumin carrier protein, were positioned into segmental bone defects (2mm) in embryonic day 18 chick femurs. The chick femur defects were then implanted onto the chick chorioallantoic membrane, sealed and cultured for a period of 8 days. Femurs were harvested from the CAM (Figure 1A) 8 days post implantation. Samples were fixed in 4% PFA and analysed by micro-computed tomography (μCT) and histologically for proteoglycan (Alcian blue) and collagen production (Sirius red) as well as for osteoid (Goldner's trichrome), mineral (von Kossa) and for presence of osteoclasts (tartrate resistant acid phosphatase (TRAP) activity.

RESULTS: After 8 days post incubation on the CAM significant bridging of the implanted BMP-2/hydrogel within the bone defect was observed (Figure 1A). μCT analysis demonstrated bridging and mineralisation of the chick femur defect with the implanted BMP-2 hydrogel compared to the control (hydrogel only) (Figure 1B). Histological analysis of the chick femur defects revealed extensive von Kossa (mineralized tissue) staining (Figure 1C) within the implanted BMP-2 hydrogel with evidence of remodeling (Figure 1D).

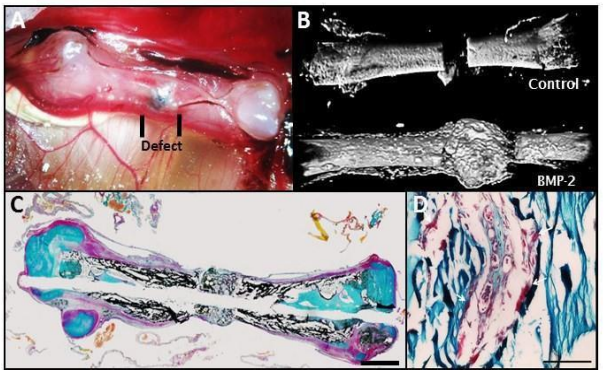

Fig. 1A: Implanted E18 chick femur defect/BMP-2/hydrogel within the vascularized bed of the CAM after 8 days culture. Fig. 1B: μCT scans of E18 chick femur defects; control and implanted BMP2/hydrogel. Von Kossa (C) and TRAP (D) staining of BMP-2/hydrogel within the defect region of the chick femur post 8 days culture within the CAM.

DISCUSSION & CONCLUSIONS: The current studies have highlighted the importance of an active vascular support network combined with delivery of BMP-2 releasing alginate/ bECM hydrogel to initiate bone repair in a critical sized femur defect. Furthermore, this chick femur defect culture system can be applied as a high-throughput test model for many new therapies in early development for orthopaedic reparation as well as

ACKNOWLEDGEMENTS: This work was supported by a strategic longer and larger grant (LOLA) from the BBSRC, UK-grant number BB/G010579/1.

Transport phenomena in perfused 3D bioartificial liver systems

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INTRODUCTION: Hepatocytes provide a wide range of metabolic functions, which gives rise to a high oxygen uptake and accumulation of cellular products. Furthermore, peculiar characteristics and intricate requirements of primary hepatocytes strongly challenges their long-term functionality and viability in vitro. 3D cell culture systems reportedly improve hepatocytes' functionality, the complexity of which further accentuates the need for efficient mass transfer in such systems. Current study investigates mass and momentum transfer in a hollow fibre membrane bioreactor (HFMBR) hosting hepatocyte spheroids.

METHODS: Provision and removal of culture medium in our HFMBR [1] is realized through separate polyethersulphone (PES) hollow fibre (HF) bundles (Pattern 1) in crossed configuration, and the spheroids are trapped within the crossed network. Additional flow patterns were also considered: both bundles supplying (Pattern 2) or removing (Pattern 3) the medium. Oxygen (nutrient) and urea (product) concentrations were modelled using COMSOL Multiphysics. Due to the complexity of the system, two geometries were considered: (i) single-spheroid study in a small segment within the bioreactor, used for efficient model setup and for parametric study (perfusion rate, oxygen tension, effective diffusion coefficients, reaction rate, spheroid size, HF size), (ii) scaled-down version of the whole bioreactor.

RESULTS: The convection-enhanced perfusion system improved mass transport, and physiological oxygen concentrations were successfully maintained. This enabled application of spheroids with radii over 200 µm (size depending on the operational conditions), while in diffusive-controlled systems this should not exceed 100-150 µm. Hypothetical cellular masses resulting from merging spheroids were also proved to be well-supplied. The results from the scaled-down bioreactor model under the same operational conditions showed higher oxygen concentrations compared to single-spheroid model. Additionally, pattern 2 was more favourable in terms of mixing, oxygen supply and urea removal. However, in this case the cellular compartment is no longer isolated by membranes. Maximum shear stress tolerated by hepatocytes was respected throughout the study.

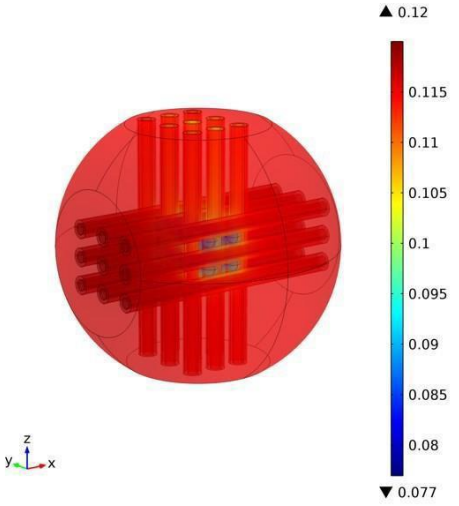

Fig. 1: Oxygen concentration profile (mol.m^{-3}) inside the scaled-down bioreactor (Pattern 1), with 2x9 PES HFs of 500µm OD and 300µm ID, hosting 12 spheroids (400µm diameter). Medium is provided by HFs in x-direction and removed by HFs in z-direction. Perfusion rate 0.015 ml/min, inlet oxygen concentration 120 µmol/L.

DISCUSSION & CONCLUSIONS: The HFMBR presented here provides efficient mass transfer rates to maintain large hepatocyte spheroids in physiological-like conditions. Additionally, modelling the full, scaled-down system potentially provides a more comprehensive understanding of the transport phenomena within the bioreactor, accounting for additional geometrical aspects. Considering small segments or using Krogh cylinder approach highly reduces the computational burden; however, it is more challenging to evaluate whether the model properly predicts the real behaviour of the system, e.g. in terms of mixing or even suitability of boundary conditions.

ACKNOWLEDGEMENTS: Current research is funded by Marie Skłodowska-Curie Actions, Initial Training Networks, project BIOART (grant no.316690, EU-FP7-PEOPLE-ITN-2012). The authors declare they have no conflict of interest.

Chondrogenically differentiated human mesenchymal stem cells induce dendritic cell maturation *in vitro*

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INTRODUCTION: Dendritic cells (DCs) are highly specialised antigen presenting cells with a fundamental role in priming T cell responses [1]. Mesenchymal stem cells (MSCs) have been shown to inhibit DC maturation *in vitro* [2], however, the effect of differentiated MSCs on DC maturation is not clear. Chondrogenically differentiated MSCs have been shown to form bone *in vivo* [3]. The success of this bone formation relies on the host's endogenous cells, however, little is known about the specific immune cells involved in this process. The aim of the current research was to investigate the interactions between donor-mismatched chondrogenic hMSCs and DCs *in vitro*.

METHODS: hMSCs were differentiated in chondrogenic medium supplemented with TGFβ3 for 10 days. DCs were isolated from the CD14+ population of peripheral blood mononuclear cells and cultured in medium containing GM-CSF (50ng/ml) and interleukin-4 (10ng/ml) for 5 days. DCs were matured using lipopolysaccharide (100ng/ml) for 24 hours. Immature and mature DCs (1 x 10^6) were co-cultured with chondrogenic hMSCs (0.2 x 10^6) for 24 hours. The DCs were characterised by flowcytometry. The mean fluorescence intensity (MFI) of FITC-dextran was measured to quantify antigen uptake capacity. DC migration to CCL21 was performed in transwells. CD11c immunostaining was performed on chondrogenic hMSCs pellets. All data was analysed using a linear mixed model with Bonferroni post-test. $P<0.05$ was considered to be statistically significant.

RESULTS: Flow cytometric analysis of the DCs in suspension revealed induction of CD80, CD86 and HLADR expression in immature DCs co-cultured with chondrogenically hMSCs. No significant effect was observed in mature DCs cultured with chondrogenic hMSCs. Immature DCs cultured with chondrogenic hMSCs had higher FITC-dextran uptake. No difference was observed in co-cultures with mature DCs. Immunohistochemistry identified CD11c positive cells on the surface of co-cultured chondrogenic hMSCs (Figure 1).

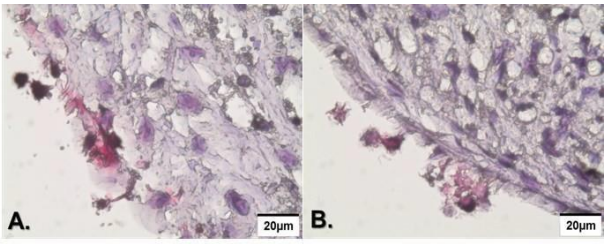

Fig. 1: Immunohistochemistry revealed CD11c positive staining in chondrogenic hMSCs cultured with immature (A.) and mature (B.) DCs. Scale bar: 20µm

DISCUSSION & CONCLUSIONS: In conclusion, chondrogenic hMSCs induce the expression of CD80, CD86 and HLADR in immature DCs, suggesting that chondrogenic hMSCs are inducing maturation of DCs *in vitro*. In addition to the fact that the capacity for antigen uptake is also enhanced in immature DCs cultured with chondrogenic hMSCs, a greater number of CD11c+ cells were also observed on the surface of chondrogenic hMSCs co-cultured with immature DCs compared to those cultured with mature DCs. Collectively these findings demonstrate that chondrogenic hMSCs are inducing maturation of DCs *in vitro*. There was no significant effect of chondrogenic hMSCs on mature DCs observed in any of the above assays. Future experiments will determine the effect of chondrogenic hMSCs on the migratory capacity of both immature and mature DCs. This research will provide the basis in attempting to determine the exact role of DCs in allogeneic MSC-mediated endochondral bone formation.

Investigating the importance of collagen type X in endochondral ossification

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INTRODUCTION: Endochondral ossification (EO) is the process by which the long bones of the body are formed developmentally. This process relies on the presence of a cartilaginous matrix and important cell signalling molecules, crucial for recruiting cells responsible for matrix remodelling and bone formation. By understanding the process by which EO occurs we can improve current tissue engineering (TE) approaches and ultimately improve bone formation [1]. Collagen type X (COLX), a collagen associated with hypertrophy, has been shown to be involved in calcium binding and matrix vesicle compartmentalisation, however a clear role for COLX in development remains to be determined [2,3]. How COLX affects TE chondrogenesis and subsequent bone formation also remains unclear. Here we investigate COLX using lentiviral knock-downs to understand its role in EO.

METHODS: MSCs were isolated from bone marrow aspirates, expanded, and cultured in αMEM containing 10% serum, 1ng/mL FGF2, and 25ug/mL ascorbic acid. Lentivirus particles were produced by combining Mission TRC shRNA (Sigma) plasmids with ViraPower™ Lentiviral Expression (Invitrogen) plasmids in HEK293FT cells. Viral particles, equivalent to 20ng/ml of p24 antigen (p24 ELISA), and 40µg/ml protamine were used to transduce MSCs followed by selection with 5µg/ml puromycin. $2x10^5$ MSCs were differentiated in chondrogenic medium (high-glucose DMEM, 50µg/mL gentamycin, 1.5µg/mL fungizone, 1mM sodium pyruvate, 40µg/mL proline, 1:100v/v ITS, 10ng/mL TGFβ3, 25µg/mL ascorbic acid, and 100nM dexamethasone), and maintained for 21 days. Samples were taken for histology, glycosaminoglycan(GAG)/DNA, PCR and ELISA. Endothelial migration/angiogenic assays were performed. Samples were subcutaneously implanted in nude mice for 8-12 weeks.

RESULTS: shRNA against COLX showed a 90% decrease in gene expression versus controls, this knock-down was also evident in COLX immunohistochemical staining (Fig.1). COLX knock-down pellets were smaller volume compared to controls however GAG content appeared unaffected (Fig.1). Although genes associated with early chondrogenesis (COLII, SOX9, RUNX2 (Fig.2)) were unaffected, differences were observed in the expression of cell signalling and recruitment genes.

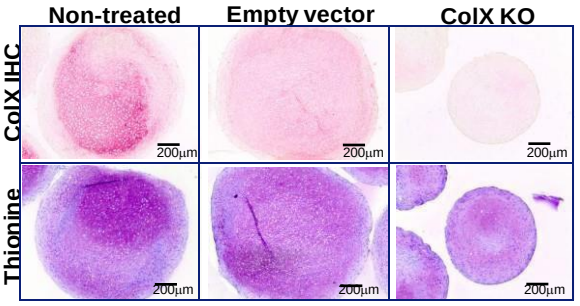

Fig.1: COLX immunohistochemistry showing successful knock-down of COLX on protein expression. Thionine staining reveals a similar GAG production between conditions.

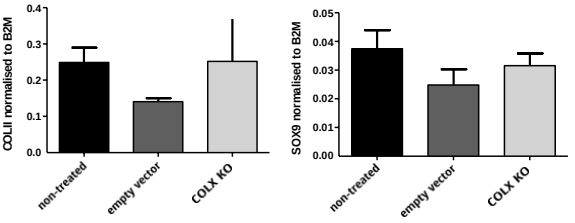

Fig.2: Gene expression of COLII and SOX9 remain relatively stable in COLX knock-downs compared to controls.

DISCUSSION & CONCLUSIONS: COLX is a definitive marker associated with hypertrophic chondrocytes, hypothesised to be essential for proper EO in TE. Although it does not appear COLX affects the expression of genes normally associated with chondrogenesis, we have shown COLX not only affects construct morphology, but also affects the production of signalling molecules important for recruiting cells which are vital for proper endochondral bone formation highlighting the importance of this collagen in TE EO.

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Large scale production of recombinant Cripto protein for the treatment of degenerative muscle diseases

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INTRODUCTION: Muscular dystrophies (MDs) are genetic disorders characterized by progressive muscle wasting, leading to limitations in motor capacity and in many cases to progressive paralysis and death [1]. Such degenerative muscle diseases can be treated with recombinant Cripto, an extracellular protein that has been recently found to have great therapeutic potential. Specifically, Cripto was found to regulate muscle regeneration and satellite cell progression toward the myogenic lineage [2]. This complex recombinant protein is produced using anchorage-dependent mammalian cell culture methods, and because protein therapies may require large doses over a long period of time, the manufacturing capacity can become a bottleneck to clinic. Protein production using anchorage dependent mammalian cell systems still need further optimization to meet the large scale demand for commercial clinical application. Herein, we improve upon the production capacity of exogenous therapeutic Cripto by using suspension bioreactors in combination with a hydrogel microcarrier 3D cultivationsystem.

METHODS: We applied a semi-synthetic hydrogel as a microcarrier that provides precisely controlled environmental conditions for Cripto-expressing anchorage-dependent HEK cells, allowing these mammalian cells to survive, proliferate, secrete Cripto protein and keep up their metabolic activity. The semi-synthetic hydrogel was comprised of nature protein (fibrinogen) which was conjugated to a synthetic polymer, polyethylene glycol (PEG). The hydrogel provides the optimization platform for the microcarriers by controlling both the mechanical properties and degradation rate of the HEK cell microenvironment. HEK cells expressing secretory recombinant Cripto are encapsulated in the PEG-Fibrinogen (PF) microcarriers and cultivated in stirred suspension bioreactors. PF precursor was produced, and HEK cells were encapsulated by polymerizing the PF precursor solution, as detailed elsewhere [3]. Cell development was followed using confocal microscopy. Cripto protein was purified from the suspension culture medium using Centromate™PE Holder (PALL) and Ni-NTA Superflow Cartridge (Quiagen). Protein extract was analyzed by Western blot and ELISA.

RESULTS: We demonstrated the capability of the PF microcarriers to support optimal conditions for cells growing in suspension bioreactors using cell staining assays at different time points. We performed cell cultivation in microcarriers and harvested proteins from the culture medium during 2 weeks in culture. The PF microcarriers exhibit high mechanical strength in order to resist shear forces and biodegradation associated with long–term suspension culture in these stirred bioreactors. We also cultivated the HEK cells on TCP and harvested the Cripto from this medium as a control group. We purified Cripto from both experiments and compered the results using ELISA. Protein yield was significantly higher from the bioreactor system.

DISCUSSION & CONCLUSIONS: The PF provides a controllable environment that can support cell cultivation and protein production in large scale suspension bioreactors. The viability, development and size of cell colonies indicate the effective cultivation of cells and diffusion into and out of the microcarrier. Moreover, the microcarriers provide mechanical protection against shear forces during cultivation in the stirred bioreactors. The synthetic component, i.e. the PEG, allows control over mechanical properties of the hydrogel, in order to support long term cultivation of the microcarriers. Therefore, we conclude that the suspension bioreactor system with PF microcarriers is important in providing the means to produce the Cripto protein in large scale required for commercial applications.

Bactericidal effect of non-thermal atmospheric plasma and its use in wound healing

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INTRODUCTION: Non-thermal plasmas (NTPs) are generated from a flow of neutral gas in a locally high-strength electric field, while the gas remains at atmospheric pressure and near ambient temperature. NTPs have nonspecific bactericidal, fungicidal and virucidal effects, which offer considerable opportunities for their use in medical sterilization and the treatment of wounds and multiple skin pathologies. However, the physical mechanisms and biochemical pathways underlying the effects of NTPs on bacteria and cell fate have still not been fully explored. Here we report on an analysis of the influence of different NTP systems on bacterial and mammalian cell cultures as well as on skin wound healing.

METHODS: To produce uniform NTP for biological applications we utilized a single jet NTP system using helium or atmospheric air as a working gas. The effects of plasma were studied on Gram-negative and Gram-positive bacterial strains, as well as on mammalian cell cultures (3T3 fibroblasts) by determining cell viability, ROS production, apoptosis, and mitochondrial membrane potential. A full-thickness skin wound model in rat was used for the *in vivo* evaluation of the effects of air plasma on wound healing.

RESULTS: Using scanning electron microscopy, we demonstrated that plasma treatments completely destroyed Gram-positive and Gram-negative bacteria within one minute of exposure, when the antimicrobial efficacy was nearly 99.99% for both strains (Fig.1) [1]. We demonstrated that depending on the exposure time plasma induces either direct physical destruction of bacteria or triggers programmed cell death that exhibits characteristic features of apoptosis [2]. The interaction of plasma with mammalian cell cultures induced cell death via the accumulation of multiple intracellular reactive oxygen/nitrogen species. In addition, the effects of ozone, a considerable component of non-thermal plasma, have been simultaneously evaluated and have revealed much faster and higher cytotoxic effects [1]. On the other hand, when tested on rat intact skin, histopathological analysis did not reveal any harmful effects on plasma–treated animals in comparison to controls. Repeated plasma-treatment (1 or 2 min) of a skin wound for 3 or 7 days did not affect the wound contraction rate or the number of macrophages within the scar, but modulated the expression of inflammatory markers.

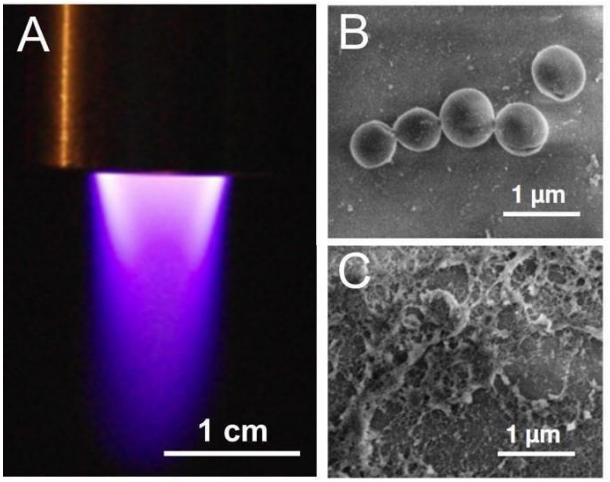

Fig. 1: (A) Plasma torch. Scanning electron micrographs of S. aureus; (B) untreated bacteria and (C) bacterial disruption after 60s non-thermal plasma treatment.

DISCUSSION & CONCLUSIONS: Our findings demonstrate the bactericidal effects and safe use of non-thermal atmospheric air plasma treatment for wound healing, highlighting the favourability of plasma applications for chronic infected wound therapy. Preliminary results of the atmospheric plasma device applications in veterinary medicine for the treatment of infected wounds in soft tissues and other pathologies in small animals and sport horses are also presented.

ACKNOWLEDGEMENTS: Supported by: Technology Agency of the Czech Republic TA04010449 and MEYS of the Czech Republic NPU I: LO1309.

Serum from platelet rich fibrin enhance proliferative potential of human mesenchymal stem cells

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INTRODUCTION: The role of Human Mesenchymal Stem Cells (hMSC) in tissue regeneration is unquestionable, but still there are problems with the variability in terms of cell proliferation and differentiation time among patients. There is need to find an effective differentiation induction method that reduces culture time while enhancing tissue quantity and quality[1]. We compared the properties of serum gained from platelet rich fibrin (SPRF), platelet rich plasma (PRP), and standard culture medium containing fetal calf serum (FCS) in proliferation and adipogenic differentiation assays.

METHODS: Metabolic activity and proliferation rate of hMSCs (Lonza, Walkersville, US) were investigated through XTT assay (Roche, Mainheim, Germany) in 96-well cell culture plate based monolayer culture, in DMEM (Gibco, Paisley, Scotland) supplemented with 10% SPRF, 10% PRP, 10% FCS or 10% FCS + bFGF. Experiments lasted 5 days and measurements were taken at three different time points (days 0, 2, 5).

The adipogenic differentiation experiment was performed in 6-well cell culture plates. Cells were incubated in DMEM with 10% FCS + bFGF for 4-6 days, until they reached 100% confluency. Differentiation process lasted for 14-20 days, cells were cultured in six different treatment groups. Three gropus with differentiating media: DMEM with adipogenic differentiation factors (dexamethasone, L-glutamine, h-insulin, isobutylmethylxanthine, indomethacin) (Lonza, Walkersville, US)) with 10% SPRF, 10% PRP, or 10% FCS and three groups with basal media: DMEM supplemented with 10% SPRF, 10% PRP, or 10% FCS. To analyse the effectiveness of the differentiation process, cells were fixed and stained with Oil Red O dye (Sigma-Aldrich, St Louis, US).

RESULTS: The most intensive rate of proliferation was observed in groups where cells were supplemented with SPRF. The hMSC cell numbers in group with SPRF were comparable to the positive control with 10% FCS and bFGF growth factor and in the same time it was observed that with PRP the cell number was significantly lower.

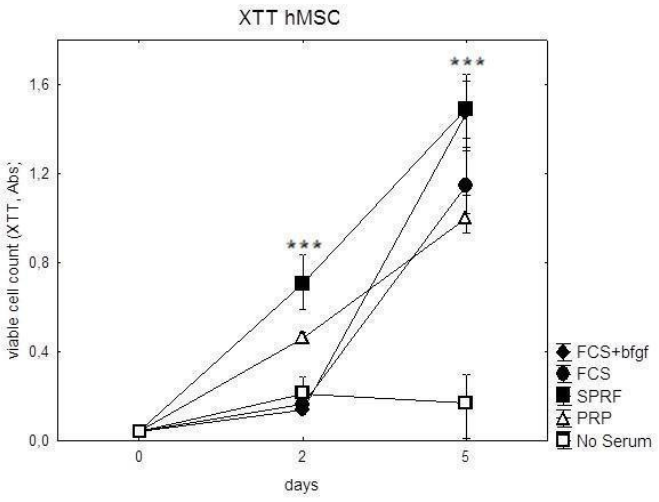

Fig. 1: Proliferation-supporting capacity of FCS+bFGF, FCS, SPRF, PRP on hMSC cells. SPRF group presented significantly higher cell number after 2 and 5 days (Two way ANOVA, $p<0.001$).

After staining with Oil Red O we discovered that in groups, where cells were stimulated with adipogenic differentiation factors and supplemented with SPRF or PRP, the differentiation potential was preserved.

DISCUSSION & CONCLUSIONS: Our results show that SPRF stimulates mesenchymal cell proliferation and at the same time preserves adipogenic differentiation potential. SPRF is a product of human origin, and as such it may replace fetal calf or bovine serum in tissue engineering applications. Since the absence of platelets and fibrinogen, it does not require the addition of heparin or other anticoagulants, making it an optimal choice in ex vivo cell and tissue cultures.

ACKNOWLEDGEMENTS: Sponsored by a FFG Basisprogram research grant to OrthoSera GmbH.

Forces and regulation of vascular network

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INTRODUCTION: Creation of vascular networks is a complex and fascinating biological process, with implications on various disciplines, such as ischemic diseases, cancer and inflammatory disorders. In the field of regenerative medicine, techniques for generating vascularized tissues bear significant clinical value, as they ensure sufficient oxygen and nutrient supply within the host tissue, cardinal to transplant integration and survival[1] _ENREF_1. However, engineering such vascularized tissues demands controlled patterning of the resulting network properties such as maturity, morphology. Recent studies attempted to regulate network properties by supplementing cultures with biological factors, biomaterials and geometrical constraints. Mechanical force production and transduction by growing tissues during development has been demonstrated to control cell signaling and gene expression and to regulate tissue homeostasis in adult organisms [2]. In this study we aimed to investigate vascular morphogenesis and to further understand the impact of tensile forces on vascular network morphology and properties.

METHODS We used the EBERS TC-3 load bioreactor for applying mechanical stimulation such as stretching on three dimensional constructs embedded with cells. Gelfoam scaffolds were used due to their ability to stand strains of more than 30% without failure; scaffolds were co-cultured with GFP- expressing HUVECs and HNDFs, supporting cells, for 14 days in the TC-3 bioreactor with strains of 10% and 1Hz frequency. Constructs were imaged every 7 days.

RESULTS: Our results demonstrate that vessel network within the stretched scaffold matured faster and prospered over time as opposed to the free floating control scaffolds. Additionally we observed that under cyclic stretching fibroblasts were elongated perpendicular to the stretching direction followed by the elongation of vessel-like structures toward the same direction. Different orientations of endothelial vessel structures were observed under static and cyclic uniaxial tensile forces. Our results present an evidence that the actomyosin pathway, responsible for force generation, with a direct impact on cell shape and tissue remodelling, guides vascular network assembly.

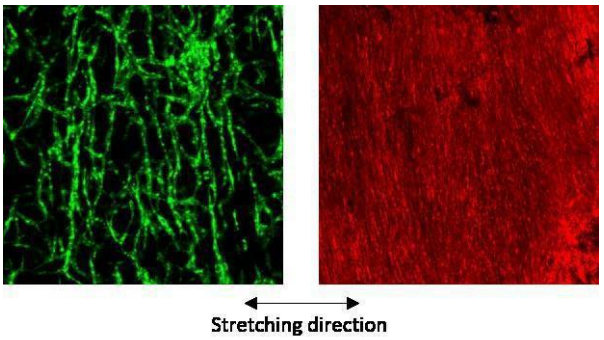

Fig. 1: cellular and vascular organization within stretched scaffolds. HUVEC-GFP in green and HNDF-RFP in red

DISCUSSION & CONCLUSIONS: We demonstrated a means of directing endothelial cell tube orientation using tensile forces, as well as the importance of cell orientation in network structuring. Overall, this study sheds light on the ability to direct tube structure using external forces allowing us to construct rationally designed vascular networks, resulting in improved integration upon implantation.

ACKNOWLEDGEMENTS: ERC Grant Agreement no. 281501 – ENGVASC for funding this project.

Silencing HLA class-I expression in allogeneic endothelial cells maintains morphological und functional properties necessary for vascular tissue engineering

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INTRODUCTION: Tissue engineered vascular grafts generated from autologous tissue sources are promising alternatives to allogeneic or xenogeneic approaches. Peripheral blood is an easily accessible autologous cell source for endothelial cells (EC) which are essential for vascular grafts due to their anti-thrombogenic and vessel tone regulating properties. However, their expansion is restricted to young and healthy donors [1] which limits their suitability for clinical use. Thus, allogeneic EC sources like the umbilical cord vein and umbilical cord blood have to be considered which however bear the risk of rejection after allotransplantation. Human leucocyte antigen class I silencing (HLA-I-sil) has been shown to minimize allogeneic rejection by cytotoxic T-lymphocytes [2]. However, it still remains unclear whether HLA-I-sil interferes with EC functionality. Thus, we here investigate whether HLA-I-sil of EC affects EC morphology and physiological functions important for vascular tissue engineering. Therefore, EC from peripheral blood (PB-EC), umbilical cord blood (UC-EC) and the umbilical cord vein (HUVEC) were HLA-I silenced and their morphological and functional properties were compared.

METHODS: PB-EC were derived from magnet-sorted CD34+ peripheral blood mononuclear cells (MNC) and UC-EC were derived from umbilical cord blood MNC after 5-14 days of cultivation. HUVEC were purchased from Pelo Biotech. HLA-I expression was silenced in PB-EC, UC-EC and HUVEC using a lentiviral vector system that delivers short hairpin RNA to silence the β-2 microglobulin domain of the HLA-I complex [2]. Before and after silencing EC were characterized for EC markers by immunostaining, their ability to form capillary-like structures in co-culture with adipose-derived mesenchymal stem cells (ASC) in a three-dimensional fibrin gel and their orientation under laminar flow.

RESULTS: Non-silenced PB-EC, UC-EC and HUVEC expressed CD31, CD144, vWF and eNOS, formed capillary-like structures in a fibrin gel (Fig. 1) and orientated themselves under laminar flow at moderate (25 dyn/cm^2) and high physiological arterial (42 dyn/cm^2) shear stress. Flow cytometric analysis of silenced EC showed a HLA-I-sil efficiency of 85% (PB-EC), 90% (UC-EC) and 80% (HUVEC). Either EC type showed an unchanged expression of each characteristic EC marker, tube formation capacity and orientation under laminar flow indicating an intact functionality aftersilencing.

Fig. 1: Capillary formation of non-silenced HUVEC (red, A) and silenced HUVEC (green, B) in the presence of ASC (green resp. red).

DISCUSSION & CONCLUSIONS: HLA-I-sil of allogeneic EC represents a promising technique to generate EC that bear a highly reduced risk of immunological rejection after transplantation while keeping their original physiological functions necessary for vascular grafts. In particular, silenced UC-EC might be of interest due to the quick and easy isolation process, the high cell yield and high proliferation capacity.

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ACKNOWLEDGEMENTS: This project was funded by Biofabrication for NIFE (supported by the german Lower Saxonian Ministry for Science and Culture and the VolkswagenStiftung).

DISCLOSURE: No conflict of interest exists.

Potential of spheroids made of cryopreserved primary human hepatocytes for bioartificial liver

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INTRODUCTION: Fresh primary human hepatocytes (PHH) are still considered as the gold standard source for bioartificial liver (BAL) applications but their restricted availability and the rapid deterioration of their metabolism hampered their use. Indeed, BALs request several billions of cells for a single treatment. Among the numerous attempts to avoid loss of differentiation and functions, 3D culture under spheroids shape have been investigated with freshly isolated hepatocytes with good success regarding functional outcomes despite a large inter-individual variability [1]. These approaches did nevertheless not solve the availability issue. In this context, we propose to reconsider the use of cryopreserved PHH (CPHH). Indeed, if large pieces of human liver (from non-transplantable organs) are scarce, resections are often performed leading to a broad source of PHH that can be easily sorted and stored. Regarding the amounts requested for BAL treatment, we aimed at evaluating the feasibility for low plateable PHH to form functional spheroids with such type of cryopreserved cells. Therefore, we developed a specific method of 3D culture based on non-adherent Petri dishes under orbital agitation. This method allows working either with small amounts of cells (≤300 000) or large ones (≥100x106) [2], in opposition with other methods (bioreactor, hanging drop, etc).

METHODS: Studies were conducted on three batches collected by Kaly-Cell (three different donors) of CPHH with only 50% plateable efficiency after thawing. We compared several liver specific functions including protein synthesis (albumin secretion), carbohydrate metabolism (glycogenolysis rate), detoxification (Cyp 1A (EROD), uridine diphosphate glucuronoltransferase activity (UGT) and transporters activities (clearance of indocynanine green) and waste removal (urea synthesis) on three different culture conditions:
(1) cells suspension after thawing ($C0_{sus}$), (2) after 24h plating on collagen-coated dish ($C1_{ad}$), (3) on spheroids in stirring conditions at different time points (4, 7, 10 days) (Sph).

Statistical analysis was performed by one way ANOVA for multiple comparisons. The Student t test was used for the comparison using GraphPad software (Prism 6).

RESULTS: After 4 days on culture in stirring conditions, spheroids formed by CHHP exhibited a mean diameter < 200 µm (45±4, 59±10, and 72±5 µm respectively on day 4, 7, and 10) and acceptable viability (Figure 1).

The comparison of the metabolic functions among the three groups presented similar profiles for the three batches despite the basal inter-donor variability: culture under spheroids conditions allow the maintenance or the increase of all metabolic functions over the ten days. ICG clearance also demonstrated apical and basolateral polarization illustrated by the presence of functional transporters.

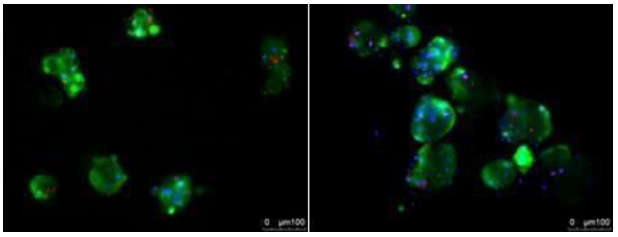

Fig. 1: Viability staining (live and dead® of CHHP spheroids after seven (left) and ten days (right) of culture. Live cells fluoresce green, and dead nucleus cells fluoresce red.

DISCUSSION & CONCLUSIONS: The very promising results and the improvement of independent liver-specific functions strengthen the use of CPHH as a possible source of cell for BAL application or for drug screening.

ACKNOWLEDGEMENTS: The study was supported by French ANR TecSan SUPPLIVER.

Promotion of robust and functional spheroid organization of human salivary gland epithelial cells cultured on hydrogel-micropatterned nanofibrous microwells

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INTRODUCTION: Development of a tissue-engineered, salivary bio-gland will benefit patients suffering from xerostomia due to loss of fluid-secreting acinar cells. This study was conducted to develop a bioengineering system to obtain a functional salivary gland (SG) organoid cultured on hydrogel-micropatterned nanofibrous polycaprolactone (PCL) microwells and to compare the efficacy of PCL microwells with a three-dimensional (3D) extracellular matrix (ECM), Matrigel, for assembly of functional SG organ-like transplants.

METHODS: Human SG epithelial cells (hSGECs) were obtained from parotid glands, expanded in vitro, and then plated on plastic dishes, Matrigel, or PCL microwells. Microwells were fabricated by photopatterning of poly(ethylene glycol)(PEG) hydrogel in the presence of an electrospun PCL nanofibrous scaffold. Morphology, cell proliferation and viability, phenotypic gene and protein expression, and polarity of 3D assembled salivary organoids were evaluated. To assess the secretory function, transepithelial electrical resistance (TER), the level of excretory protein (α-amylase), and intracellular calcium concentration ([Ca2+]i) after stimulation were measured.

RESULTS: Although hSGECs cultured on both Matrigel and PCL microwells aggregated to form 3D acinar-like spheroids, the aggregating potency was greater on the PCL microwells than on the Matrigel. The proliferation rate of hSGECs was higher when cultured on the PCL microwells than the Matrigel, without any significant cell death. The 3D-assembled spheroids expressed higher levels of salivary epithelial markers (α-amylase and AQP5), tight junction proteins (ZO-1 and occludin), and adherence proteins (E-cadherin) on PCL microwells than on the Matrigel. Furthermore, the 3D-assembled spheroids on the PCL microwells showed higher levels of transepithelial electrical resistance (TER), α-amylase secretion and intracellular calcium concentration ([Ca2+]i) than those on the Matrigel, suggesting more robust and functional organization of polarized hSGECs.

DISCUSSION & CONCLUSIONS: We established a bioengineering 3D culture system to promote robust and functional acinar-like organoids from hSGECs. PCL nanofibrous microwells can be applied in the future for bioengineering of an artificial bio-salivary gland for restoration of SG function.

1

Incorporation of selenium-based antioxidants into a calcium phosphate cement

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INTRODUCTION: Natural bone regeneration may not occur after extensive trauma or degenerative diseases such as osteoporosis. Functional repair requires surgical intervention and the implantation of osteoconductive scaffolds. Calcium phosphate cements (CPCs) have been frequently used as the basis for bone regenerative scaffolds. However, one caveat to using these scaffolds is the occurrence of chronic inflammation due to surgery or degradation products which can then lead to device failure. Selenium (Se) based bioorganic compounds have excellent anti-oxidative behaviours due to their properties as glutathione peroxidase (GPx) mimics [1]. In the current study, we have screened a library of Se containing compounds for hydrogen peroxide, H_2O_2, scavenging activity and are explored the outcomes of their delivery from CPCs for better device integration and patient outcome.

METHODS: H_2O_2 release was measured with luminol amplified chemiluminescence using primary human monocytes. Briefly, cells were suspended in a white 96 well plate ($2*10^5$ cells/well) in a luminol solution (500 µM) that was prepared from a stock of 50 mM luminol (3-aminophthalhydrazide) diluted in 0.2 mM NaOH along with Se compounds. Cells were induced to release ROS using phorbol-12-myristate-13-acetate (PMA; 500 nM). Luminescent signal was recorded every 2 minutes using a microplate reader at 37°C for 120 minutes. CPCs were prepared and loaded with Se compounds as previously described [2]. Mechanical testing was performed using a universal material testing machine equipped with a load cell of 5 kN at a cross-head speed of 1mm/min.

RESULTS: Screening of the Se bioorganic library revealed several compounds which potently reduced H_2O_2 generated by the mononuclear cells (Fig 1). A brushitic CPC showed no significant changes in its compressive strength after being doped with the Se bioorganic compound (Fig 2.)

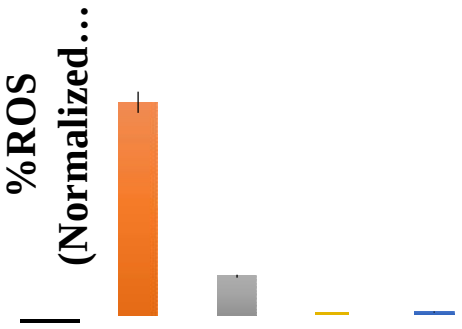

Fig. 1: Luminol signalling showing generation of ROS from monocytes after exposure to PMA and Se containing compounds (25µM).

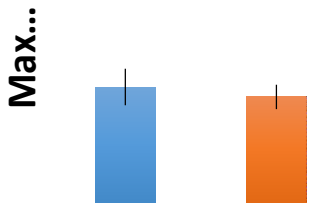

Fig. 2: Compressive strength after setting cement in PBS for 24h

DISCUSSION & CONCLUSIONS: The Se compounds were able to scavenge H_2O_2 (most likely via a GPx-like scavenging mechanism) without compromising mechanical properties of the CPCs. Based on these results, we are continuing our investigations and assessing the *in vitro* effects of these antioxidants released from CPCs in both bone and immune cell models. Functionalizing CPCs or other scaffolds with these antioxidants may improve patient outcome by modulating the inflammatory reactions.

ACKNOWLEDGEMENTS: CarlTryggers Stiftelse (15:363), Magnus Bergvalls Stiftelse (2015-01111) and Ollie & Elof Ericssons Stifelse för Vetenskaplig Forskning

Repairing injured articular cartilage: investigation of potential repair mechanisms using mesenchymal stem cells

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INTRODUCTION: Traumatic cartilage injuries are a significant risk factor for the development of osteoarthritis. Classically, cartilage is considered to have poor repair capacity such that cartilage injuries do not heal. However, recent evidence suggests that cartilage injuries in very young animals can repair [1]. The mechanism of this repair is still unknown. The stem cells found in the joints might be involved in this repair mechanism. The aim of this project was to investigate if mesenchymal stem cells (MSCs) in the joint tissues respond to articular cartilage injury and are involved in potential repairmechanisms.

METHODS: Articular cartilage, synovial fluid (SF) and bone marrow (BM) MSCs were isolated from bovine metacarpophalangeal joints and the cell numbers expanded in monolayer culture. Cartilage pieces were incubated 72h in culture medium [Dulbeccos modified Eagles medium, DMEM] containing 10% foetal calf serum and 10^{-7} M dexamethasone to obtain 'non-injured' cartilage. Cartilage was devitalized by microwave irradiation to obtain 'dead' cartilage. 'Injured' cartilage was prepared by cutting living cartilage several times with a no.11 scalpel blade. To investigate whether MSC migratory factors were released upon cartilage injury, cartilage explants were cut into fine pieces and incubated in serum-free DMEM containing 0.1% bovine serum albumin (BSA) for 24h. This 'Injury-conditioned medium' (IJCM), was used for cell migration assays (Transwell™, classical scratch and cell exclusion zone assays). To identify potential factors released from the extracellular matrix, heparin-binding proteins were removed from IJCM using immobilized heparin. Potential 'injury' factors released by chondrocytes (CIJM) were investigated by injuring monolayers of chondrocytes with a pipette tip and incubating the injured chondrocytes in serum-free DMEM containing 0.1% BSA for 4h. One-way ANOVA was used to calculate statistical significance of cell migration.

RESULTS: SF-MSCs showed significant migration to injured cartilage (1.5-fold increase; $P< 0.001$) but not to non-injured and dead cartilage (1-fold and 1-fold respectively; $P> 0.05$; $P>0.05$). Fluorescently-labelled SF-MSCs specifically adhered along the edges of injured tissue of freshly injured cartilage. SF-MSCs demonstrated a 1.6-fold increase and BM-MSCs a 1.9-fold increase in migration to IJCM compared to control (DMEM+0.1% BSA) ($P<0.05$). IJCM significantly reduced wound width by 75%±5.7% ($P<0.001$) compared to 14%±1.2% in control (DMEM+0.1% BSA) in scratch migration assays. Heparin-treatment of IJCM significantly reduced MSC migration into the scratch; only 31%±4.8% ($P<0.05$) reduction in wound width was observed. These migratory effects of IJCM and heparin-treated IJCM were not observed with chondrocytes or MG63. In contrast to classical scratch assays, no migration of SF-MSCs was observed in response to control and IJCM in migration assays where no cell injury occurred such as the cell exclusion zone assay. However, migration in response to IJCM was observed in this system when cell injury was created (by scraping off cells) close to the cell exclusion zone. Moreover, addition of both CIJM and IJCM stimulated SF-MSC migration into the cell exclusion zone of non-injured MSC cultures. The identity of the injury factor(s) in CIJM and IJCM is being investigated.

DISCUSSION & CONCLUSIONS: The results suggested that injured cartilage released migratory factors which targeted migration of MSCs to the site of injury. Some these factor(s) appeared to be heparin-binding proteins. Lack of migration in response to IJCM in cell exclusion zone assays where no cell injury occurred suggested that in addition to injury factors released into the IJCM, a short-lived factor(s) released from the cells is needed to activate/initiate MSC migration. Moreover, following partial-thickness defects, where BM-MSCs do not have a direct access to the injury site, SF-MSCs could be involved in the homeostatic reparative mechanisms of injured cartilage seen in young animals.

Rat subcutaneous and perigonadal adipose tissue-derived mesenchymal stem cell spheroids: investigation of differentiation and co-culturing potentials

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INTRODUCTION: Tissue specific cells and stem cells are the major components of tissue engineering and regenerative medicine applications. Accordingly, mesenchymal stem cells (MSCs) are regarded as a promising population of cells in these fields owing to their unique properties. Three dimensional (3D) culturing of MSCs provide highly *in-vivo* condition similarity to *in-vitro* model systems. In this study, rat subcutaneous and perigonadal adipose tissue-derived MSCs were isolated using primary explant culture technique and cultured with/without human umbilical vein endothelial cells (HUVEC) and L929 mouse fibroblast cells as spheroidal shape aiming at preparing an alternative MSC transplant system for stem cell based therapies.

METHODS: Hanging drop technique was used to prepare homotypic and heterotypic cell spheroids from single cell suspensions (Foty, 2011). Spheroid formation potential of adipose tissue-derived MSCs was examined comparatively using fluorescent staining, histological staining and light microscopy. Cellular organization and morphology of homotypic/heterotypic spheroids were visualized by scanning electron microscope and fluorescence microscope. Differentiation characteristics of MSC spheroids were determined colorimetricly with Von Kossa staining, Alcian blue staining and Oil Red-O staining at specified intervals.

RESULTS: Optimization studies have showed that the sizes of the spheroids were dependent on the density of cells suspended in the drops. The sizes of the spheroids are ~200 µm in diameter when the number of cells per spheroid is optimized 25×10^3 cell/10µl drop (Fig. 1).

Co-culturing of MSCs as spheroidal form promote favorable biological responses of cells including enhanced cell-cell attachment, extracellular matrix (ECM) synthesis, differentiation and angio-regulatory properties.

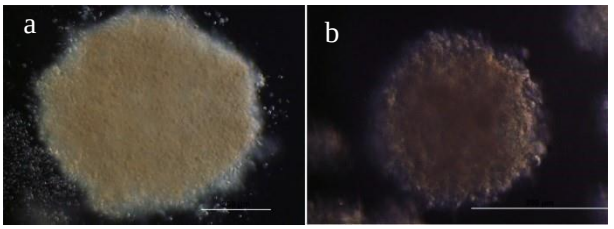

Fig. 1: Inverted microscope images of subcutaneous (a) and perigonadal (b) MSC spheroids. Scale values (white lines) are: 200 µm and magnifications are: 40× (a) 10× (b)

DISCUSSION & CONCLUSIONS: Cells in a 3D culture environment show different characteristics from cells in 2D monolayer culture (Fennema *et al.*, 2013). It is reported that culture of MSCs as 3D spheroids or aggregates enhances their therapeutic effects (Bartosh *et al.*, 2010). Insights gained from this presented study will help improve current scaffold-free stem cell transplantation techniques.

Evaluation of "stemness" properties of different types of oral stem cells expanded under serum/xeno-free GMP-compliant culturing conditions

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INTRODUCTION: Development of clinical-grade, xeno-free cell preparations is central to cGMP (Good Manufacturing Practice compliant) conditions to meet regulatory requirements for clinical application of cellular therapies [1]. This study aimed to investigate the potential of two serum/xeno-free, cGMP culture media to maintain "stemness" of human Stem Cells (SCs) compared to a conventional serum-containing medium.

METHODS: Dental Pulp Stem Cells (DPSCs) cultures were established from pulp biopsies of third molars of healthy individuals after routine extraction; Bone-Marrow Mesenchymal SCs (BM-MSCs) from osseous biopsies during dental implant placement; and Salivary Gland SCs (SG-SCs) from minor salivary glands of lower lip biopsies diagnosed as negative for Sjögren syndrome. The enzymatic dissociation method was used for cell culture establishment in all cases [2]. Patients (six for each cell type) signed an informed consent. Three different media were used: StemPro MSC SFM XenoFree (Life Technologies, Carlsbad, CA); STEM MACS MSC, XF (Miltenyi Biotek, Bergisch Gladbach, Germany) and a-MEM (Invitrogen, Karlsruhe, Germany) supplemented with 15% FBS, L-ascorbic acid phosphate and antibiotics/antimycotics (=Complete Culture Medium-CCM). Cells were characterized for mesenchymal (CD90, CD73, CD29, CD49f, CD81, CD166, CD146, STRO-1, CD34), endothelial (CD105, CD106), hematopoietic- (CD45) and embryonic (SSEA-1, 3, 4, 5) SC markers at passages 2, 6 and 10 by flow cytometry. Real time PCR was used to assess spontaneous up- or down-regulation of osteogenic (ALP, BMP-2), adipogenic (LPL, PPAR) and chondrogenic (ACAN) differentiation markers during continuous *ex vivo* expansion.

RESULTS: Oral SCs expanded with both serum-free media showed lower proliferation rates compared to expansion with CCM, while in some cases serum-free expanded BM-MSCs ceased to proliferate, acquiring a senescent-like appearance. Expansion with the serum-free media induced a uniform spindle-shaped phenotype in all SC types, in contrast to CCM which preserved the differential morphological characteristics of the heterogeneous DPSC, BM-MSC and SG-SC populations. All types of oral SCs at passage 2 were entirely positive for CD90, CD73, CD29, CD81, CD49f, CD166 and partly for CD105, CD106, CD146, STRO-1, CD34 and SSEAs, while negative for CD45. After expansion with both serum-free media a pronounced down-regulation of CD146, Stro-1, CD105, SSEA-4 and occasionally other SSEAs with parallel upregulation of CD34 and CD106 was observed in all cases. Similar trends, although to a minor extent were found for CCM-expanded SCs. Expansion under serum-free conditions caused pronounced up-regulation of ALP and BMP-2, with parallel complete elimination of the baseline expressions of PPAR, LPL and ACAN; therefore, showing a preferential SC shift towards osteogenic phenotypes. In contrast, expansion with CCM caused upregulation of PPAR (all 3 types) and ACAN (SG-SCs) and down-regulation of ALP and BMP-2 (all 3 types).

DISCUSSION & CONCLUSIONS: These findings indicate there is still a vacant role for the optimal cGMP serum-free medium to maintain "stemness" of oral SCs. Selection of different stem/progenitor populations may occur under various culturing conditions, which might be of major importance depending on the targeted clinical application. Further investigation with functional assays in preclinical models is necessary to validate efficacy of cGMP media to meet clinical-grade standards.

A novel human plasma-derived medium supplement for xeno-free culture of microvascular endothelial cells

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INTRODUCTION: Although endothelial cell-based therapies were proven feasible and effective in various studies, broader translation from research to clinic in the field is still being hampered by the inconsistencies of the protocols used for the isolation and culture of these cells [1]. The majority of growth media used for endothelial cell propagation are based on xenogeneic components of animal origin, such as FBS (fetal bovine serum). That may compromise the post-translational safety of patients and has several other reverberations [2]. SCC (supplement for cell culture) is a novel xeno-free supplement that was already reported to support the growth of iPSC (induced pluripotent stem cells) and hMSC (human mesenchymal stem/stromal cells) [3]. SSC is obtained from well-controlled human plasma starting material through Cohn-Oncley industrial plasma fractionation and manufactured under GMP rules. In the present study, we show its application in human microvascular endothelial cells (HMVEC) culture.

METHODS: HMVEC were purchased from a commercial supplier (Lonza). Cells were grown in a xeno-free (XF) medium, consisting of basal medium (MCDB 131) with SCC (Grifols) and different supplements. Establishment of the most applicable formulation was preceded by screening of over 50 formulations, complemented with different combinations of commonly used growth factors. This optimization process was based on 7-day-metabolic activity assays (resazurin) and morphology observation. Subsequently, HMVEC were grown in the optimized SCC-based formulation and their phenotype was determined qualitatively by immunofluorescence stainings and quantitatively by flow cytometry, using standard endothelial markers. Furthermore, a functional Matrigel sprouting assay was used to assess the ability of XF-expanded HMVEC to form tubular structures.

RESULTS: HMVEC cultured in media supplemented with SCC were able to proliferate, maintained correct cobble-stone morphology and the expression of a set of characteristic surface markers (Fig. 1). They were positive for CD31, CD146, CD144, von Willebrand factor (vWF) and acetylated-LDL uptake, whereas negative for CD90. The observed marker expression levels were in general comparable for the cells cultured in XF formulation and the ones grown in non xeno-free media – either commercial or home-prepared. SCC-cultured cells were capable of forming tubular-like structures in Matrigel.

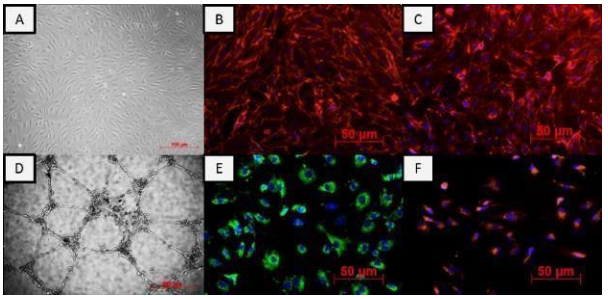

Fig. 1: Phenotype of HMVEC grown in SCC-supplemented medium. A. Cell morphology; Immunofluorescent staining for: B. CD31 C. VE-Cadherin E. vWF; D. Tube formation in Matrigel F. Ac-LDL uptake.

DISCUSSION & CONCLUSIONS: Results suggest that basal medium complemented with standard supplements, a growth factor cocktail and SCC is suitable for the culture of HMVEC. Further characterization of a long-term XF culture is being performed, along with verification of this formulation in endothelial progenitor cells system.

ACKNOWLEDGEMENTS: The project is a part of a Marie Curie Initial Training Network – European Industrial Doctorate (ITN-EID) IB2 (317052), and has been funded under the European Community 7th Framework Programme.

Short term response of mesenchymal stem cells against chondrocyte apoptosis

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INTRODUCTION: Mesenchymal stem cells (MSCs) are an essential component for tissue engineering applications and regenerative medicine due to their multipotent differentiation capacity, ability to response environmental biosignals, immunomodulatory and immunosuppressive properties [1]. To date, MSCs have been used for treatment of many diseases. One of the fields which these cells are used is rheumatologic disorders [2] which are related to chondrocyte apoptosis. Apoptosis of chondrocytes may occur due to either age related degenerations -like in osteoarthritis- or autoimmunity such as rheumatoid arthritis. In the present study, it was aimed to discover the short term effects of MSCs against chondrocyte apoptosis via using an indirect co-culture method.

METHODS: MSCs were isolated from human bone marrow. The cells at passage 3 were used for the characterization studies including the cell morphologies, differentiation capacity and surface markers. Human articular chondrocytes (hCA) were obtained by purchasing. A cytokine cocktail which contains TNF-α, IL-1β and IFN-γ was made to drag hCAs into apoptosis. Different concentrations of compositions were created and cultured with hCAs. Apoptosis was detected by WST-1 analysis. Then hCAs were seeded to 6 well culture plates, MSCs were seeded onto inserts with 3 µm pore size and placed to hCA seeded 6 well culture plates. hCAs and MSCs were co-cultured in the presence of apoptosis signals in this co-culture system (*Fig. 1*). At the end of 1st, 3rd and 7th days of culture, hCAs were enzymatically detached from plate surface. Apoptosis level and cell cycle attributions determined by annexinV-PI staining and cell cycle analysis in the flow cytometry, respectively.

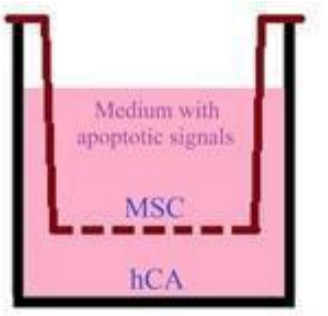

Fig. 1: Co-culture design used in the study

RESULTS: According to annexinV-PI staining results, it is observed that MSCs support the proliferation of hCAs. Cell cycle analysis also showed that these cells maintain proliferation and number of the hCAs which stays in S phase are increased by MSCs.

DISCUSSION & CONCLUSIONS: By taking all the data into consider these results show that MSCs can protect chondrocytes against apoptosis indirectly. With this indirect co-culture method, it is demonstrated that this anti-apoptotic effect is not related with the direct cell-to-cell contact but biosignal molecules secreted by these cells. In this study, maximum period of exposure with apoptotic signals is 7 days. From this point of view, the experimental model does not completely resemble the diseases, because there is a prolonged continuous apoptotic attack *in vivo*. In this context, it is necessary to do further experiments with longer exposure time, *in vitro* and *in vivo*.

ACKNOWLEDGEMENTS: This study is supported by The Scientific and Technological Research Council of Turkey (TUBITAK) with the project number of 114S154.

Management of facial hypertrophic scars by using fractional CO2 laser, adipose-derived stem cells, regenerative epithelial suspension and lipoinjection combination

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INTRODUCTION: Facial burns often get an inextricable situation in terms of healing and long-term morbidity. Many different procedures were used to overcome these challenging topics. Although results were more promising in terms of surgeons, is inadequate for patients. We present the efficiency of fractional CO2 laser(FL), Adipose-Derived stem cells(ADSC), Regenerative Epithelial Suspension(Recell®) and lipoinjection combination treatment on facial hypertrophic scars due to burns(FS) in this paper.

METHODS: 18 patients(M: 10, F: 8) among the ages of 18-35 years(Mean:25) between 2012-2015 were divided into three groups. Skin thickness and perfusion were measured by ultrasonography, Vectra® computer simulation was performed for symmetric lipoinjection, biopsies were performed and hematoxylin-eosin and Movat pentachrome staining were carried out in all patients on preoperative and postoperative 12. months. ADSC-enriched lipoinjection was performed in all groups. The mechanical dermabrasion was applied in Group1, dermaroller was done in Group 2, and FL was performed in Group3, Recell® was administered following in all groups. All of the patients underwent a satisfaction questionnaire.

RESULTS: Mean follow-up was 18(14-24) months. A significant improvement in skin softness, thickness, elasticity, color and symmetry was obtained in all patients. An increase in Keratinocyte, type 1 collagen; a decrease in nodular type 3 collagen and elastin, epidermal rete ridges, proteoglycan, fibronectin, neurofilament, T cells, macrophages and mast cells was observed in the histopathological studies in Group 3 compared to other groups (Fig 1.). A significant reduction in skin thickness, scar microcirculation and an increase in fatty tissue rates were obtained from USG in Group 3. Group 3 had higher scores in satisfaction questionnaire.

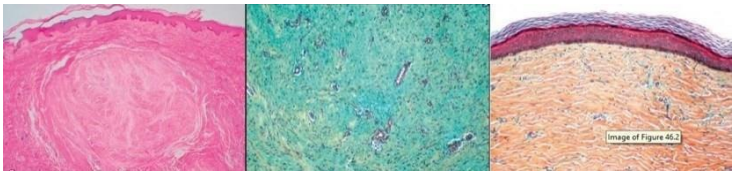

Fig. 1: Patients postoperative histopathological findings.

DISCUSSION & CONCLUSIONS: FL has an active role on smoothing and regression of FS. ADSC-enriched lipoinjection is effective in the long term management of facial asymmetry. The Recell® application increases the amount of keratinocytes and provides significant skin quality.

ACKNOWLEDGEMENTS:

Conflict of Interest: None

The efficiency of the bone marrow aspirate concentrate on healing of the corneal injuries due to acid burns

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INTRODUCTION: Acid burns often get an inextricable situation in terms of healing and long-term morbidity. Especially, the most important result is cornea and eye trauma. Corneal disease is a common cause of blindness, and keratoplasty is considered as an effective treatment method. However, there is a severe shortage of donor corneas worldwide. We present a simple, effective and cheaper method ‘ bone marrow aspirate concentrate usage’ (BMAC) in the treatment of corneal defects due to acid burns.

METHODS: An acid burn victim 2 year-old boy was admitted to our burn centre, and consulted for third degree facial burns. There was bilateral corneal damage and partial loss of vision on physical examination. Written informed consent was obtained from patient’s family for the surgical procedure. 30 cc of bone marrow was harvested from from anteromedial face of the tibia and processed using the SmartPReP BMAC® system (Harvest Technologies Corp.) to obtain BMAC. Bmac was injected into the conjunctival areas of both eyes. Postoperative results were archived by using Lifecell SPY device (SPY).

RESULTS: There were bilateral (Blood supply to the right eye was 50%, and left 40%) decreases in blood flow to the orbital, and periorbital conjunctiva. On postoperative period, blood supply has increased markedly (Blood supply to right eye was 65 %, and left 60%), and there was bilateral corneal healing in the postoperative 1. month eye exam.

DISCUSSION & CONCLUSIONS: Despite keratoplasty being the first choice in the repair of corneal defects, there is a severe shortage of donor corneas worldwide. We also argue that BMAC therapy may be a powerful alternative treatment for corneal injuries due to acid burns.

ACKNOWLEDGEMENTS:

Conflict of Interest: None.

Use of soft substrates to promote and maintain high plasticity in epigenetically erased fibroblasts

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INTRODUCTION: Terminally differentiated cell phenotype is achieved through complex epigenetic mechanisms, that regulate the chromatin structure as well as specific gene transcription programs. It has been recently demonstrated the possibility to interact with the epigenetic signature, switching the original cell phenotype into a different one, through the use of epigenetic erasers, readers or writers. Here we drive terminally differentiated cells into a high plasticity state, through the use of the epigenetic eraser, 5-aza-cytidine (5-aza-CR) [1-2]. We demonstrate that the simultaneous use of a "low stiffness" substrate, that match pluripotent cell intrinsic softness [3], is able to boost the induction and maintenance of a high plasticity state in epigenetically erased cells.

METHODS: Human skin fibroblasts were plated either on standard plastic dishes (group A) or on polyacrilammide gels (PAA) of "low stiffness" (1kPa) (group B). 24 hours after plating, cells were erased with 1 micromolar 5-aza-CR for 18 hours and subsequently cultured in Embryonic Stem Cell (ESC) medium for 17 days. Morphological analysis were carried out during the entire length of the experiment. OCT4, NANOG and VITRONECTIN expression levels were assessed by Real-time PCR.

RESULTS: Morphological evaluations showed that 5-aza-CR treated cells lost the fibroblast's elongated morphology and appeared rounded, with larger and granulated nuclei. After 7 days of culture in ESC medium, group B cells formed three-dimensional spherical structures, while group A cells remained in monolayer for the entire length of the experiment. These morphological modifications were accompanied by gene expression changes. Indeed, cells after 5-aza-CR treatment actively expressed the pluripotency markers, OCT4 and NANOG, both in A and B group. However, while group A cells progressively down regulated their expression by day 6, group B cells steadily transcribed for the pluripotency factors until day 17, when cultures were arrested. VITRONECTIN, that supports the maintenance of pluripotency and self-renewal [4], was expressed at higher levels in group B cells than those of group

A.

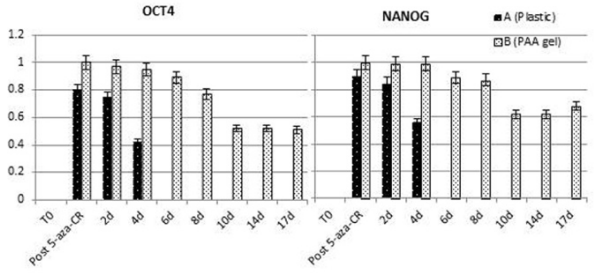

Fig. 1: Pluripotency gene expression changes in human erased skin fibroblasts plated on standard plastic dishes (A- Plastic) or on polyacrilammide gels of "low stiffness" (B- PAA gel), at different time points. Gene expression levels are reported with the highest expression set to 1 and all other times relative to this.

DISCUSSION & CONCLUSIONS: The results obtained confirm that epigenetic erasing induces a high plasticity state in terminally differentiated fibroblasts, with striking morphological changes and up-regulation of pluripotency related genes. The use of an adequate soft substrate promotes and stabilizes the maintenance of the acquired plasticity, suggesting a correlation between mechano-sensing and epigenetic regulation of cell phenotype.

ACKNOWLEDGEMENTS: Supported by Carraresi Foundation. Authors are members of the COST Actions FA1201, BM1308 and CM1406.

Autologous mesenchymal stem cell for neurological disorders: what happens during in vitro expansion?

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INTRODUCTION: Bone marrow (BM) mesenchymal stem cells (MSCs) have been successfully tested in preclinical models of dopaminergic damage [1]. Their ability to reduce inflammation and apoptosis in damaged neurons might be of interest in no-option neurological disorders. One of the potential targets of MSC therapy is Progressive Supranuclear Palsy (PSP), a sporadic, severe and rapidly evolving neurodegenerative disease which is not responsive to L-DOPA. Our hospital-based GMP-approved facility provides autologous BM-MSCs manufactured as advanced therapy medicinal products (ATMPs) in the context of a phase I clinical study (NCT01824121) with the aim to evaluate the safety of intra-arterial infusion of autologous BM MSC in patients with PSP. The study was authorized by the local Ethics Committee, and approved by the Italian Medicinal Agency (AIFA). In connection with this project, we set up a parallel *in vitro* study authorized by the local Ethics Committee, to investigate DNA damage and replicative senescence in BM-MSCs from PSP patients.

METHODS: BM from twelve PSP patients was directly seeded in alphaMEM (MacoPharma) supplemented with 10% FBS (Life Technologies). The cells were cultured until reaching the plateau phase. At different passages (P0-P1-P2) the cells were tested for the following quality controls: cell number, viability (Annexin V Apoptosis Detection Kit FITC, eBioscience), purity (CD90+/105+/45-, Beckman Coulter), sterility, CFU-F, karyotype and comet assay. In particular karyotype and comet assay were evaluated to asses the genomic stability and DNA damage during *in vitro* propagation. For karyotype, colcemid was added to cells in culture to arrests mitotic cycle in metaphase. Chromosomes staining with quinacrine mustard was performed and at least twenty metaphases were analyzed using Ikaros program (Metasystems). Comet assay combines alkaline DNA gel electrophoresis with fluorescence microscopy to visualize migration of DNA strands from individual agarose-embedded cells. The results were compared to those obtained from age-matched healthy donors.

RESULTS: BM-MSCs from nine patients passed the quality controls. Two BM-MSCs developed karyotype alterations at different passages during in vitro expansion: one of them had also altered cell growth. The remaining BM-MSC line had a low proliferation rate. DNA damage, as measured by the comet assay, was significantly more evident in MSCs from PSP patients compared to age-matched healthy donors ($P<0.05$).

DISCUSSION & CONCLUSIONS: There are sporadic evidences that MSC from patients affected by neurological diseases might somehow behave differently in culture compared to those generated from normal healthy donors [2]. We demonstrated that it is possible to obtain normal BM-MSCs from the majority of PSP patients. Nevertheless, in our study BM-MSCs from a subgroup of patients have altered cell growth and/or karyotype abnormalities. Therefore, we strongly suggest an accurate pre-screening procedure before treating patients in clinical trials for neurodegenerative disorders. A deeper understanding of the mechanism underlining the different cell behaviour of MSCs from neurological patients is needed.

ACKNOWLEDGEMENTS: The project is supported by Lombardy Region (Independent research, call 2012 "Terapia a base di cellule staminali mesenchimali autologhe nella paralisi sopranucleare progressiva: studio clinico randomizzato, controllato, in doppio cieco di fase I e II") and by "Fondazione Grigioni per il morbo di Parkinson".

Enchancement of cord blood primitive hematopoietic progenitors: impact of stromal support and hypoxia

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INTRODUCTION: Umbilical cord blood mononuclears (cbMNCs) are currently considered as an alternative source of hematopoietic stem and progenitor cells (cbHSPCs) [1]. Effective and physiologically-based hematopoietic cell expansion remains among the most important goals in cell therapy and regenerative medicine [1]. The HSPC milieu is specified by the supportive stroma and hypoxic O_2 level. Recently we have proposed a functional approach to enrich cbHSPC population due to adhesion to adipose tissue MSCs (ASCs) and further expansion of the progenitors of adherent primitive cells under normoxia (20% O_2) and "physiologic" hypoxia (5% O_2) [2].

METHODS: ASCs were isolated from human adipose tissue. cbMNCs was obtained from "CryoCenter" CB Bank in frame of scientific collaboration. cbMNCs were added to ASC monolayer and cocultured under 20% either 5% O_2. After 72 h unattached cbMNCs were removed. Newely arising HSPCs were FACS-analysed using antibodies to mature MNC and primitive HSPC antigens. Data are considered statistically significant after Student's t-test ($P \leq 0.05$).

RESULTS: Attached HSPCs were giving rise to floating (Fig. 1A) and ASC-associated HSPCs (Fig. 1B) both at 20% and 5% O_2. This interaction was accompanied with up-regulation adhesion molecule genes ICAM-1, VCAM-1 and down-regulation of genes involved in matrix remodeling (COLs, MMPs, TIMPs, ADAMs, HAS1) and cell-matrix contacts (ITGs) as was demonstrated with RT- PCR (Fig 1 C,D). These data indicate on activation of ASCs, which more effectively increased at 5% O_2. HSPCs were enriched with $CD34^+$ cells up to 6 (20% O_2) and 8 (5% O_2) times. Functional analysis of HSPCs revealed cobble-stone area forming cells (CAFCs) and lineage-restricted colony-forming cells (CFCs), the number of which was 1.6 times higher 5% O_2 (Tabl. 1). This increase was related to a rise in the number of multipotent precursors- BFU-E, CFU-GEMM and CFU-GM. These changes were

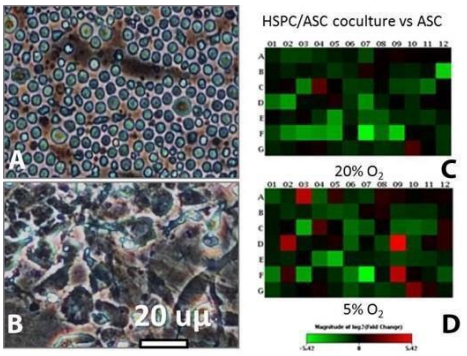

Fig. 1: Floating (A) and ASC-associated cbHSPCs (B)after 7 days of expansion in coculture. Differential expression of genes involved in cell adhesion (C,D)

were at least partly ensured by the increased concentration of MCP-1 and IL-8 at 5% O_2.

Table 1. Enrichment of cbHSPC population with primitive hematopoietic precursors

	$CD34^+$/ ml	$CD34^+$, %	$CD133^+$, %
Unmanipulated cbMNCs	6000±1900	0.31±0.1	-
cbHSPCs-1, 20% O_2	41200±6700	30±10	20±9
cbHSPCs-1, 5% O_2	62600±6300*	55±15*	26±15

DISCUSSION & CONCLUSIONS: Our data demonstrate that ASCs in hypoxia significantly modulate their physiology and ensure a better maintenance/expansion of cbHSPCs, pointing to the hypoxia as a physiological regulatory factor as well as a cell engineering tool.

ACKNOWLEDGEMENTS: This work was supported by BRMT Program of Presidium of RAS.

DISCLOSURES: The authors declare that they have no competing interests.

Progesterone prevents epithelial-mesenchymal transition of ovine amniotic epithelial cells and enhances their immunomodulatory properties A

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INTRODUCTION: Ovine Amniotic Epithelial Cells (AEC) are a subset of placental stem cells and object of intense study due to their regenerative and immunomodulatory properties. Freshly-isolated AEC express the typical epithelial markers such as Cytokeratin-8. Nevertheless, after several cultural passages AEC undergo to a phenotypical and functional shift, the epithelial-mesenchymal transition (EMT). Indeed, AEC undergone EMT lose their epithelial markers starting n to d express) mesenchymal ones (e.g. Vimenti an α-SMA and EMT-related genes such as Snail and Twist [1]. Unfortunately, EMT is accompanied by a reduction of regenerative and immunomodulatory potential of AEC [2,3]. This phenomenon does not occur during pregnancy, likely because AEC are exposed to a well-defined environment characterized by low oxygen and high levels of steroids (primarily progesterone, P_4). Starting from these premises, the present research has been designed in order to verify the role of P_4 during the *in vitro* AEC amplification.

METHODS: Amniotic membrane was collected at the slaughterhouse and AEC were isolated using a validated protocol [2]. AEC were incubated in presence of different concentration of P_4 (from 100 μM to 0.01 μM) with or without its nuclear receptor inhibitor (RU-486). Thiazolyl blue tetrazolium bromide (MTT) assay was used to measure cell proliferation. Cytokeratin-8 and α-SMA were evaluated by immunofluorescence. EMT-related genes and cytokines expression were assessed by Real-Time qPCR. Gene expression analysis were performed on CTR and P_4-treated AEC with or without LPS stimulation for 24h.

RESULTS: AEC exposed to 25 μM P_4 retained an epithelial phenotype for four passages by conserving an adequate proliferation activity differently from the other P_4 concentrations. This is confirmed by Cytokeratin-8 expression (Fig.1) and by the down-regulation of EMT-related genes (Snail and Twist). As CTR cells, AEC exposed to RU-486 undergo EMT. Moreover, P_4 increased the expression of Oct-3/4, Sox2 and Nanog after the first passage.

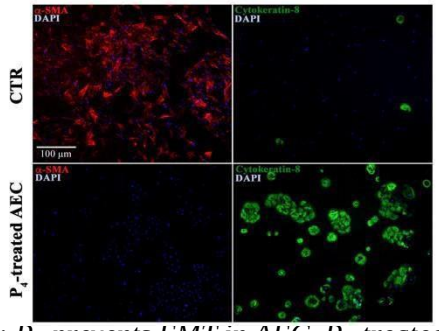

Fig. 1: P_4 prevents EMT in AEC. P_4-treated AEC retained th e expression of Cytoke in-8 whereas rat CTR start to express α-SMA, at the third passage.

P_4-treated AEC stimulated with LPS increased the expression of immunomodulatory cytokines such as IL-10, -13, -4, TGF-β, PIBF and Gal-1. On the contrary, CTR cells did not modify cytokines expression though LPS-stimulated.

DISCUSSION & CONCLUSIONS: P_4 preserves epithelial phenotype of AEC by preventing the *in vitro* EMT. P_4 exerts these morphological effects acting through its nuclear receptor. Furthermore, P_4 induces a re-expression of epithelial markers in AEC underwent EMT, simulating the physiological process of mesenchymal-epithelial transition (MET). Intriguing, the preservation of epithelial morphology is, in parallel, accompanied by an increase of AEC biological properties amplified *in vitro*, confirmed by the up-regulation of stemness and cytokines gene expression. In particular, P_4 enhances the immunomodulatory potential of AEC. Therefore, P_4 treatment could represent an innovative approach to preserve the epithelial phenotype of AEC which is essential to maintain unaltered the high regenerative potential of these cells, during the *in vitro* amplification.

1

Immunomodulatory properties of equine adipose tissue-derived mesenchymal stem cells

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INTRODUCTION: Mesenchymal stem cells (MSCs) are multipotent cells, capable of differentiation into multiple tissues, such as bone, cartilage, tendon, muscle and adipose tissues. The use of MSCs has been the focus of attention in clinical practice to aid bone repair and tendon healing in horses. Recently, MSCs are reported to decrease stimulated T cell proliferation. However, details of the immune modulation of action remain unknown. In this study, we isolated equine MSCs from adipose tissue (eAD-MSCs) and then investigated the characteristics of the eAD-MSCs and their immunomodulatory properties.

METHODS: The eAD-MSCs isolated from tissues were collected from racehorse tail head. The eAD-MSCs were examined for MSC surface markers, proliferating abilities, and differentiation potentials of the cultured cells at passage 3. Next, equine peripheral blood mononuclear cells (ePBMCs) were isolated by Ficoll-Paque density gradient centrifugation from the blood of racehorses. eAD-MSCs (4×10^5 cells/well) were plated into the inner transwell membrane (1 μm pore size) of a 6 well plate for ePBMCs (2×10^6 cells/well) stimulated by mitogen (LPS, 1 μg/mL). After co-culturing for 72 hr, eAD-MSCs and ePBMCs were harvested and evaluated by quantitative real-time RT-PCR (qPCR) for the mRNA expression of inflammatory factors. To examine the immunomodulatory effects of eAD-MSCs in a co-cultured condition, ePBMCs were cultured with the eAD-MSCs and stimulated with LPS (1 μg/mL).

RESULTS: As a results, the mRNA expression levels of pro-inflammatory factors (CCL2, CCL5, COX2, IL-1β, IL-4, IL-6, IL-8, PGE2 and TNF-α were significantly increased in LPS-stimulated ePBMCs when compared to LPS-unstimulated ePBMCs; and these were decreased by eAD-MSCs. Also, the mRNA expression level of anti-inflammatory factors (IL-10, TGF-β1, and VEGF) was decreased in LPS-stimulated ePBMCs, when compared to LPS-unstimulated ePBMCs; and these were increased by eAD-MSCs.

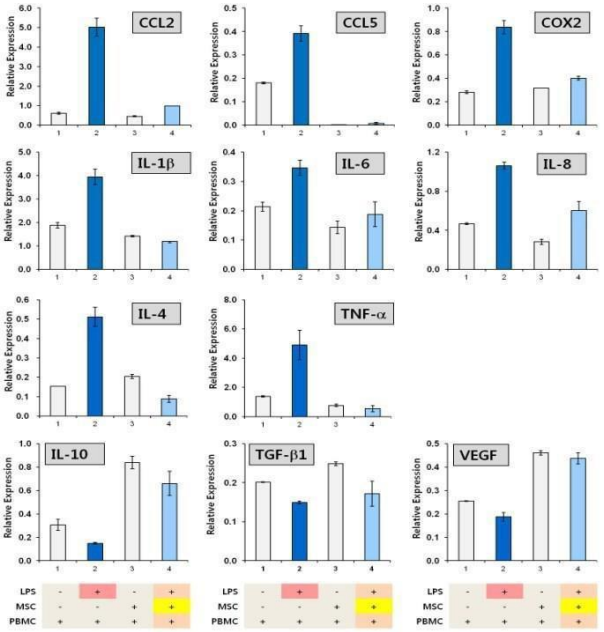

Fig. 1: Anti-inflammatory effects of eAD-MSCs in a co-cultured condition with ePBMCs

DISCUSSION & CONCLUSIONS: We demonstrated that eAD-MSCs have a capability of a principal mediator of immunomodulation. We found that the gene expression of several molecules associated with anti-inflammatory responses was evident in ePBMCs co-cultured with eAD-MSCs. Further studies are needed to determine the necessity of cell-to-cell contact between MSCs and T cells for immunomodulatory function and to gain a fuller understanding of the immunomodulatory effects of eAD-MSCs.

ACKNOWLEDGEMENTS: This work was funded by the Animal and Plant Quarantine Agency, the Republic of Korea.

Stem cell therapy for healing racehorse's bone chip fractures

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INTRODUCTION: Bone chip fractures in the carpal joint are most often seen in racehorses because of the high level of intensity in racing. These issues are the major cause of decreased performance in racehorses. Mesenchymal stem cells (MSCs) are essential for the repair and regeneration of damaged tissues. MSCs also are reported to possess immunomodulatory functions. MSCs have been explored to improve intra-articular therapy in racehorses. In this study, the effect of intra-articular injections of MSCs in racehorses with carpal bone chip fractures was investigated.

METHODS: Before accessing the MSC therapy, we obtained synovial fluids from the fracture site of the racehorses. Also, we collected adipose tissue for MSC isolation and peripheral blood from racehorses. Using the MSC specific marker, adipose tissue-derived MSCs were identified. The racehorses received intra-articular injection of autologous MSCs (or allogeneic) (3×10^7 cells/total 3 mL). After 2 weeks, we collected samples (synovial fruids and peripheral bloods) from the racehorses. To test the effect of MSC therapy by using the ELISA, quantitative real-time RT-PCR and postoperative clinical monitoring, we analyzed inflammatory factors from the untreated samples compared to MSC-treated samples of racehorses.

RESULTS: The level of pro-inflammatory factors (IL-1β, IL-2, IL-4, PGE2 and TNF-α) was significantly decreased in MSC-injected racehorses' samples, compared to when previously accessing the MSC therapy. Whereas, the mRNA expression levels of anti-inflammatory factors (IL-10) were higher than prior to accessing the MSC therapy. Next, we analyzed clinical results from the untreated racehorses compared to MSC-treated racehorses. Pain scores are consisting of lameness grade, joint effusion scale, and joint pain amount during the flexion test. Average pain score has decreased by a third of what it used to be. Racing return rates in MSC-injected racehorses is higher than untreated groups, and the average rehabilitation period in MSC-injected racehorses is shorter than untreated groups.

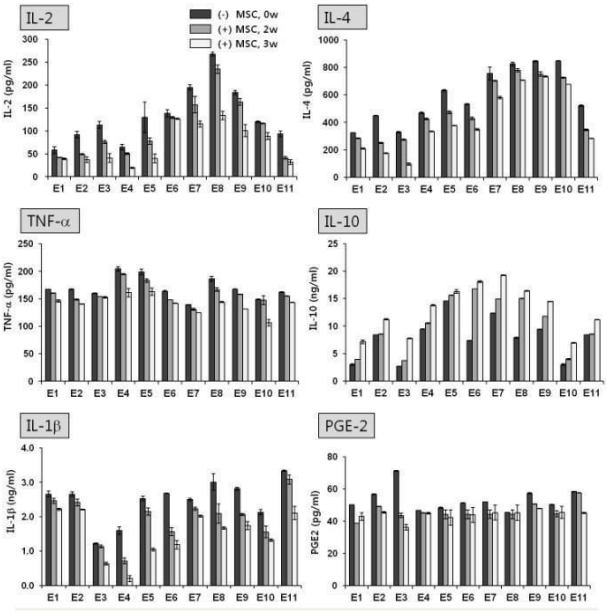

Fig. 1: Pro- and anti-inflammatory cytokines in racehorses with bone fractures

Table 1. Comparison of untreated vs stem cell injection in racehorses with bone fractures

	untreated	treated
Pain score	2.70	2.33
Rehabilitation period	208 day	192 day
Racing recovery rate	80%	91%

DISCUSSION & CONCLUSIONS: For 12 months durability and performance of racehorses that came back were monitored. During the serial clinical exam, pain scores are lowered in most of the MSC-treated racehorses. Further studies are needed to investigate the anti-inflammatory mechanisms of MSC in racehorses.

ACKNOWLEDGEMENTS: This work was funded by the Animal and Plant Quarantine Agency, the Republic of Korea.

Engineered mesenchymal stem cell therapy in tumor ablation

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INTRODUCTION: Both human bone marrow mensenchymal stem cells (hMSCs) and BMP-2 protein play an important role in the development and growth of human malignancies by effecting on angiogenesis and cell migration. While the antitumor activities were demonstrated in our research.

METHODS: The replication defective adenovirus vector containing human BMP-2 gene infected hMSC (advBMP-2/hMSCs) was injected to the Hep3B tumor. The antitumor activities were observed in a coculture model in vitro and in a xenogenic subcutaneous hepatocellular carcinoma models in vivo including human Hep3B HCC tumors in the back of the BALB/c athymic (nu/nu) mice.

RESULTS: We observed that inhibitory of proliferation via enhanced apoptosis when Hep3B cocultured with advBMP-2/hMSCs. The regional injected of advBMP-2/hMSCs induced tumor apoptosis and prolong animals' life span.

DISCUSSION & CONCLUSIONS: Although complete tumor regression was not achieved in both treatment groups, the cell-based delivery was superior in terms of prolonging animals life span and compromised tumor development. MSC would not only be an effective platform for the delivery of therapeutic proteins to cancer sites, with synergic effects of autocrine BMP-2 excretion to enhance the apoptosis in Hep3B tumors. The result showed a compromised strategy of anticancer therapy, instead of oncolytic effect, to make animals live longer. Repetitive regional injection or injection via the feeding vessels would be attempted in the future. These results suggest a palliative cancer therapy that hMSCs possess intrinsic antineoplasticproperties.

ACKNOWLEDGEMENTS: We acknowledge professor Jueren Lou, Shanghai Institute of Biological Products, China National Biotec Group, for the gift of adenovirus. The authors are thankful to Dr. I-Ping Chiang for assisting in reading the pathologic examinations. This research was supported by the China Medical University Hospital, Taiwan, contract numbers DMR-96-123, and DMR-98-114.

The influence of cell sheet formation of human adipose-derived stem cells on stemness and differentiation capabilities

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INTRODUCTION: The abundance and easy accessibility of adipose-derived stem cell (ASC) have made it a promising candidate for stem cell therapies. It has been proposed that the use of cell sheets is beneficial for ASC transplantation, so we aimed to explore the stemness and differentiation capabilities of human ASCs within cell sheets.

METHODS: We stimulated extracellular matrix (ECM) secretion of human ASCs and fabricated cell sheets by treatment with ascorbate 2-phosphate (A2-P), a stable form of ascorbic acid. Further treatment with antioxidants or collagen synthesis inhibitors were employed to elucidate the mechanism of stemness and differentiation enhancement induced by A2-P.

RESULTS: We found enhanced expression of stemness markers (Nanog, Oct4 and Sox2) in ASCs within A2-P-induced cell sheets. We further demonstrated that ASCs within cell sheets exhibited significantly enhanced neurogenic (ectoderm) and hepatogenic (endoderm) transdifferentiation capabilities as comparing to monolayer ASCs, demonstrated by upregulated *Nestin* and *Albumin* genes when cultured in appropriate induction media. Meanwhile, adipogenic and osteogenic (mesoderm) differentiation capacities of ASCs were still maintained after cell sheet formation. Moreover, A2-P treatment along with two types of collagen synthesis inhibitors, l-2-azetidine carboxylic acid (AzC) or cis-4-hydroxy-d-proline (CIS), significantly inhibited the A2-P-mediated stemness enhancement, whereas treatment with the antioxidants N-acetyl cysteine (NAC) or L-Glutathione reduced (GMEE) showed no effect. These findings demonstrated that A2-P enhances stemness and differentiation capabilities of ASCs through promoting ECM synthesis, not antioxidation.

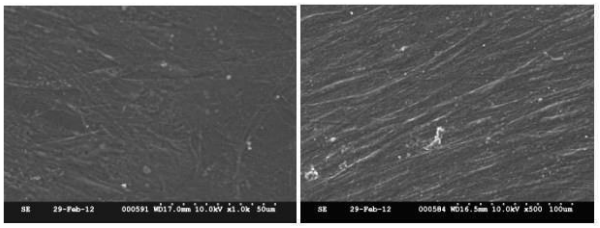

Fig. 1: Electronic microscopic images of ASCs cultured without (left) or with (right) A2-P for 7 days. Cell sheet formation with A2-P treatment was noted.

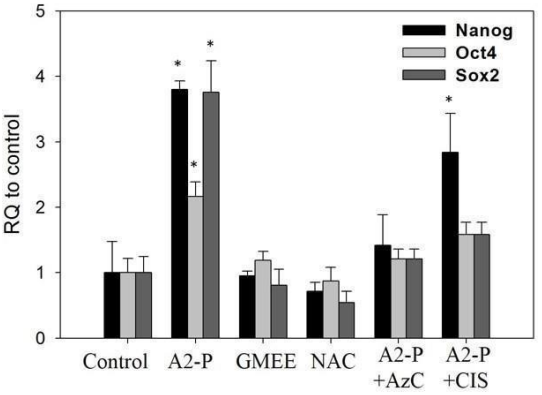

*Fig. 2: RT-PCR measurements for pluripotent marker genes in ASCs after treatment with A2-P, GMEE, NAC, A2-P+AzC and A2-P+CIS. *p < 0.05 from the same gene in the control group.*

DISCUSSION & CONCLUSIONS: Stemness enhancement in ASC sheet could be mediated through physical cell support and control of cell shape and geometry as a function of surface topography, substrate stiffness, and biochemical signaling [1]. In this study, we demonstrated the importance of the ECM within cell sheet on stem cell fate determination. Therefore, the use of cell sheets should be beneficial for ASC-based tissue engineering.

ACKNOWLEDGEMENTS: The study was supported by National Health Research Institutes, Taiwan (EX103-10312EC)

Xeno-free strategy for expansion of human mesenchymal stem/stromal cells for regenerative therapies

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INTRODUCTION: Human mesenchymal stem/stromal cells (hMSC) have generated great interest mainly because of their multidifferentiation potential and immunomodulatory role [1]. For cellular therapies, high cell numbers are required, and thus cells must be expanded *ex vivo*. Most of the current protocols make use of fetal bovine serum (FBS) as nutrient-rich supplement. However, regulatory guidelines encourage novel xeno-free alternatives to define safer and standardized protocols for hMSC expansion. A new pharmaceutical-grade Cell Culture Supplement medium (SCC) has been recently described as a possible candidate for xeno-free supplementation of hMSC expansion media [2]. The behavior of attachment-dependent cells, such as hMSC, is mediated not only by soluble factors present in the medium, but also by cell adhesive components that adsorb to the culture surface [3]. This work aims to evaluate the effect of different soluble and adsorbed bioactive factors in hMSC growth under xeno-free conditions in SCC supplemented medium.

METHODS: BM-derived hMSC (Lonza) were plated in 24 well tissue culture polystyrene (TCPS) plates and cultured in media supplemented with 15% v/v SCC (Grifols) or 10% FBS MSC qualified (Hyclone). Plates were pre-coated with different xeno-free coatings before culture and media supplemented with different human recombinant growth factors, alone or in combination. Metabolic activity was assayed by Resazurin assay, while cells morphology was monitored through phase contrast optical microscopy. Next, the efficiency of hMSC expansion on SCC formulation was evaluated relative to traditional 10% FBS medium and commercial available serum free defined medium.

RESULTS: While xeno-free coatings preserve cell morphology, they do not significantly improve cell growth, as compared to the FBS supplemented medium. The addition of individual human recombinant growth factors to the SCC-supplemented medium did not show any improvement, but when different growth factors were used simultaneously they led to a substantial increase in cell metabolic activity levels. This effect was further improved with the use of a xeno-free coating. Under these conditions (GF cocktail plus coating), cumulative net cell yield/cm^2 was significantly higher for SCC medium, compared to 10% FBS medium and one commercially available xeno-free medium.

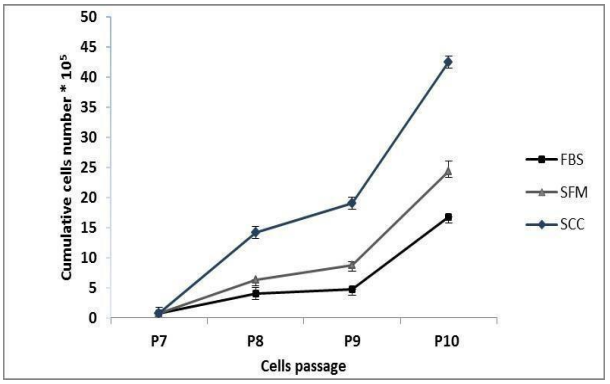

Fig. 1. hMSC expansion along 4 passages in SCC, FBS and commercial available xeno free media (SFM).

DISCUSSION & CONCLUSIONS: SCC appears to be a promising candidate to be used as cell culture supplement in xeno-free hMSC expansion

ACKNOWLEDGEMENTS: This project is part of a Marie Curie Initial Training Network - European Industrial Doctorate (ITN-EID) IB2 (317052), and has been funded under the European Community
7th Framework Programme.

Impact of an alginate encapsulation system on the angiogenic and wound healing potential of hBM MSC cultured as spheroids or single cells

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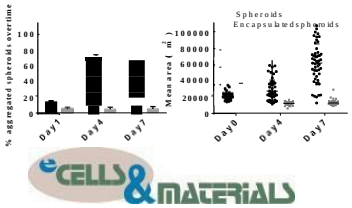
INTRODUCTION: Wound healing is a complex process that involves inflammation and tissue remodeling. Mesenchymal stem/stromal cells (MSC), particularly MSC spheroids, have been reported to evidence enhanced expression of factors with a key role on wound healing[1]. In order to potentiate MSC response on regenerative processes, delivery strategies that maximize function and retainment at injured sites would be valuable. By encapsulating hBM MSC within alginate microbeads, cellular-mediated paracrine activity can be exploited.

METHODS: MSC cultured as single cells or as spheroids were suspended in alginate and extruded into a 100 mM calcium chloride bath (Fig. 1A and C, respectively). To assess the wound healing and angiogenic potential of MSC, expression of relevant genes, namely VEGF, HGF and TSG-6, normalized to monolayer, was analyzed by RT-PCR, as well as scratch and tube formation assays with fibroblasts L929 and in co-culture with HUVECS, respectively, were performed.

RESULTS: Encapsulation of spheroids in alginate beads hinders their aggregation, with consequent maintenance of more controlled spheroid size overtime (Figs. 1D and E, respectively). Contrarily to DMEM 1% A/A, which lacks the paracrine factors secreted by MSC, conditioned media collected from culturing MSC as monolayer, encapsulated single cells, spheroids or encapsulated spheroids in DMEM 1% A/A for 24h favored migration of fibroblasts into a wounded area. Particularly, encapsulated spheroids evidenced higher closure of the scratched area (Fig. 2A) as well as increased the formation of more interconnected tubelike structures when co-cultured with HUVECS relatively to encapsulated single cells (Fig. 2B). In addition, enhanced expression of the anti-inflammatory gene TSG-6 was observed (Fig. 2C).



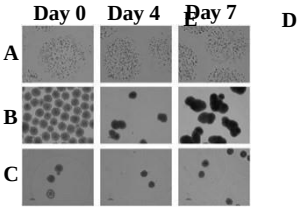


Fig. 1: Encapsulated spheroids (C) evidence reduced agglomeration (D) and maintenance of a more uniform size (E) overtime relatively to unencapsulated spheroids (B).

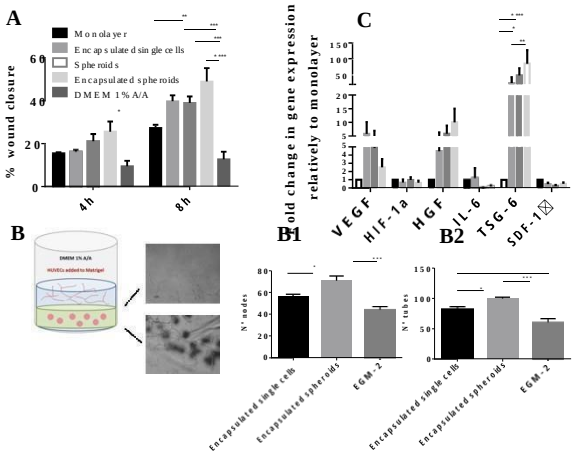


Fig. 2: The wound healing (A) and angiogenic (B) paracrine activity of hBM MSC is potentiated by culturing these cells as encapsulated spheroids. Enhanced gene expression of TSG-6, an anti-inflammatory cytokine was also observed (C).

DISCUSSION & CONCLUSIONS: The combined use of spheroids with an alginate encapsulation system potentiates the proangiogenic, anti-inflammatory and chemotactic capabilities of hBM MSC.

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ACKNOWLEDGEMENTS: Funding by FCT SFRH/BD/52000/2012 and UID/BIO/04565/2013 and by PORL2020 (No 007317).

A novel biomaterial-based approach for therapeutic angiogenesis

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INTRODUCTION: Cardiovascular disease is a leading cause of death worldwide. Promoting regeneration of tissue damaged by ischemia poses a significant challenge. Therapeutic angiogenesis aims to promote the formation of new blood vessels enabling vascular reperfusion of ischemic tissue.

Hierarchical structures composed of synthetic polymers can be used to provide scaffolds for targeted cell delivery. Their physical characteristics may also influence cell behaviour. Surface topography is known to influence cell alignment, morphology and differentiation and rough surfaces have been shown to effect cellular expression of biomarkers and growth factors.

Control of angiogenic growth factor secretion using cell substrates with bespoke topography is an innovative concept. The aim of this study was to investigate the effects of a novel biodegradable polymer substrate, exhibiting unique hierarchical surface topography, on the secretion of angiogenic factors from adipose-derived mesenchymal stem cells (ADMSCs).

METHODS: Hierarchically structured substrates were prepared from a biodegradable polymer (poly-DL-lactide-co-glycolide) using thermally induced phase separation (TIPS). Surface topography was characterised using scanning electron microscopy, atomic force microscopy and Dektak surface profilometer. The biological effect of surface topography on ADMSC-derived angiogenic secretome was assessed using an *in vitro* model of angiogenesis (V2a Kit™ - Vasculogenesis to Angiogenesis), Proteome Profiler™ Human Angiogenesis Array Kit, and ELISA for vascular endothelial growth factor (VEGF). Cell viability was assessed by LIVE/DEAD staining and cell proliferation measured using CyQUANT® NF Cell Proliferation Assay.

RESULTS: TIPS PLGA substrates exhibited a hierarchically structured porous and rough topography compared with control surfaces coated with the same polymer (Fig. 1). ADMSCs cultured on TIPS polymer coated surfaces produced increased angiogenic activity compared with PLGA and TC plastic control samples (Table 1). Semi-quantitative profiling of the angiogenic growth factor secretome showed differential expression between the substrate groups. VEGF secretion per cell was significantly increased in the TIPS group compared with PLGA and TC plastic control groups.

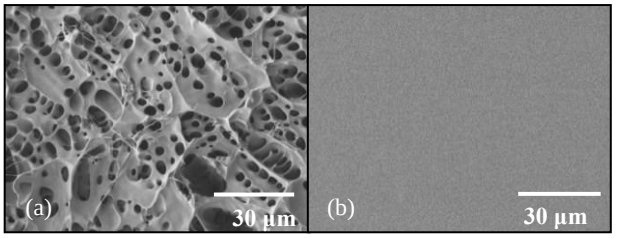

Fig. 1: Scanning electron microscopy images of (a) TIPS PLGA and (b) control PLGA surfaces used as a substrate for ADMSC culture.

Table 1. Quantification of endothelial tubules in the in vitro model of angiogenesis. Tubules were stained with CD31, imaged and analysed using AngioSys 2.0 software following incubation with ADMSC-conditioned culture medium.

	TIPS PLGA	Control PLGA	TC Plastic
Tubule Length	11732 ± 1502 P<0.05*	8404 ± 752	8607 ± 389
No. Tubule Junctions	547 ± 123 P<0.05*	274 ± 47	339 ± 11
No. Tubule Branches	748 ± 66 P<0.01**	405 ± 53	442 ± 14

DISCUSSION & CONCLUSIONS: Hierarchically structured polymer substrates produced using TIPS stimulated an increase in angiogenic growth secretion capable of stimulating angiogenesis *in vitro*. The findings from this study offer a novel approach for using clinically ready biomaterials to simulate the secretion of angiogenic growth factors. Future work aims to test whether the response is replicated *in vivo* using suitable models of tissue ischaemia.

ACKNOWLEDGEMENTS: Supported by grants from UK Medical Research Council, the British Heart Foundation and The Wellcome Trust.

Impaired osteogenic differentiation and upregulation of receptor activator of nuclear factor kappa-B ligand in bone marrow stromal cells grown in uremic microenvironment

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INTRODUCTION: The loss of renal function in Chronic Kidney Disease – Mineral and Bone Disorder (CKD-MBD) is associated to bone alterations and an increased fracture risk [1]. The study evaluated the effect of uremia on bone marrow mesenchymal stromal cells (BMSCs), as possible active participants in the pathogenesis of bone disease. Furthermore, because BMSCs are widely studied for applications of bone tissue engineering, it is important to understand if uremia may spoil their regenerative potential. This study thus investigated *in vitro* the effects of a serum pool from hemodialysis patients on BMSCs, to understand how it may affect osteogenic differentiation.

METHODS: A pool of uremic serum (HUS) was prepared from samples derived from forty CKD-MBD patients undergoing hemodialysis. HUS was added to media either for BMSC maintenance cultures or for osteogenic differentiation. Morphological alterations were evidenced with Neutral Red and phalloidin-FITC staining. Cytotoxicity of uremic serum was assessed with LDH assay and cell viability was tested with Alamar Blue Assay. The osteogenic differentiation ability in FBS or HUS cultures was determined with Alizarin Red staining for the evaluation of calcium deposition, determination of ALP activity, gene expression analysis and ELISA.

RESULTS: The results showed an altered spatial organization of cells and signs of cytotoxicity, along with hyperproliferation. Moreover, gene expression analysis suggested an impairment in the osteogenic differentiation, with a partial upregulation of *ALPL*, *BGLAP* and *SPP1* genes, whilst a strong decrease in *COL1A1* levels. More importantly, Receptor activator of nuclear factor

kappa-B ligand (*RANKL*) was upregulated with a mild reduction in osteoprotegerin levels.

*Fig. 1: Heat map summarizing the results of gene expression analysis for BMSCs grown in HUS vs. FBS. *: p<0.05. **: p<0.01. Yellow: unaltered expression. Red: gene downregulation in HUS vs. FBS. Green: gene upregulation in HUS vs. FBS.*

DISCUSSION & CONCLUSIONS: Uremic serum impairs the osteogenic differentiation of BMSCs and BMSCs may enhance osteoclastogenesis through RANKL activation, therefore contributing to the alterations in bone remodeling in CKD-MBD patients.

ACKNOWLEDGEMENTS: Financial support by the Italian Ministry of Education, Universities and Research (Project FIRB no.RBAP10MLK7) and Rizzoli Orthopaedic Institute and 5×1000 funds (years 2011-2012).

DISCLOSURES: The authors declare no conflict of interest.

Cell engraftment mediated by hyalgan to cartilage: implications for tissue repair

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INTRODUCTION: The identification of key factors influencing cell homing following transplantation could provide important insights for the comprehension of the biological mechanisms leading to tissue regeneration [1]. To shed some light on this important aspect, we investigated the migration ability of Bone Marrow Concentrate (BMC) and Mesenchymal Stem Cells (MSC) alone and in combination with Hyalgan (HA) as innovative therapeutic strategies for osteoarthritis (OA). We assessed what are the post-transplant effects of the different treatments on cartilage, synovium and menisci. Moreover, we monitored cell biodistribution and examined whether HA combination and OA features are key factors in modulating cell migration.

METHODS: OA was induced bilaterally by Anterior Cruciate Ligament Transection in rabbit. MSC and BMC alone and mixed with HA were intra-articularly administered into the hind limbs. The effects of treatments were observed on cartilage, synovium and menisci at 2 months follow-up using Kruskall Wallis followed by the post-hoc Dunns test. To assess local biodistribution, cells were labelled with a fluorescent dye (CM-DiI) and their presence was monitored on the explanted tissues using an epi-fluorescence microscope at 7 and 14 days from their delivery. Spearman's rank-order correlation method was used to assess the relationships between cell migration and OA features in the different tissues using specific histological scores.

RESULTS: Both MSC and BMC homed considerably to cartilage tissue where noticeable OA features were evident. A minor percentage of CM-DiI$^+$ cells was observed in the meniscus and the lowest cell concentration was found in the synovial membrane regardless the experimental time (Fig.1). Cell clusters within the articular tissues were considered among the critical factors responsible for cell homing. By contrast, a negative correlation between OA changes and cell migration was noticed in the synovium only when cells were mixed with HA. However, a decrease of CD-44 and M1 macrophage subset was noticed in these groups. HA treatment alone and combined with cells lead to a reduction of OA features, such as hyperplasia and inflammatory cells, especially in the synovium at 2 months follow-up.

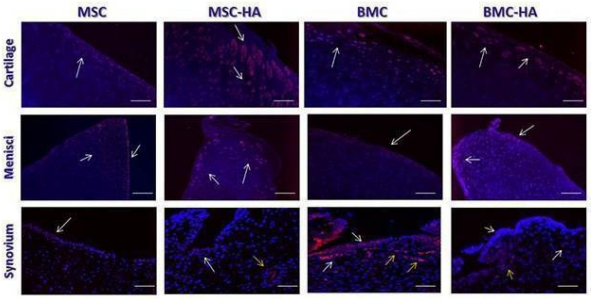

Fig. 1: Biodistribution of CM-DiI$^+$ cells. Representative specimens of different treatments under epi-fluorescence. Red staining: CM-DiI$^+$ cells. Scale bar: 100 µm.

DISCUSSION & CONCLUSIONS: Findings from cell biodistribution studies suggest a direct role of HA in driving cell homing towards cartilage and meniscus through the inhibition of cell recruitment at synovial level. Cell clusters together with fibrosis processes and M subsets are among the critical factors modulating cell migration and tissue repair.

DISCLOSURES: All Authors, except for AS and CS, did not report conflict of interest. AS and CS are employed of Fidia Company involved in the field of research.

Opposite early SOX9 response of human iPS cells compared to MSCs during in vitro chondrogenesis

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INTRODUCTION: Induced pluripotent stem cells (iPS cells) are highly attractive for cartilage tissue engineering. Their unlimited proliferative capacity makes them available in vast amounts, while articular chondrocytes – the current gold standard – can only be harvested in extremely limited quantities. Being progenitors of all tissues, iPS cells comprise the intrinsic ability to form stable hyaline cartilage. Thus, they promise to overcome mesenchymal stromal cells (MSCs) that differentiate in vitro along the undesired endochondral pathway [1]. Indeed, in vitro iPS cell chondrogenesis has been suggested to yield stable chondrocytes. In order to illuminate mechanistic differences during in vitro chondrogenesis of iPS cells versus MSCs, we investigated the early regulation of the chondrogenic master transcription factor SOX9, whose upregulation has often been considered as success indicator for cartilage formation.

METHODS: Two human iPS cell lines were induced to form intermediate mesenchymal progenitor cells (iMPCs), which were subsequently differentiated into the chondrogenic lineage. SOX9 gene expression and protein levels were quantified prior and 4 days after chondrogenic stimulation. Collagen type II and proteoglycan deposition were assessed histologically after 6 weeks in chondrogenic micromass culture.

RESULTS: Compared to MSCs, independently derived iMPC populations expressed highly variable SOX9 protein levels prior to chondrogenic stimulation. Upon induction of chondrogenesis, Smad proteins in iMPCs were phosphorylated in response to treatment with TGF-β as efficiently as in MSCs, thus indicating that iMPCs were able to adequately respond to chondrogenic stimulation. Strikingly, SOX9 protein levels in iMPCs were significantly lower on day 4 of chondrogenic stimulation than on day 0, while MSCs showed upregulated SOX9 protein at this time (fig 1A). Along with an altered condensation behaviour compared to MSCs and despite early SOX9 downregulation, iMPC pellets developed collagen type II positive chondrocytes (fig 1B). Chondrogenesis was better in the iPS cell line with higher SOX9 levels prior to chondrogenic stimulation. Chondrogenesis was further improved by co-treatment with TGF-β + BMP-4 which appeared to shorten the duration of the SOX9 protein decline. However, this came at the expense of undesired hypertrophy.

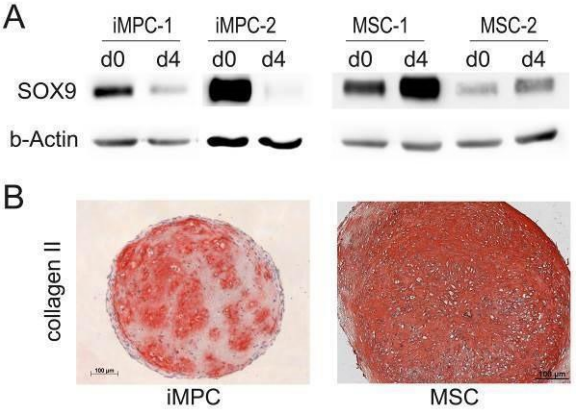

Fig. 1A: SOX9 protein levels (Western blot) of 2 independent iMPC populations versus 2 MSC donors at days 0 and 4 of chondrogenic stimulation. B: Collagen II immune histochemistry after 6 weeks of chondrogenic micromass culture.

DISCUSSION & CONCLUSIONS: In conclusion, there is a negative SOX9 response to TGF-β stimulation in iMPCs which is opposite to MSCs and may explain the inadequate chondrogenesis of iPS cells. The surprising opposite regulation of SOX9 protein in iMPCs compared to MSCs clearly demonstrates that the role of this transcription factor for the induction and progress of chondrogenic differentiation is far from understood. What influence the initial transient downregulation of SOX9 protein has on in vitro chondrogenesis and whether the early SOX9 upregulation in MSCs should be suppressed are interesting questions for further studies.

Assessment of jaw periosteal cell mineralization by Raman microspectroscopy

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INTRODUCTION: Previously, we demonstrated that MSCA-1 (mesenchymal stem cell antigen-1) positive cells represent the osteoprogenitors within the entire cell population derived from the jaw periosteum (JPC). In order to examine whether the marker-free technology of Raman spectroscopy can be used to determine the efficiency of the osteogenic differentiation process, MSCA-1 positive cells were investigated using Raman microspectroscopy under serum-containing and xeno–free culture conditions.

METHODS: MSCA-1+ cells were osteogenically induced under FCS-containing (DMEM) and xeno-free culture (MC) conditions for 20 days. Raman spectra were measured from 100 random points within each cell culture dish. Principle component analysis (PCA) of Raman spectra was performed and cell mineralization in either one or two spectral components (PCs) for three donors was detected. PCA was carried out to identify the spectra containing Raman signals from bone material. The quality of formed mineral crystals was assessed by calculating following ratios: phosphate/hydroxyproline (961/884), phosphate/ phenylalanine (961/1003), phosphate/amide III (961/1280), carbonate-to-phosphate (1075/961), and hydroxyapatite crystallinity (the inverse of full-width half maximum (FWHM) of the hydroxyapatite peak at 961cm^{-1}).

RESULTS: The quantity of cell mineralization detected by Raman microspectroscopy in cell view dishes with glass bottom was consistent with the results obtained by fluorescent OsteoImage or Alizarin stainings. However, the degree of cell mineralization was not always consistent with calcification detected within cell culture plates of other format. In depth analysis of the Raman data revealed higher crystallinity, higher collagen maturity and higher carbonate content following differentiation under FCS-containing compared to xeno–free culture conditions. Additionally, significant higher phosphate to protein ratios were detected under xeno-free culture conditions.

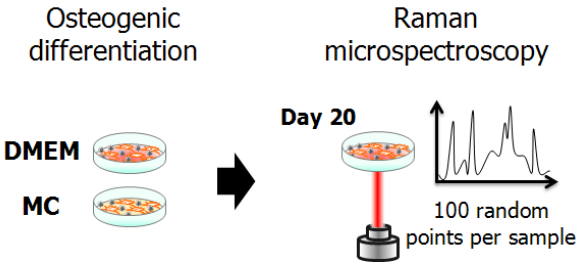

Fig. 1: Design of the performed experiments. MSCA-1 positive jaw periosteal cells were cultured under FCS-containing (DMEM) and xeno-free (MC) culture conditions. After osteogenic induction for 20 days, Raman microspectroscopic analyses from 100 random points within the dish were performed.

DISCUSSION & CONCLUSIONS: Raman spectroscopy represents a suitable technology for studying the mineral quantity and quality of MSCA-1+ periosteal cells. Future studies should include analyses of mechanical properties of the formed crystals in order to be able to make a reliable statement about the quality of in vitro JPC mineralization.

The effects of adipose derived stromal vascular fraction and platelet rich fibrin on experimental colonic anastomosis

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INTRODUCTION: Intestinal anastomosis is an important issue of gastrointestinal system procedures. The anastomotic leaks have still been problem by increasing morbidity and mortality. It was shown that the adipose tissue was rich in mesenchymal cells [l]. They have also reported that isolation of stem cells from adipose tissue had less morbidity when compared to the bone marrow. The adipose derived stromal vascular fraction (SVF) cells were indicated to comprise multipotent mesenchymal stem cells and were proven to be as effective as the cultured mesenchymal stem cells [2, 3]. We performed an experimental study to find out the effects of platelet rich fibrin (PRF) and adipose stromal vascular fraction on colonic anastomosis model.

METHODS: In this study, 80 female Sprague-Dawley rats were used. Each three group consisted with 20 rat. Also 10 rats were used for harvesting stem cells and platelet rich fibrin. Descending colons were completely transected and anastomoses were performed in all groups. No additional process is applied on anastomosis of the control group while platelet rich fibrin, platelet rich fibrin+stem cells were applied on anastomosis of other 2 groups respectively. All rats were sacrificed under anesthesia by the 7th day of the experiment. All the three groups had 2 subgroups. One subgroups was planned to measure the anastomotic bursting pressure and histopathologic examination, second subgroup was planned for angiographic examination of the anastomotic angiogenesis. Adipose tissue derived stem cells were labeled with DiIdye.

RESULTS: There were no significant differences in weights of the rats between 3 groups. There were no perforation, fistulisation nor intraabdominal abscess in any group. Anastomotic bursting pressure and angiogenesis was compared between groups. Statistically significant difference was determined between PRF+stem cell group and other two groups. Mean bursting pressure was calculated 159±20 mmHg in the control group, 176±24 mmHg in PRF group, 191±20 mmHg ($p<0.05$) in the PRF+stem cell group.

Vessel per millimeter square was calculated from the angiographic examination. In the control group 17±4, in the PRF group 25±3 and in the PRF+stem cell group 33±4 vessel ($p<0.05$) were counted. There were no significant difference between groups when compared anastomotic reepithelisation, fibrosis and necrosis. But there were significant in the vascular proliferation ($p<0.05$) and inflammation ($p<0.05$) when compared to control group. Stem cells were observed as endothelial cells and was seen among the villous components in the flourescent microscope examination.

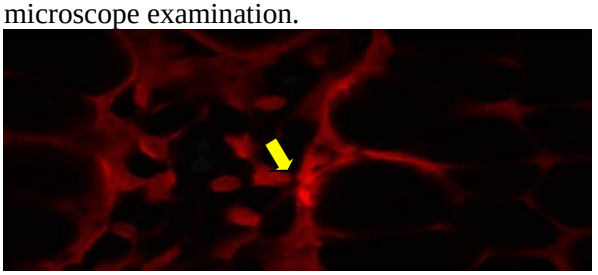

Fig. 1: Stem cell closed to intestinal villus (Stem cells is shown by arrow).

DISCUSSION & CONCLUSIONS: Adipose derived stem cells have been utilized with low donor site morbidity and with high potential in regenerative medicine. Increased vascularization and increased durability at the colon anastomosis were observed. We used PRF as a carrier for stem cells in our study and when we labeled the stem cells and found out the in-vivo differentiation of these cells both into endothelial and into villous structures. The paracrine, indirect effect of adipose stromal vascular fraction were indicated to be helpful in healing at the anatomsis site. Adipose stromal vascular fraction cells can be an alternative treatment modality with the effects on wound healing of anastomosis. Further studies are needed to determine the long term effects.

Effects of Adipose Derived Stem Cell and Platelet Rich Fibrin in Postsurgical Adhesion Prevention in a Rat Model

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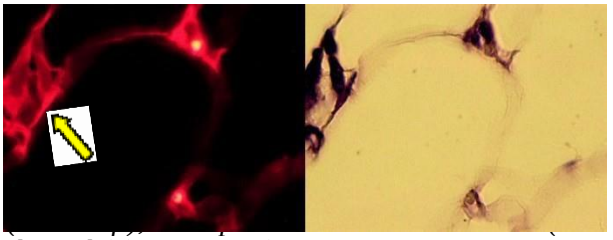
INTRODUCTION: Intra-abdominal adhesion is the most common postoperative complication with life threatening and debilitating consequences [1]. The surface lining of the peritoneum is the key site in adhesion formation and prevention. A suitable barrier that separates damaged peritoneal surfaces for the entire re-epithelialization is likely to be effective in reducing adhesion formation [2]. The adipose derived stromal vascular fraction (SVF) cells were indicated to comprise multipotent mesenchymal stem cells and were proven to be as effective as the cultured mesenchymal stem cells [3, 4]. The aim of this study is to investigate if adipose derived stem cells (ADSCs) and platelet rich fibrin (PRF) can prevent post-surgical adhesion in a rat model.

METHODS: We have enrolled 30 Sprague-Dawley male rats (350-400 gr) in our study. All of the rats underwent laparotomy. All rats were underwent operation for traumatization of peritoneum by incision in the right lateral abdominal wall after midline laparotomy and a peritoneal defect was created. In addition to that, we traumatized anti-mesenteric side of the cecum to induce adhesions. Rats were separated into three groups. In control group (group 1, n=10), after traumatization we have closed the abdomen without any other application. In group 2 (n=10) PRF; in group 3 (n=10) PRF+ADSCs were administered intraperitoneally just before abdominal closure. Adhesions were scored on the seventh day and all of the rats were sacrificed. We performed histopathological examination of intra abdominal adhesions. In pathological investigations, we have evaluated the adhesions by scoring fibroblasts, granulation tissue and collagen production. Adipose tissue derived stem cells were labeled with DiI dye.

RESULTS: We found statistically significant difference between three groups with regard to macroscopic adhesion scores and histopathological findings ($p<0.05$). In addition, macroscopic adhesion scores were significantly lower and histopathological findings of adhesions were better in group 3 when compared to group 1 ($p<0.05$). There was no statistical difference in adhesion scores and histopathological findings of group 1 vs group 2 and group 2 vs group 3. However, adhesion scores were higher in control group. Besides adhesion scores of group 2 were higher than group 3.



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DISCUSSION & CONCLUSIONS: In our study, we observed that intraperitoneal administration of PRF+ADSCs decreased adhesions. Some of the stem cells were differentiated to the epithelial cells. This is the direct effect of stem cell therapy. We thought that the decreased adhesion formation would be the indirect (paracrine) effect of the stem cells. The efficacy of PRF+ADSCs in adhesion prevention should be investigated in further studies.

Human adipose stem cells cultured in a novel nanofibrillar cellulose membrane bandage, enable remodelling of wounded areas

Carmen Escobedo-Lucea[1,2], Andrés Sanz-García[1,2], Vincenzo Cerullo[1], Deborah Burks[3] and Marjo Yliperttula[1]

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INTRODUCTION: The field of wound care is always expanding with new advances but still none avoids the inflammatory reaction post-trauma. Given their immunomodulatory properties, human adipose stem cells (hASC) have emerged as efficient candidates to reduce inflammation. The main roadblocks to increase their success in clinical applications are the risk carried by animal reagents used in hASC culture, as well as the the lack of appropriate vehicles for deliver the cells to the injured area. To fight zoonosis, we have developed an animal-free protocol for hASC isolation and maintenance. Wood-derived nanofibrillar cellulose (NFC) is a natural promising material [1]. We propose the use of xenogeneic-free hASC to relax chronic dermal inflammation, using as vehicle for cell delivery a removable NFC dressing and helping the auto regeneration.

METHODS: hASC isolated and maintained in xenogeneic conditions [2] were seeded on NFC membrane (n=6). Different coating conditions Laminin (5 μg) or CellStart (1/50) were tested for evaluate adhesion. After 7 days and 3 passages in culture, in vitro assays were carried out to study if the culture on NFC affects different parameters of hASC as morphology (by SEM and TEM), viability (Alamar Blue), multipotent gene profile expression, cytokine release and proliferation by Ki-67 (Invitrogen). Cell toxicity was also evaluated by tunnel assay [3]. Wound repair was measured by using SCID mice. Immune rejection against dressings was studied *in vitro* and *in vivo* using NGS hu-PBMSC humanize mice (Jackson).

RESULTS: After 7 or 10 days in culture, hASC cells continued growing and their morphology remained normal when we compare with their counterparts seeded on plastic. Ki-67 analysis demonstrated that proliferate with higher rate using plastic than over membrane. Protein array studies in vitro revealed that the culture over NFC does not alter the cytokine and chemokine hASC release pattern. Additionally, in vitro immune studies co-culturing PBMSC with the bandages demonstrated that hASCs maintained their immunomodulatory abilities when cultured over NFC. The use of NFC-hASC bandages in wound treatment of SCID mice wound healing model, allowed faster and mature healing. Immunological studies in vivo using NGS humanized mice model confirmed the absence of rejection at the site of injury and also systemically.

DISCUSSION & CONCLUSIONS: Membrane is not triggering any toxic effect over hASC cells and is able to preserve their bioactivity and immuno-modulatory properties [4]. Nanocellulose membranes are not toxic and do not induce any damage, ulcer or tumour after being applied on animal models. The use of this treatment help to improve wound healing processes, enhancing faster recovering of the injured area in nude mice. NFC-hASC functionalized membranes are a promising candidate to promote wound healing.

ACKNOWLEDGEMENTS: This work was partially funded by TEKES, Academy of Finland (AOF) Research Fellowship. C.E.L. and A.S.G. are funded by AOF under Research Fellow Program (266486) and the FINSKIN Project associated (273689), respectively. It made use of the Central services of the University of Valencia and Centro de Investigacion Principe Felipe - Valencia. We would like to thank Mrs. Teresa Minguez, Mrs. Viviana Bisbal, Dr. Vicente Mirabet, Professor J.M. Garcia-Verdugo and Dr. Angeles Asensi for their technical support.

Tenogenic phenotype maintenance and differentiation using macromolecular crowding and mechanical loading

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INTRODUCTION: Tissue engineering strategies for tendon repair have limited clinical applicability due to delayed extracellular matrix (ECM) deposition and subsequent prolonged culture periods, which lead to tenogenic phenotypic drift. Deposition of ECM *in vitro* can be enhanced by macromolecular crowding (MMC), a biophysical phenomenon that governs the intra- and extra-cellular milieu of multicellular organisms, which has been described to accelerate ECM deposition in human tenocytes. A variety of cell sources have been studied for tendon repair including tenocytes, dermal fibroblasts and mesenchymal stem cells (MSCs) and various biophysical, biochemical and biological tools have been used to induce phenotype maintenance in long term cultures or differentiation. Therefore, we propose to assess the combined effect of macromolecular crowding and mechanical loading on different cell sources to determine their suitability for the in vitro fabrication of tendon-like tissue.

METHODS: Human dermal fibroblasts, tenocytes and bone marrow mesenchymal stem cells were cultured for 3 days with carrageenan (MMC) under static and dynamic culture conditions. Cyclic uniaxial strain was applied using a MechanoCulture FX (CellScale) at 1 Hz and 10% strain for 12 hours a day. Cell morphology and alignment were evaluated by fluorescein isothiocyanate (FITC) labelled phalloidin and 4',6-diamidino-2-phenylindole (DAPI) staining. Extracellular matrix composition was evaluated by immunocytochemistry. Cell phenotype maintenance/differentiation (tenogenic, chondrogenic and osteogenic lineages) were assessed by gene and protein analysis.

RESULTS: After 12 hours of exposure to the uniaxial load, cells are strictly aligned in the direction perpendicular to the load, as seen in Figure 1 for the dermal fibroblasts. Similar behaviour was seen for tenocytes and MSCs. ECM deposition is increased in the presence of MMC and this effect is maintained under mechanical loading. On Figure 2 it can be seen an increase in deposition of collagen I for all different cell types. Other ECM molecules have been assessed and a similar behaviour was observed. Markers for osteogenic and chondrogenic lineage have been assessed and were shown not to be expressed.

Fig. 1: Dermal fibroblast morphology after 12 hours of static or dynamic conditions with (MMC) or without macromolecular crowding (No MMC). Arrow indicates direction of applied load.

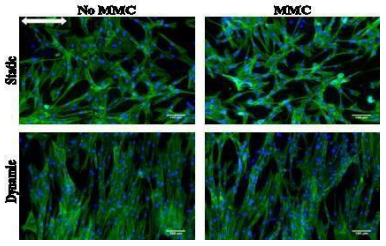
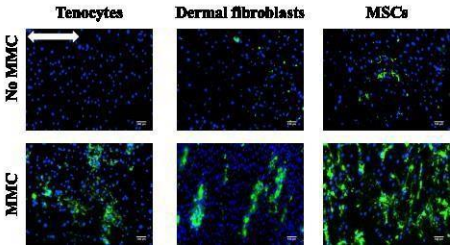


Fig 2: Collagen type I deposition under uniaxial strain in the absence (No MMC) and presence (MMC) of macromolecular crowding. Arrow indicates direction of applied load.



DISCUSSION & CONCLUSIONS: Mechanical loading and macromolecular crowding can induce cell and ECM alignment and increased ECM deposition without affecting cell metabolic activity or viability. ECM composition and tenogenic marker expression suggest this approach might be suitable to maintain or differentiate towards tenogenic lineage.

ACKNOWLEDGEMENTS: The authors would like to acknowledge the Irish Research Council (RS/2012/82), the Health Research Board (HRA_POR/2011/84) and Science Foundation Ireland (SFI) Centre for Research in Medical Devices (CÚRAM) (13/RC/2073) for financial support.

Tenomodulin subpopulation of human adipose stem cells as a promising source of tendon progenitor cells

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INTRODUCTION: Cell based strategies envision promising insights for tendon therapies due to the naturally limited cellularity and regeneration of these tissues. Human stem cells from the stromal vascular fraction of adipose tissue (hASCs) have shown their tenogenic potential for tendon strategies[1]. However, the heterogeneous populations within the hASCs pool may hinder their use in specific applications. Tenomodulin (TNMD) has been recognized as an important marker of tendon progenitor cells[2], thus herein we hypothesized that TNMD positive (TNMD+) cells are more prone to differentiate into tendon-like cells. Therefore, a distinct TNMD+ subpopulation was isolated from hASCs by immunomagnetic cell separation[3]. Subsequently, the tenogenic differentiation capacity of TNMD+ cells was evaluated (towards the heterogenous hASCs population obtained from the SVF) in the presence of growth factors (GFs) associated to development, growth and healing of tendon tissue[1]. Cell response to biochemical stimuli was studied analysing cell morphology, tendon-related markers expression, and extracellular matrix (ECM) formation.

METHODS: TNMD+ cells were sorted using Dynabeads® M-450 Epoxy (Invitrogen) coupled with TNMD antibody (Santa Cruz Biotech). Cells isolated without antibody coupling were considered the control population (CTR). Tenogenic differentiation capacity of both TNMD+ and CTR populations was evaluated for up to 28 days in α-medium or with: EGF, bFGF, PDGF-BB or TGF-β1. The time-related effects of GFs supplementation on the expression of tendon-related markers was evaluated by RT-PCR and flow cytometry, while the ability of cells to synthetize a Collagen I and III ECM was analysed by immunocytochemistry.

RESULTS: A TNMD+ subpopulation was successfully obtained by immunomagnetic separation as demonstrated by TNMD deposition in sorted cells in comparison to CTR cells (Fig.1). TNMD+ cells showed enhanced genetic expression of tendon markers, namely SCX, TNMD, and DCN as well as Col I and III over CTR cells for up to 28 days.

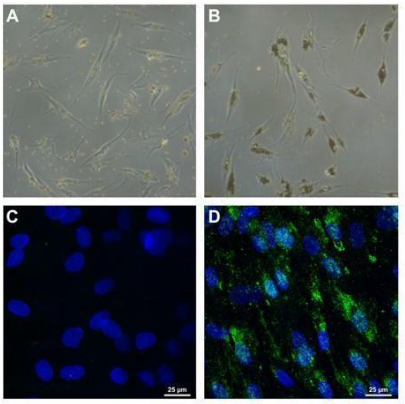

Fig. 1: Micrographs of hASCs: CTR cells (A) and TNMD+ cells (B) 3 days after of immunomagnetic isolation; TNMD immunolocation (green) in CTR (C) and TNMD+ cells (D). DAPI (blue) stains cell nuclei.

SCX and TNMD protein expression by flow cytometry was also increased in TNMD+ cells. Although gene and protein expression were influenced by supplemented GFs with a tendency to increase the expression of tendon markers in TNMD+ cells, no evident differences between supplemented and α-medium were found.

DISCUSSION & CONCLUSIONS: TNMD+ cells show a markedly positive tenogenic response in comparison to CTR cells. This response is supported by the enhanced genetic and protein expression of tendon markers. Thus, the TNMD+ subpopulation is predisposed towards tenogenic lineage and therefore can be used more efficiently as source of tendon progenitor cells.

ACKNOWLEDGEMENTS: Funds from **(FCT/MCTES)** and **(FSE/POCH), PD/59/2013.**

Mesenchymal stem cells in combination with Cyclosporine A for the modulation of immune response

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INTRODUCTION: Mesenchymal stem cells (MSC) represent a heterogenous population of multipotent stem cells that can be isolated from various tissues. Due to their immunomodulatory, antiapoptotic, cytoprotective and differentiation capabilites, MSC hold a great promise for treatment of many inflammatory diseases and for use in a regenerative medicine. There are numerous studies describing the use of MSCs in combination with immunosuppressive drugs [1, 2] but the exact mechanism of observed synergism has not yet been described.

We have demonstrated previously a successful incorporation of non-specific immunosuppressive drug Cyclosporine A (CsA) into nanofiber scaffolds [3]. The aim of this study was to analyze the therapeutic effect of MSCs applied locally on nanofiber scaffold with or without incorporated CsA in a mouse model of allogeneic skin transplantation.

METHODS: MSCs-loaded nanofiber scaffolds with incorporated CsA were applied topically to skin allografts. The fluorescence microscopy and flow cytometry were used to trace the fate and survival of PKH-26 labelled MSCs. The local immune response was determined by characterisation of immune cells infiltrating the skin graft and lymph nodes using flow cytometry, expression of proinflammatory genes was detected by PCR. The production of nitric oxide (NO) was evaluated by Griess reaction, interleukin-10 (IL-10) and interferon-γ (IFN-γ) were determined by enzyme-linked immunosorbent assay (ELISA). The statistical significance of differences between the means of individual groups was calculated using one-way analysis of variance (ANOVA), $p \leq 0,05$ was considered statistically significant.

RESULTS: We observed that the treatment with MSCs and CsA significantly decreased the amount of graft-infiltrating macrophages and the production of NO. These changes were accompanied with the considerable lower production of IFN-γ as a cytokine priming classically activated macrophages.

Simultaneously, the significant upregulation of CD206 expression and increased production of interleukin-10 by graft infiltrating macrophages as markers of alternatively activated macrophages were detected).

DISCUSSION & CONCLUSIONS: Our study revealed that the local application of MSCs-seeded and CsA-loaded nanofiber scaffolds modulate the macrophages towards alternatively activated phenotype. This phenotypic switching may represents a new potential therapeutic option for tissue regeneration and suppression of the local inflammatory reaction.

ACKNOWLEDGEMENTS: This study was supported by the Grant Agency of Charles University (Grant Nos 546613 and 80815).

DISCLOSURES: Authors have nothing to disclose.

Application of bone marrow derived mononuclear cells (BMC) for bone tissue engineering – bench to bedside experience

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INTRODUCTION: The gold standard for the treatment of large bone defects are autologous bone chips, taken from the iliac crest. However, this procedure is fraught with donor site complications. Tissue engineering approaches might circumvent those limitations. The combination of endothelial progenitor cells (EPC) and marrow stromal cells (MSC) with a β-tricalciumphosphate (β-TCP) scaffold was effective in experimental bone healing models [1]. However the use of precultured cells is strongly restricted by EU law. In contrast, BMC can be harvested and reintroduced to the patient within hours which is more compatible with the clinical requirements. BMC are already used in cardiology and are ideally suited for regenerative medicine due to their regenerative potential and safety profile [2]. Here, we will report about our long lasting experience using BMC from *in vitro* to *in vivo* experiments up to a phase-I clinical trial.

METHODS: Human BMC were isolated from anonymized bone marrow samples (Ethical vote: 329/10, Hospital of the Goethe-University) by density gradient centrifugation for preclinical research projects. *In vitro* experiments: Human BMC were seeded on three different scaffolds (β-TCP, demineralised bone matrix (DBM), bovine hydroxyapatite (BHA)), seeding efficacy, progenitor cell adherence, survival and VEGF synthesis were analysed. *In vivo* experiments: BMC seeded on β-TCP ($1.33*10^6/cm^3$ β-TCP) were placed in a stabilized bone defect (5 mm) created in the femur of male athymic rats. Bone healing was assessed in comparison to animals treated with MSC and EPC after eight weeks. Clinical phase-I trial: Generation of formal study protocol, IMPD, IB and application for §40 AMG permission from the *Paul Ehrlich* Institute (PEI), ClinicalTrials.gov Identifier: NCT02153372.

RESULTS: BMC demonstrate high adhesion, viability and VEGF secretion on uncoated bone β-TCP and human DBM and to a lesser extent on BHA. Progenitor cell populations were found in comparable percentages on all scaffolds [3]. When human BMC, seeded on a β-TCP scaffold, were implanted into a critical size bone defect of the athymic nude rat, similar good bone healing was seen compared to rats that received a combination of precultured MSC and EPC [1]. Based on these results, a clinical phase-I trial was successfully applied for at the PEI. Ten consecutive patients (9 female, 1 male; mean age 69 yrs) with a proximal humerus fracture received angle stable plate fixation and purified autologous BMC on β-TCP ($1.33*10^6/cm^3$ β-TCP, granule size 1.4-2.8 mm). All fractures healed within 3 months, no secondary dislocations were observed. Neither morbidity at the harvest site nor at the surgical wound site was observed. Neither local nor systemic inflammation was noted [4].

DISCUSSION & CONCLUSIONS: We demonstrated in consecutive experimental approaches and a clinical phase-I study that BMC is an effective and safe cell source suitable to support the therapy of large bone defects. However, further research is necessary to optimize relevant parameters e.g. density of BMC, type of scaffold and timing of BMC implantation.

ACKNOWLEDGEMENTS: This work was funded by the LOEWE Center for Cell and Gene Therapy Frankfurt funded by "Hessian Ministry of Higher Education, Research and the Arts".

Maintaining of induced pluripotent stem cells through culture on nanopattern polydimethylsiloxane membrane

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INTRODUCTION: During natural tissue development, cells are interacting with various nanoscaled topographical and biochemical cues in their microenvironment [1]. Recently up-and-coming study is nanotopography as an important physical parameter in stem cell niche for regulating cell fate [2]. In this study, we determined effects of various nanopattern polydimethylsiloxane (PDMS) membrane as a culture substrate in promoting the proliferation and maintenance of human induced pluripotent stem cells (hiPSCs) in feeder free conditions.

METHODS: Irregular (IPM), groove (GPM), post (PPM) nano-pattern membranes were casted on PDMS in each other molds. Flat (FPM) pattern membrane is none pattern to the mold by using a silicon wafer (Fig. 1). We used the hiPSC line (SBI, cat# SC802A-1) generated by direct delivery of four proteins fused to a cell penetrating peptide. Single hiPSCs were seeded at $1x10^4$ cells/cm^2 on the nanopattern plates pre-coated with 15 μg/ml poly-L-ornithine /1 μg/ml fibronectin in mTeSR1 medium supplemented with 10 mM Y-27632, which was removed after 2 days. Cells were passaged every 5 days for 50 days (ten passages) in the same method and culture medium as used in hiPSCs plating.

RESULTS: Population doubling level showed that cell proliferation was significantly greater in the PLO/FN coated GPM or PPM than in FPM and IPM at 10 passages. The cell proliferation marker Ki67 mRNA level and percentages of Ki76-expressing cells were also significantly increased during the ten times of passages in GPM or PPM. These result means that GPM and PPM influences hiPSCs positively to maintain or increase cell proliferation potential. Also, undifferentiated ESC marker Oct3/4 and nanog gene and positive cells were significantly greater in the PLO/FN coated GPM or PPM than in FPM and IPM at 10 passages (P10). The result of RT-PCR and western blotting showed that the gene expression of a-actinin, vinculin, paxillin increased with passaging of hiPSCs in all patterns. However, phosphyrylation of FAK and paxillin were higer in GPM and PPM at P10.

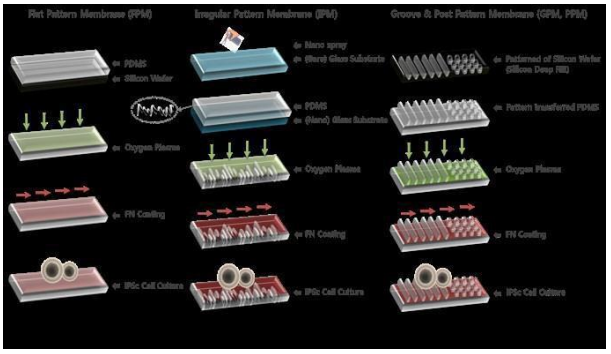

Fig. 1: Schematic diagram of the fabrication process.

DISCUSSION & CONCLUSIONS:

These findings suggest that nanopatterns, particularly regular patterns such as grooves and posts, contribute to the enhancement of stemness of hiPSCs. Also, continuous passaging of hiPSCs on regular nanopatterns, GPM and PPM, influence the adhesion of hiPSCs and increase the expression focal adhesion molecules. It is postulated that hiPSCs on their own increased the expression of focal adhesion molecules to adapt to the stress and survive on regular nanopatterns.

ACKNOWLEDGEMENTS: This study was supported by the National Research Foundation (NRF) funded by the Korean government (2015R1A2A1A09002793 and 2013R1A1A2062961).

An easily accessible adult stem cell pool may be suitable for tissue engineering-based regeneration of articular cartilage defects

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INTRODUCTION: Cartilage defects due to traumatic injuries or also in degenerative diseases like osteoarthritis lack appropriate self-repair and require therefore cell based therapies for tissue regeneration. Cartilage tissue engineering based on isolated human chondrocytes has been widely studied. However, detailed investigations have been hampered by a number of factors such as the scarce availability of human cartilage and its weak cellularity. Moreover, primary chondrocytes have a short *in vitro* life span and undergo a rapid shift of their collagen phenotype. Therefore, adult stem cells are an alternative cell source and in particular stem cells of the dental pulp (DPSCs) of adult individuals provide an easily accessible cell source to be used for new cartilage tissue engineering approaches.

METHODS: Third molar derived DPSCs from three different donors were characterised via flow cytometry and expanded in monolayer cell culture. For chondrogenic differentiation the cells were transferred to a scaffold-free 3D-culture in the presence, or absence, of dexamethasone, TGF-β and BMP-2. After three weeks in 3D-culture the cartilage-like microtissues were harvested for isolation of RNA or for cryosectioning. The tissue maturation process was analysed via TaqMan-based qPCR (for gene expression of collagen type II, aggrecan, Sox9, and Twist1) and on tissue cryosections using histology (Alcian Blue to detect proteoglycans) as well as fluorescence-based immunohistochemistry (for protein expression of Sox9 and COMP).

RESULTS: Flow cytometry analyses of DPSCs revealed a high expression of common mesenchymal stem cell markers in all of the three donors. In detail, CD29, CD73, CD90, CD146, and CD166 were expressed by 85 – 99% of analysed DPSCs. Stable dental pulp stem cell aggregates were formed already after three days in 3D- culture independent from medium composition. Chondrogenic microtissues cultured in medium supplemented with TGF-β and BMP-2 showed a proteoglycan rich extracellular matrix indicated by Alcian Blue staining. Furthermore, cells in these microtissues expressed the cartilage transcription factor Sox9 and synthesized cartilage oligomeric matrix protein COMP. However, no collagen type II expression was detectable via immuno-fluorescence. Corresponding results were obtained at the transcriptional level using qPCR. These analyses showed gene expression of Sox9, comparable to that of primary chondrocytes as positive control, and in one TGF-β1 + BMP-2 condition a measurable induction of aggrecan expression. However, collagen type II gene expression was not detectable in any differentiation approach. In addition, we detected Twist1 gene expression.

DISCUSSION & CONCLUSIONS: The use of new stem cell sources for cartilage tissue engineering in order to repair articular cartilage is of growing interest. One promising cell source are DPSCs as they can be easily obtained from human third molars that are routinely extracted because of orthodontic reasons, show a high proliferation capacity *in vitro* and multilineage differentiation potential. Flow cytometry analyses indicated a considerably high percentage of mesenchymal stem cells within all cell populations used for this study. The present study has shown that DPSC are able to undergo chondrogenic differentiation and growth factors enhance this process. Although the chondrogenesis of cells in the generated micro-tissues seemed to be just at the beginning and needs to be optimized, DPSCs represent a suitable potential cell source for future cartilage tissue engineering

DISCLOSURES: The authors declare that they have nothing to disclose.

Tuning the biomechanical properties of novel hydrogels for use in tissue engineering

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INTRODUCTION: Fibrin-based hydrogels have previously demonstrated high biocompatibility and regeneration capabilities both in vitro and in vivo. However, the biomechanical properties of these biomaterials are very poor, and novel strategies are in need in order to improve the biomechanical and rheological behaviour of these hydrogels. Addition of agarose or other long-chain polymers and 3D tuning by nanostructuration proved to be efficient ways to improve the mechanical properties of fibrin hydrogels. The objective of this work is to determine the role of the concentration of agarose and the level of nanostructuration on the biomechanical properties of fibrinhydrogels.

METHODS: Fibrin hydrogels were generated from human plasma as previously described [1]. Briefly, plasma was mixed with DMEM medium, tranexamic acid -as antifibrinolitic agent- and calcium chloride -as inductor of the polymerization reaction-, and gels were allowed to jellify at 37ºC. In gels containing agarose, melted type VII agarose diluted in PBS was added to a final concentration of 0.1%, and 0.3%. After gelation, all biomaterials were subjected to nanostructuration in a bioreactor of controlled pressure and hydration level, and tissues with different degrees of nanostructuration (soft and strong nanostructuration) were generated by controlling pressure and time. All tissue constructs were biomechanically evaluated by using an Instron electromechanical material testing machine. Histological assessment was carried out by glutaraldehyde fixation and analysis in a scanning electron microscope (SEM).

RESULTS: First, tensile tests demonstrated that tissues with the highest hydration levels had low tensile modulus of Young, and strong nanostructuration resulted in significantly improved values for this parameter ($p<0.05$). The concentration of agarose was significantly correlated with lower values of this modulus, and the highest Young's modulus corresponded to fibrin gels with no agarose ($p<0.05$). Then, analysis of the elastic modulus under compression showed a significant increase of this parameter with increasing concentrations of agarose ($p<0.05$), and with strong nanostructuration methods ($p<0.05$). For soft nanostructuration, G' values for fibrin gels containing 0, 0.1 and 0.3% agarose was 16, 37 and 99 Pa, respectively. For tissues subjected to strong nanostructuration, G' values were 280, 1211 and 2610 Pa, respectively. For the G'' modulus, values were 3, 5 and 17 for soft nanostructuration and 56, 192 and 284 for strong nanostructuration. SEM analysis showed a differential fibrillar surface pattern in the different tissues, with strong nanostructuration resulting in a denser fibrillar mesh, and agarose was associated to higher porosity (Figure 1).

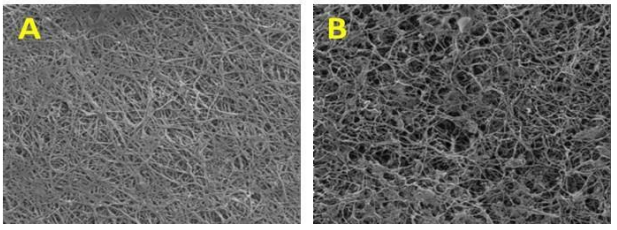

Fig. 1. SEM evaluation of fibrin hydrogels (A) and fibrin hydrogels with 0.1% agarose subjected to strong nanostructuration methods.

DISCUSSION & CONCLUSIONS: These results demonstrate that the biomechanical properties of fibrin hydrogels can be modulated and tuned by controlling the concentration of agarose and the level of nanostructuration applied to the biomaterial. By using these methods, bioengineered tissues could be used for cornea, nerve, oral mucosa and cartilage tissue engineering.

ACKNOWLEDGEMENTS: This study was supported by the Spanish Plan Estatal de Investigación Científica, Desarrollo e Innovación Tecnológica (I+D+I) 2013-2016 from the Spanish Ministry of Economy and Competitiveness (Instituto de Salud Carlos III), grants FIS PI14/0955, FIS PI14/1343, FIS PI14/2110 and FIS PI15-2048 (co-financed by FEDER funds, European Union).

Non-mulberry silk fibroin grafted poly(Є-caprolactone)/nano hydroxyapatite nanofibrous scaffold for osteoconduction and osseointegration

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INTRODUCTION: Nanofibrous scaffolds of mulberry silk fibroin are widely accepted as bone scaffolds. Nonmulberry silk protein fibroin of *Antheraea mylitta* has better mechanical properties over mulberry silk with improved cell proliferation due to built-in RGD sequences in fibroin, which could unveil new horizons for its application in regenerative medicine[1]. This study aims at developing biodegradable, mineralized, nanofibrous scaffolds for use in bone regeneration.

METHODS: Nano-hydroxyapatite (nHAp) was synthesized by precipitation reaction. To prepare nHAp doped poly(Є-caprolactone) (PCL) (reported as osteogenic material) nanofibrous scaffold, a defined amount of nHAp (25 wt% and 50 wt%) was suspended in chloroform by ultra-sonication and vigorous stirring before adding to the 10 wt% polymer solution prepared in chloroform. Dioctyl sulfosuccinate sodium salt served as a surfactant for stabilizing the suspended nHAp in the polymer solution. E-spinning was carried out using at 11 kV (DC) supply with flow rate set at 1 ml/h at 30°C maintaining 15 cm distance from the capillary tube to collector. These matrices were stripped off the aluminum foils upon reaching a thickness of about 1 mm. Immobilization of 2 wt% non-mulberry silk fibroin (NSF) on fabricated nHAp doped PCL nanofibrous scaffold was carried out using aminolysis[2]. After grafting of protein the matrices were finally dried off and treated with ethanol for a couple of minutes before carrying out further studies. The NSF grafted PCL nanofibrous matrix (without nHAp) was also prepared to act as a control.

RESULTS: The nHAp particles showed rod like characteristics with length and width of ~70 and 24 nm, respectively. Analysis of FESEM images using Image J®, diameter of the different nanofibrous scaffold were estimated to be 456nm (NSF-PCL/n25) and 462 nm (NSF-PCL/n50). As per expectation, adding nHAp slightly increased the diameter of fibers. The TEM micrographs showed the nHAp particles evenly distributed in the polymer fibers. XRD, FTIR and TGA analysis revealed the effective blending of nHAp and grafting of NSF on nanofibrous matrix. The results of contact angle measurement showed that incorporation of nHAp reduced the hydrophobicity. Mechanical strength of the nanofibrous matrices was increased with addition of nHAp; NSF-PCL/n25 (16.06 MPa, 168.42%) exhibited greater strength over NSF-PCL/50 (12.7 MPa, 159.03%). Confocal microscopy (Fig.1) of the constructs after 7days *in vitro* culture exhibited more intensive and homogenous distribution of actin filaments for nHAp doped scaffolds along with better ALP activity and promoted significant expression of genes related to bone growth over 2 weeks of cell culture.

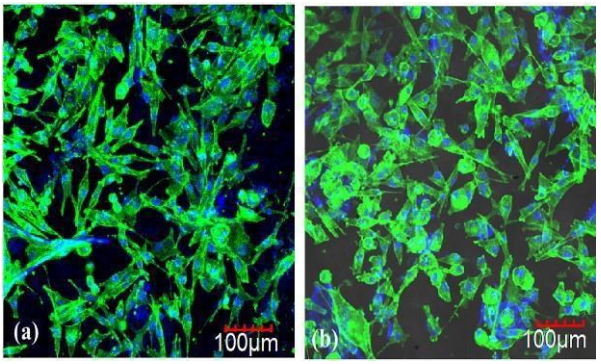

Fig. 1: The actin organization of MG-63 cells grown on (a) NSF-PCL/n25 and (b) NSF-PCL/n50 nanofibrous scaffolds at day-point 7.

DISCUSSION & CONCLUSIONS: The results indicate that incorporation of nHAp on NSF grafted PCL nanofibrous matrix are suitable as scaffold for bone tissue engineering; while the matrix with a high nHAp loading density is found to be weaker than one with low nHAp loading density due to reduction of dissipate energy of nHAp particles. The current *in vitro* study serves as a starting point for design and testing of clinically relevant orthopedic grafts *in vivo*.

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ACKNOWLEDGEMENTS: Supported by DBT and ICMR, Govt. of India.

Biodegradable 3D scaffolds based on chitosan-*g*-polylactide copolymers for bone tissue engineering

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INTRODUCTION: Biodegradable polymeric scaffolds play a pivotal role in regenerative medicine. Chitosan (CS), a natural polysaccharide, is a biocompatible biodegradable polymer widely used in tissue engineering. However, CS is slightly soluble in an aqueous culture medium with poor mechanical properties [1]. On the other hand, polylactide (PLA), a biocompatible biodegradable synthetic polymer has excellent mechanical properties [2]. Grafting a synthetic polymer along the CS backbone is an attractive way to regulate the physical and mechanical properties of CS. In this work, we present the preparation of two CS-*g*-PLA graft copolymers, CS-*g*-PLA(20) and CS-*g*-PLA(50), with 20 wt% and 50 wt% PLA content, respectively, as well as the evaluation of the morphology, viability and proliferation of pre-osteoblastic cells on both copolymers. Exploration of their osteogenic potential is in progress.

METHODS: The CS-*g*-PLA copolymers (*Figure 1*) were synthesized by grafting PLA chains, bearing a terminal carboxylic acid group along the CS backbone [3].

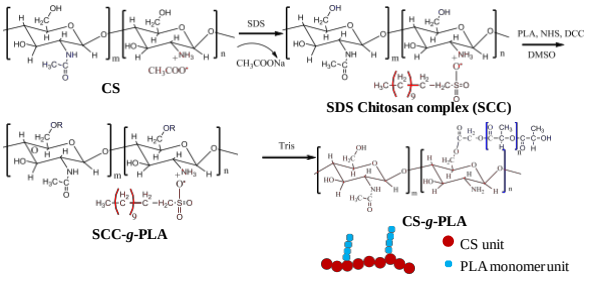

Fig. 1: Schematic of the procedure followed for the preparation of the CS-g-PLA copolymers.

Compressed discs of the CS-*g*-PLA(20) and CS-*g*-PLA(50) copolymers were used to study the degradation profile of the copolymers in the cell culture médium, as well as the adhesion and proliferation of MC3T3-E1 pre-osteoblastic cells. Moreover, 3D scaffolds were prepared by lyophilization of the copolymer from aqueous solutions. The morphology of the cells on the copolymer surfaces was examined by scanning electron microscopy (SEM). The cell viability and proliferation on the CS-*g*-PLA copolymer was assessed by the PrestoBlue® assay.

RESULTS: We prepared CS-*g*-PLA graft copolymers with PLA content varying from 20 wt% to 50 wt%. FTIR and ^{1}H-NMR spectroscopies verified the presence of both the CS and PCL segments in the copolymer. Analysis of the ^{1}H NMR data allowed to calculate the copolymer grafting density, which was found to be one PLA chain every 180 CS monomer repeat units for CS-*g*-PLA(20) and one PLA chain every 25 CS monomer repeat units for CS-*g*-PLA(50). Degradation studies showed a total weight loss of 9% after 21 days in cell culture for both copolymers. Cell viability experiments showed that the CS-*g*-PLA copolymers promote cell adhesion and proliferation. MC3T3-E1 cells cultured on the two material surfaces retain their characteristic elongated morphology *(Figure 2).*

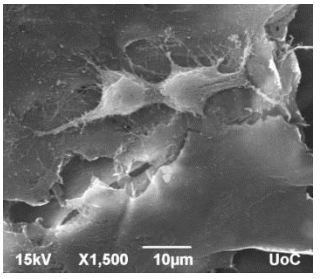

Fig. 2: SEM image showing the morphology of MC3T3-E1 cells cultured on CS-g-PLA(50).

DISCUSSION & CONCLUSIONS: We have successfully synthesized novel CS-*g*-PLA copolymers by grafting end-functionalized PLA chains onto the hydroxyl groups of CS, varying the PLA content from 20 to 50 wt%. The copolymer structure was verified by FTIR and ^{1}H NMR. Cell culture experiments show strong cell attachment and good cell proliferation of MC3T3-E1 cells on the CS-*g*-PLA scaffolds. Ongoing cell differentiation studies are expected to demonstrate the copolymers osteogenic potential.

Alendronate functionalized mesoporous bioactive glass nanospheres

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INTRODUCTION: Osteosarcoma (OS) is the most common primary malignant bone tumor in children and adolescents, with a second peak in incidence in adults over the age of 50 [1]. The mainstay of curative osteosarcoma treatment is surgery (often amputation). However, the survival of patients with OS treated with surgery alone is less than 20% [2]. However, although little is known on the mechanisms by which OS destroys the hard matrix of the skeleton, the role of osteoclasts, primary cells involved in bone matrix solubilization, seems to be fundamental in the cancer progression. Recent findings have shown that the induction of osteoclast activity by tumor cells is particularly increased in those patients with aggressive OS [3]. Recent studies in *in vivo* models indicate that bisphosphonates (BPs), commonly used in osteoporosis treatments, can inhibit the tumor local expansion and the formation of metastases, and act as anti-angiogenetic factors [4]. In this study we prepared nano-sized bioactive glass nanospheres (nMBG) loaded with the most potent amino-bisphosphonates, alendronate (AL). The combined capability of the developed drug system as inhibitor on cancer cells viability and as inhibitor of bone resorption was investigated *in vitro* through determination of tumor cell proliferation/survival, and osteoclast activity.

METHODS: nMBG were synthesized by a hydrothermal method using cetyltrimethylammonium bromide (CTAB) and poly (vinylpyrrolidone) (PVP) as co-templates. Loading of alendronate on nMBG was carried out in MilliQ water at different concentrations of sodium alendronate trihydrate. Alendronate content was determined spectrophotometrically. The nMBG, alone or drugged, were characterized using several techniques (e.g. X-ray diffraction assay, TEM). *In vitro* tests were performed using a human osteosarcoma cell line (MG63) and a murine monocyte/macrophage cell line (RAW 264.7). Cells viability assay, cells morphology assays and TRAP activity evaluation were performed up to 7 days. Gene expression of two principal osteoclastic markers (Oscar and CtK) were performed at day 7.

RESULTS: The results of the chemical and structural characterization show that the nMBG display a relatively high surface area (528 m^2/g) and a mean pore volume of 0.63 cm^3/g, both of which decrease on increasing alendronate content. It is possible to modulate the amount of AL loaded into the nanospheres up to a maximum value of about 17 wt%. The results indicate that even the lower concentration of alendronate provokes decreased tumor cell viability (≈50%) for AL-nMBG groups respect to nMBG group alone at day 3 and day 7 without any statistical differences among the group. Moreover the osteoclast activity exhibits an alendronate dose-dependent inhibition as shown by TRAP and Oscar and CtK analysis.

DISCUSSION & CONCLUSIONS:
The approach developed in this study allows to load mesoporous bioglass nanospheres with increasing amounts of AL and yields materials where the bioactivity of the nanospheres is coupled with the antiresorptive and anticancer properties of alendronate. The results of *in vitro* tests prove that the proposed multifunctional system is a promising platform for controlled regulation of osteosarcoma cells and osteoclast activity, providing a new tool for OS treatment.

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ACKNOWLEDGEMENTS: The authors acknowledge Fondazione del Monte di Bologna e Ravenna (Prot. N° 434 bis/2015 del 20 Luglio 2015, "Nanoparticelle di silice porosa come vettori di rilascio controllato di farmaci in pazienti osteoporotici").

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

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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 immunohistochemistry analysis, implants were paraffin embedded and processed for 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 concordance with these studies, we used a cell free approach and obtained bone formation in a very high percentage of the constructs in a subcutaneous environment, which is very challenging to get bone formation without cells.
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. .

ACKNOWLEDGMENTS: BioInspire Marie Curie Action (FP7/2007-2013)

Conduits for peripheral nerve repair fabricated using microstereolithography and electrospinning

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INTRODUCTION: Peripheral nerve injury can result from a wide variety of injuries such as trauma (e.g. laceration) or from iatrogenic cases such as third molar removal or dental implant surgery. Peripheral nerve injuries can be treated via suturing (small gap injuries), autografts and nerve guidance conduits (NGCs.) The 'gold standard' autograft treatment for peripheral nerve injury presents significant drawbacks such as donor site morbidity and the need for multiple operation sites [1]. NGCs can be used as an alternative treatment, however current commercial products have simple designs and limited success and are only useful for small nerve gaps, up to around 25mm [2].

Here we combine microstereolithography (μSL) and electrospinning to produce advanced nerve guide conduits containing a large number of highly aligned microfibres with the aim of allowing improved peripheral nerve regeneration over larger nerve gaps.

METHODS: Poly(ε-caprolactone) (PCL) was methacrylated to allow it to be photocurable. PCL and poly(ethylene glycol) (PEG) nerve guide conduits were fabricated using μSL with a 405nm laser and a digital micromiror device.

Highly aligned PCL microfibers were fabricated via electrospinning. PCL (Mn 80,000 g/mol) was dissolved in dichloromethane and slowly dispensed from a syringe across a voltage gap onto a rotating collector. These fibres were analysed with scanning electron microscopy (SEM) and inserted into the tubes. Micro-computed tomography (μCT) was used to analyse the density and alignment of fibres within the tubes.

Cultures with the Schwann cell model RN22 were carried out to assess the infiltration of cells into the conduit and the degree of alignment along the fibres.

RESULTS: PCL tubes were fabricated to a high quality with microstereolithography. Electrospun fibres ranging between 1-10μm were fabricated with a high degree of alignment, as shown from SEM imaging. The fibres were inserted into the PCL tubes and μCT images revealed good fibre alignment and packing density within the tubes (see *Fig 1.*) RN22 cells were seen to infiltrate the tubes and align along the PCL fibres.

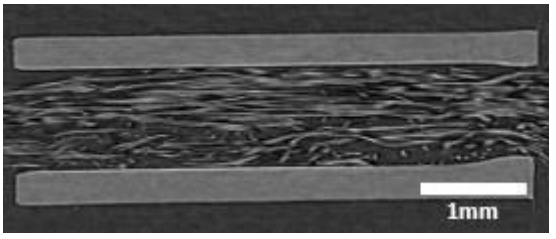

Fig. 1: μCT image of a NGC with PCL fibres.

DISCUSSION & CONCLUSIONS: Microstereolithography serves a method for the production of highly controllable NGC tubes. Together with highly aligned electrospun fibres, advanced NGCs can be produced with much more complex designs than current commercial products. Many studies show the beneficial effects of aligned fibres on cell growth and suggest that these designs may improve the function of current NGCs allowing longer nerve injuries to be repaired.

ACKNOWLEDGEMENTS:
Acknowledgments to the EPSRC for funding.

Selective cell adhesiveness on model surfaces grafted with elastin-like recombinamers

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INTRODUCTION: Nowadays there is a substantial interest in engineering solid supports to achieve functional immobilization of proteins for investigation of their biological functions and for the development of novel biosensors. [1] Elastin-Like Recombinamers (ELRs) have near ideal features because its potential to have into their backbone new functionalities conferring stimuli sensitiveness and diverse bioactivities. [2]
The aim of this work is to biofunctionalize model (gold) surfaces with several percentages of two ELRs showing different cell adhesion domains in order to obtain selective cell adhesiveness of Human Umbilical Vein Endothelial Cells (HUVECs). One of the ELRs contains a cell adhesive RGD (Arg-Gly-Asp) sequence which constitute a major recognition system for cell adhesion. The other one contains REDV (Arg-Glu-Asp-Val) sequence derived from the alternatively-spliced IIICS-5 fibronectin domain. Afterwards, the percentage composition that enhance selective endothelial cell adhesion will be employed to functionalize areas of the surface cleaned by laser ablation in order to obtain selective cell adhesiveness of a co-culture made of Human Foreskin Fibroblast (HFF1) and HUVECs.
METHODS: ELRs were constructed using standard genetic engineering techniques and purified using several cycles of temperature-dependent reversible precipitation. The surfaces were cleaned with Argon plasma that effectively removes contaminations without damaging the underneath gold coating. Immersion in recombinamer solutions for 6 hours and subsequent washing steps with milliQ water overnight were performed. The reaction between the gold surface and the SH groups present in the two recombinamers were assessed by contact angle, XPS, AFM, SEM) and FTIR. Certain areas of the surfaces having a width of 200 μm have been therefore ablated by a Maldi-Tof Ultraflextrem laser. Subsequently the biofunctionalized surfaces were submitted to cellular adhesion assays for 4 hours using a co-culture of HFF1 and HUVECs. Each of them was labeled with PKH26 (Red fluorescent dye) and PKH67 (Green fluorescent dye) respectively in order to be detected by photometer plate reader and fluorescence microscope.
RESULTS: XPS, AFM and FT-IR spectra grafting have demonstrated that the polymers are binding to the surface due to the presence of the SH group of the cysteines. Micrographies obtained with SEM confirmed the homogeneous coating of surfaces with recombinamers. The recombinamer composition 75% REDV elastin-like recombinamer and 25% RGD elastin-like reombinamer exhibited the best ability to enhance selective HUVEC adhesion. The co-culture HFF1 and HUVEC adhesion assay confirms that cells adhere biospecifically to the surfaces.
DISCUSSION & CONCLUSIONS: Our design strategy for selective cell adhesiveness on model surfaces created a favourable environment to determine the appropriate recombinamer composition for further developing a selectivity cellular system. The advantage of REDV over other popular binding sequences, such as RGD or YIGSR, is the selectivity of this ligand toward endothelial cells. Exploiting the capability of the ELR, which can be designed with specific sequences, avoiding the need of an intermediate linker before the immobilization of theprotein.

ACKNOWLEDGEMENTS: This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement number 31730.

Fabrication, characterization and osteogenic properties of 3D printed PCL and PCL-Bioactive glass composites

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INTRODUCTION: Composite scaffolds of polymers and glasses are promising for bone tissue engineering because of their tuneable properties and similarity to the native bone structure. This work describes the fabrication and characterization of scaffolds produced by additive manufacturing combining poly-ε-caprolactone (PCL) and a new class of bioactive glass (BG) particles. This new glass was self-developed using natural origin raw materials. It proved to have osteo- as well as angiogenic properties in 2D culture. Therefore, the main objective of this study was to produce a 3D scaffold composite containing the glass particles. We hypothesise that this composite material could be relevant for bone engineering and regeneration.

METHODS: PCL and PCL-BG scaffolds were prepared as previously described [1]. Briefly, BG particles (53% SiO_2, 24% Na_2O and 23% CaO, ≤ 38 µm) were incorporated into 10% (w/v) PCL solution (CAPA 6500, Perstorp, United Kingdom) at 10%wt. Scaffolds were produced by melt extrusion additive manufacturing technology at 100 Cº using a 21G nozzle with a lay-down pattern of 0-90°. Scaffolds' porosity and morphology were analysed by µCT and SEM. Bioactivity was evaluated by SEM-EDX. For mechanical testing, scaffolds were subjected to 10% compression at a rate of 1 mm min^{-1} in a zwicki1120 microtester fitted with a 2.5 kN load cell (n=8). Cytocompatibility and osteogenic properties were analysed *in vitro* by culturing human adipose mesenchymal stem cells (AMSCs) in osteogenic media in absence of dexamethasone. Cell viability was evaluated by MTT, LDH and live/dead staining. Osteogenic differentiation was analysed by osteogenic gene expression (qPCR) and mineral deposition (Von Kossa staining). Experiments were performed in triplicates of 3 independent donors (N=3, n=9). Statistical analysis was performed with GraphPad Prism using one-way Anova.

RESULTS: µCT and SEM analysis showed that the PCL and PCL-BG scaffolds had a uniform morphology with open and highly interconnected pores (table 1). The distribution of the BG particles in the PCL matrix was homogeneous (fig.1) and lead to the deposition of a calcium phosphate layer on the surface of the scaffolds. In addition, the young's modulus of PCL-BG scaffolds was increased in comparison to PCL at similar porosity. *In vitro* studies showed that incorporation of BG particles resulted in reduced toxicity and improved cell attachment to the scaffold's surface. In addition, enhanced mineral deposition was observed on the scaffolds containing BG particles. Finally, qPCR results showed that the expression of the osteogenic markers RunX2 and osteopontin and the vascularization marker VEGF was markedly upregulated in PCL-BG compared to PCL.

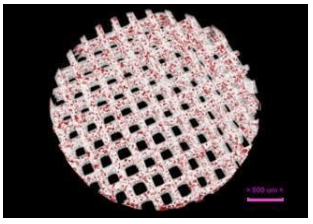

Fig. 1: µCT 3D reconstruction of a PCL-BG scaffold. The PCL matrix is shown in white and glass particles are shown in red.

Table 1. Scaffolds' mean porosity (%)

	Total porosity	Open porosity
PCL	80,05 ± 3,7	79,94 ± 3,58
PCL-BG	76,56 ± 5,98	76,44 ± 5,94

DISCUSSION & CONCLUSIONS: Additive manufacturing is a suitable technique for the fabrication of PCL-BG composites with an interconnected structure and homogenous mineral distribution. The BG particles used in this study promoted the expression of osteogenic markers in AMSCs and the deposition of a mineralized matrix. Importantly, the master vascularization regulator VEGF was upregulated at early time points suggesting that vascularization may be improved. In conclusion, the PCL-BG composite scaffolds described in this study show promise for bone tissue engineering applications.

ACKNOWLEDGEMENTS: SFT thanks the Zeidler foundation for funding her research.

In vitro evaluation of gelatin and chitosan electrospun nanofibers as artificial guide in peripheral nerve repair

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INTRODUCTION: For tissue engineering application, the distribution and growth of cells on a scaffold are key requirements. In recent years the research focused the attention on the development of artificial biomaterial scaffolds to improve peripheral nerve regeneration [1]. The major problem of using these native proteins for scaffolds preparation is that, being animal derived proteins, may cause immune-rejection problems. Gelatin (GL) and chitosan (CS) scaffolds have been used as alternative to synthetic polymers because of their biocompatibility and biomimetic properties [2]. Appropriate fibrous substrates, functioning as a temporary matrix, can be easily prepared by electrospinning, which allows the obtainment of random or aligned fibrous matrices suitable as internal filler for nerve guidance conduits (NGCs) [3,4]. GL and CS fibers with different orientation (random or aligned) were compared in order to understand which fibrous matrix is the best to be used as internal filler. The effect of fiber alignment on cell adhesion and proliferation was assessed *in vitro* by using different Schwann cell (SC) and neuronal models. Moreover, actin cytoskeleton organization, lamellipodia and filipodia formation and axon outgrowth were evaluated.

METHODS: Random and aligned GL and CS nanofibers have been prepared by electrospinning technique. Randomly oriented nanofibers were collected on a plane aluminum foil while for aligned nanofibers a rotating mandrel with a speed of 2400 rpm was used.

The random and aligned GL and CS nanofibers were characterized by scanning electron microscopy (SEM) for their morphology and diameter size (~150-200nm). Adhesion and proliferation assay were performed by culturing RT4-D6P2T SC line and primary SC purified from rat sciatic on the different substrates (GL random and aligned and CS random and aligned nanofibers) and in control condition (PLL coated coverslips). Cells were fixed, using PFA and stained for vinculin and actin. Neuritis outgrowth was evaluated by culturing dorsal root ganglia (DRG) explants on the different fibrous substrates and performing an immunocytochemistry (ICC) using anti-β-tubulin antibody.

RESULTS: GL and CS nanofibers induce similar adhesion and proliferation rate. GL and CS random fibers promote higher adhesion and proliferation rate in comparisons to the aligned ones. Cells seeded on control condition or on random GL fibers display higher cytoskeleton organization and more focal adhesion points than cells culture on GL aligned fibers or CS fibers both random and aligned (Fig. 1). However, GL and CS fiber alignment results in SC and axon oriented growth. Filipodia formation is higher on aligned fibers, suggesting that these substrates can promote higher cell migration in comparison to random ones. As regards neurite elongation no differences are observed in DRG explants model.

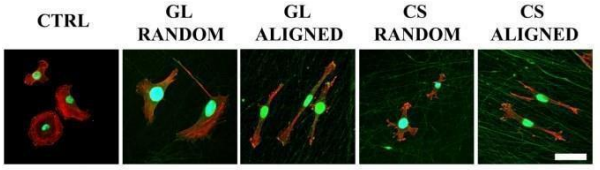

Fig. 1: Fluorescence images of RT4-D6P2T cell actin cytoskeleton and focal adhesion point organization on the different substrates. Scale bar 40 µm.

DISCUSSION & CONCLUSIONS: GL and CS fibers can induce and promote different regenerative events (i.e. SC adhesion, SC proliferation, neuronal differentiation and growth) depending on their morphology and biochemical composition. Suggesting that both GL and CS nanofibers can be promising internal filler for conduits to be used in peripheral nerve repair.

Synthesis of high purity α-calcium sulfate hemihydrate via the hydrothermal method

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INTRODUCTION: Calcium sulfate is a well-tolerated, biodegradable, osteoconductive bone graft substitute and is a reasonable alternative to autogenous bone graft for bone lesions[1]. Purity plays a significant role in the medical application of calcium sulfate. Recently, it has been reported that α-calcium sulfate hemihydrate (α-HH, $CaSO_4 \cdot 0.5H_2O$) can be prepared through a hydrothermal method, by heating gypsum (calcium sulfate dihydrate, DH, $CaSO_4 \cdot 2H_2O$), in various salt solutions near boiling point under atmospheric pressure[2]. The hydrothermal method exhibits advantages over the commonly used autoclave method in terms of purity control and energy consumption. The aim of this study was to optimize the synthesis parameters to obtain high purity α-HH.

METHODS: The synthesis was performed in a 1 L double-walled cylindrical glass reactor (Globe, Syrris, UK). A circulator was used to heat the reactor (CF41 Cryo-Compact Circulator, Julabo, Germany). In each run, 500ml 4mol/L calcium chloride solution was added into the reactor and preheated to the reaction temperature. Then, 50g commercially available calcium sulfate dehydrate (Israel Chemicals Limited (ICL), Tel Aviv, Israel) was mixed in the reactor and the slurry was kept stirring. About 100ml hot slurry was withdrawn at different intervals. Different reaction temperatures and time intervals have been tested. The solid particles was then immediately separated by vacuum filtration and quickly rinsed with 1 L boiling water and 200ml isopropanol. The powder was then dried at 60 ˚C for two hours. A SEM (LEO 1550, Zeiss, Germany) was used to observe the morphology of the synthesized material. Thermal gravity analysis (Q500, TA instrument, USA) was carried out for calcium sulfate phase identification and crystal water determination.

RESULTS:

Fig.1 shows that 105˚C/30min is the proper synthesis parameters which give 96% HH and 4% calcium sulfate anhydrite (AH). The rod-like and prismatic crystals clearly observed in Fig. 2 are α-HH crystals.

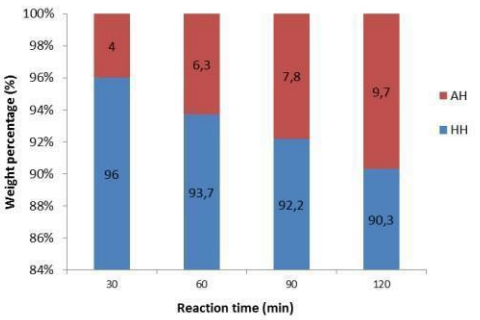

Fig. 1: Weight percentage of HH and AH after synthesis.

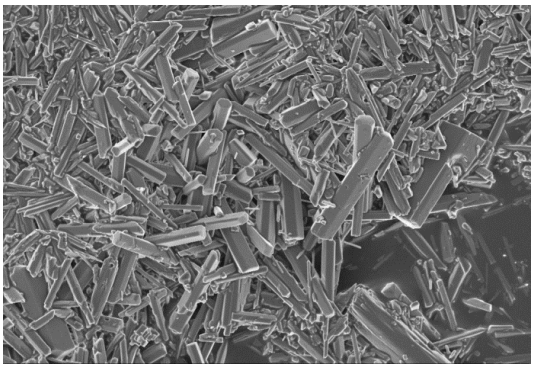

Fig.2: SEM micrographs of the synthesized α-HH

DISCUSSION & CONCLUSIONS: Synthesis results showed that preparation of α-HH with high purity from calcium sulfate dihydrate with the hydrothermal method was feasible. In this study, the proper synthesis condition was 105˚C/30min, achieving 96% of α-HH. The synthesized α-HH presented intact hexagon-prism-shaped structure.

ACKNOWLEDGEMENTS: The Swedish Innovation Agency (Vinnova) is gratefully acknowledged for financial support.

Functionalized collagen conduit implantation in a paraplegic rat model

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INTRODUCTION: Limited regeneration occur spontaneously following spinal cord injury (SCI). Biomaterials hold great promising for the regeneration of many tissues including spinal cord (SC) [1]. The Neurograft collaborative consortium proposed a novel micro-porous collagen conduit to restore SC functions. The conduit was designed to create a bridge across the lesion and provide a trophic support to the survival of neurons and axons outgrowth. The conduit was tested in a new paraplegic rat model that mimicked irreversible acquired paraplegia and in a rat transection model.

METHODS: Paraplegia was induced in a rat model by a contusion at thoracic vertebra T9. Four weeks after contusion the injured portion of the SC was (i) removed and replaced by the conduit (conduit), (ii) transected without implantation (transection control) or (iii) left untreated (contusion control). The motor functions were evaluated for 8 weeks after implantation using the Basso, Beattie and Bresnahan (BBB) rating scale. The inflammation and regeneration of the SC with/without conduits were investigated using histopathologic evaluation. The conduit was also combined with mesenchymal stem cells (MSC) and tested in single transection model. The transection was performed at T9 and the SC was implanted with (i) the conduit (conduit), (ii) the conduit combined with neural/glial-differentiated MSC (conduit + MSC) or (iii) left empty (control transection). SCI regeneration was evaluated similarly over 12 weeks. The conduit was tested for its biocompatibility following ISO 10993 standard for irritation, cytotoxicity, acute systemic toxicity, degradation kinetic and genotoxicity.

RESULTS: The conduit demonstrated its biocompatibility in all testing performed according to ISO 10993 standard. Its degradation kinetic was compatible with *in vitro* culture of MSC, allowing conduit functionalization before *in vivo* implantation (Fig. 1a). Its degradation kinetic was also compatible with the SC regeneration process, given that, after treatment period, the conduit was adherent to the surrounding spinal cord and restored the physical continuity of the spinal cord (Fig. 1b). Independently of the paraplegia model tested, BBB evaluation demonstrated no significant improvement of motor functions following implantation of the conduit, with or without MSC. The histopathologic evaluation is under process.

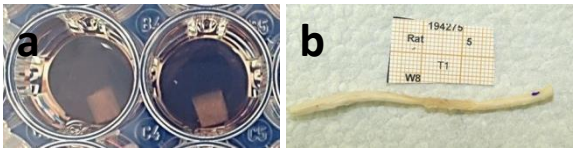

Fig. 1: Macroscopic imaging of: a) MSC-loaded conduit after in vitro neural/glial-differentiation, and before in vivo implantation; b) Rat spinal cord after 8 week implantation of conduit (post contusion + transection).

DISCUSSION & CONCLUSIONS: To achieve cellular regeneration and functional recovery upon SCI has been a demanding challenge leading to the development of highly complex therapeutic systems including a biomaterial device with specific characteristics and bioactive agents (cells/ molecules). Such systems should ensure suitable mechanical properties, cell-adhesion, electrical activity and biodegradability. While these parameters have been seized within neurograft development, no motor function was restored after transection+conduit implantation, demonstrated by the BBB rating evaluation. Independently of the *in vivo* model used, outcomes were equivalent. Regeneration of the spinal cord is still being investigated by extensive histopathologic analysis for further understanding of the ongoing repair mechanisms, and future fine-tuning of the therapeutic system. Nevertheless, the neurograft conduit has demonstrated its biocompatibility, becoming valuable as a scaffold to test other combination of active molecules and/or stem cells.

ACKNOWLEDGEMENTS:
NEUROGRAFT FP7- HEALTH- F4-2012-304936 project. Cyclosporine A was graciously provided by Sandoz-Novartis.

Characterization of multiphasic calcium phosphate cements with improved degradation rate

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INTRODUCTION: Calcium phosphate cements (CPCs), most often brushite and hydroxyapatite (HA), are interesting biomaterials thanks to their numerous advantages, e.g. self-sets in vivo, bioactive, osteoconductive, osteotransductive, and injectable. CPCs are also generally biodegradable. However, the very slow degradation rate of HA obstructs bone ingrowth and the rapid degradation rate of brushite causes mechanical instability. To solve this problem, biphasic cements composed by HA and β-TCP have been investigated [1,2]. However, no improvement in dissolution rate was found[2]. A possible solution might be the addition of an even more soluble phase, e.g. brushite. The aims of the present study were to designing and characterize novel multiphasic calcium phosphate cements (MCPCs) with enhanced degradation rate.

METHODS: All cements were based on a HA cement prepared by mixing α-TCP with a 2.5 wt% Na_2HPO_4-solution, Different amounts (0, 20, 35, 50 and 65 %) of crushed set brushite [3] particles (SBPs) were added to the powder phase. Specimens were left to set in PBS for 7 days. Setting times were determined with Gillmore needles. The porosity of the cements was assessed by a solvent exchange method. Wet specimens were tested in compression (CS) and diametral tensile strength (DTS). Phase composition was analysed with XRD and Rietveld refinement using Profex. An accelerated mass degradation was attained by placing cement samples in a 0.01 M hydrochloric acid solution for 8 h and 24h.

RESULTS: A mouldable paste was obtained for all MCPC with different SBPs content. Setting times for the MCPCs were comparable or slightly shorter than those for the HA cement (0 % SBP). The cement composition after setting was a mixture of calcium phosphates, with a constant amount of OCP and, as expected, a decreasing amount of HA and increasing amount of brushite with increasing SBPs content. Small amounts of beta-dicalcium pyrophosphate were also found, due to impurities in the ß-TCP powder, as well as unreacted α-TCP. The porosity slightly increased with SBP content (from 36.3% ± 0.5% for 0% SBP to 40.5% ± 0.4% for 50% SBP). The CS of the MCPCs was comparable to the one of pure HA up 50% SBP and then slightly lower for 65% SBP. DTS was higher for 20% SBP and then decreasing with increasing SBP content. As showed in Fig 1, the weight loss at 8h was comparable for MCPCs up to 50% SBP but at 24h there was an increased weight loss with increasing SBP content (apart an unexpected lower weight loss for 50% SBP). Ca^{2+} release followed a similar trend.

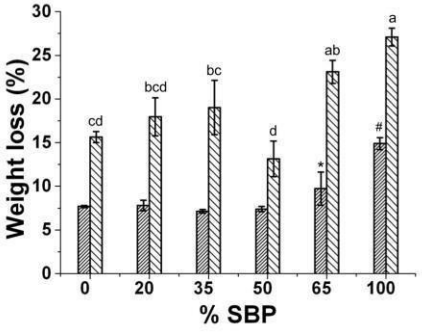

Fig. 1: MCPCs weight loss at 8h and 24h at pH=2. (Differences statistically significant at $p < 0.05$. Different series are marked with different symbols)

DISCUSSION & CONCLUSIONS: It was possible to obtain self setting MCPCs. The most interesting are the 20% SBP and 35% SBP cements since they maintained approximately the same setting times and mechanical properties as pure HA, while at the same time an increased degradation rate at longer times (24 h) was found. However, further studies to fully characterize the material are needed, e.g. specific surface area measurements, SEM and XRD after degradation

ACKNOWLEDGEMENTS: The authors acknowledge Carl Tryggers Stiftelse (CTS 15:355) for funding.

Effect of fibrin concentration on matrix deposition capacity of articular chondrocytes for nucleus pulposus regeneration

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INTRODUCTION: Nucleus pulposus (NP) degeneration of the intervertebral disc (IVD) is often associated with lower back pain (LBP) [1]. An emerging cell-based commercial approach is being developed by NuQu® (ISTO Tech., Missouri, USA) to regenerate the NP thereby restoring its functionality and is currently in phase II clinical trials (NCT01771471). This approach uses expanded juvenile articular chondrocytes combined with fibrin as a cell delivery system [2]. However, previous work has shown that fibrin concentration influences cell proliferation and matrix deposition [3]. Importantly, for IVD regeneration, the optimal concentration has not yet been identified. The objective of this work was to explore the effects of fibrin concentration to enhance NP-like tissue formation in IVD-like culture conditions (i.e. low oxygen, low glucose)

METHODS: Juvenile porcine articular chondrocytes (CCs) were isolated through collagenase digestion and culture expanded. Various concentrations of fibrinogen (Sigma) were dissolved in Aprotinin (Nordic Pharma) and combined with CCs at a density of $4x10^6$ cells/ml. Crosslinking was performed using 5U/ml of thrombin (Sigma) for 45 min at 37°C to yield final concentrations of 12.5, 25, 37.5 and 50mg/ml fibrin. Constructs (5x3mm) were cultured in 5% oxygen conditions in low glucose media for 21 days and assessed in terms of cell viability (LIVE/DEAD®), construct contraction, cell morphology, histology and biochemical analysis (DNA, sulfated-glycosaminoglycan (sGAG) and collagen content).

RESULTS: Enhanced cell viability and proliferation was observed in 25mg/ml and 37.5mg/ml fibrin hydrogels with increased cell death occurring in the highest concentration (50mg/ml). Interestingly, low concentration (12.5mg/ml) and high concentration (50mg/ml) induced cell spreading (Fig. 1A). In terms of degradation, the lowest concentration (12.5mg/ml) exhibited significant contraction compared to all other groups (Fig. 1B). In terms of matrix deposition, the lowest concentration (12.5mg/ml) yielded the highest accumulation of sGAG content (Fig. 1C).

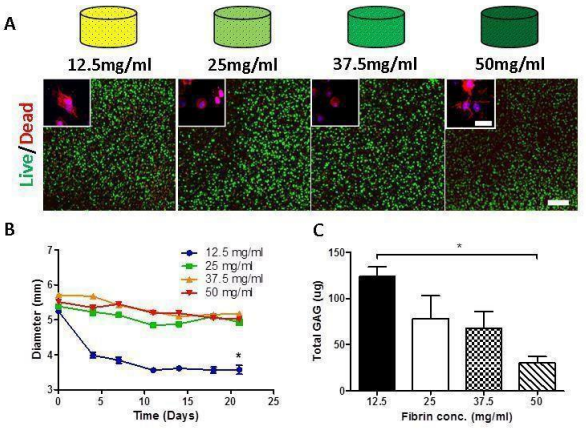

Fig. 1: (A) Fibrin concentration affects cell viability (sb=200µm) and morphology (sb=25µm) (B) Effect of fibrin concentration on hydrogel contraction (C) sGAG deposition decreases with increased fibrin concentration.

DISCUSSION & CONCLUSIONS: Modulation of fibrin concentration results in significant differences in the performance of articular chondrocytes in terms of proliferation, cell spreading and matrix synthesis with a trend towards an increased matrix deposition with lower fibrin concentrations. This work highlights the importance of fibrin concentration which needs to be tailored for successful nucleus pulposus regeneration.

ACKNOWLEDGEMENTS: Funded by the Irish Research Council Postgraduate Scholarship (2014- 2018). Grant No: 202860/13027

Cross-linked superfine electrospun tragacanth-based biomaterial as scaffolds for tissue engineering

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INTRODUCTION: Natural polymer-based nanofibrous structures promote cell adhesion and proliferation due to their high surface area/volume ratio, high porosity, and similarity to native extracellular matrix in terms of both chemical composition and physical structure [1]. Gum tragacanth (Tg) is a natural polysaccharides obtained from plants. It is a biocompatible, biodegradable and anionic polysaccharides that has been used extensively as an emulsifier in food and pharmaceutical industries [2]. Despite, its good rheological properties and compatibility, the potential biomedical applications of Tg have not been fully investigated. The objective of the present study was to explore the feasibility of combining Tg with gelatin to fabricate a scaffold that serves as a simple collagen-glycosaminoglycans analog for tissue engineering applications, e.g. as a scaffold for human skin epithelial cells.

METHODS: Tg/gelatin nanofibrous scaffolds were prepared using electrospinning techniques. Tg/gelatin was prepared by blending 10 wt% gelatin (in glacial acetic acid, and co-solvent) and 2 wt% Tg (in water). The fibres were then crosslinked to stabilize them.

RESULTS & DISCUSSION: SEM images revealed that the Tg/gelatin nanofibers were highly uniform with mean diameter of 74 ± 21 nm (Fig. 1). The nanofiber meshes were stable up to 7 days incubation in water (Fig. 2). The stability of the crosslink nanofiber was confirmed by monitoring the dissolution of gelatin from the nanofibers using BCA assay. No significant gelatin dissolution was measured in the supernatant, indicating that the crosslinked nanofibers were stable. Our results indicated the crosslinked Tg/gelatin had a relatively high degree of crosslinking of 76% with 13.11 free primary amines rather than the untreated mesh with 54.28 free amines, which result to the formation of stable nanofibers. Their performance as scaffolds to support the growth and differentiation of skin cells will be presented.

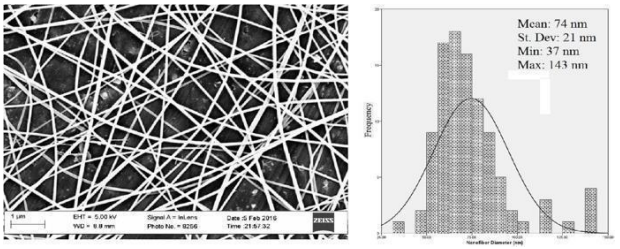

Fig. 1: The SEM images of Tg/gelatin nanofibers and its diameter distribution.

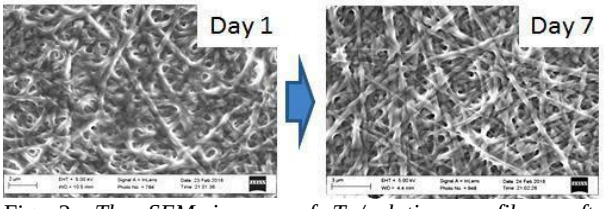

Fig. 2: *The SEM images of Tg/gelatin nanofibers after incubation in water between day 1 and day 7.*

CONCLUSIONS: We developed a stable crosslinked ultrathin Tg/gelatin nanofibrous scaffold. The morphology, fiber diameter and crosslink efficiency of the Tg/gelatin meshes were characterized. The crosslinked nanofiber showed high structural stability after incubation in water up to 7 days. We will explore this highly stable nanofibrous scaffold to support cell attachment, proliferation and subsequent tissue regeneration. The Tg/gelatin nanofiber scaffold combines the advantages of highly flexible, porous and ultrathin properties and is a promising candidate for future development of skin patchs for regenerative medicine.

Alginate hydrogels incorporating cell penetrating peptide-mediated gene delivery for cartilage tissue engineering

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INTRODUCTION: Diverse biomaterials have been proposed to enhance cartilage regeneration, with emerging evidence suggesting that the incorporation of therapeutic proteins or genes may enhance the quality of repair. Biomaterial-based gene delivery is a promising alternative to the use of recombinant proteins for the sustained and local delivery of growth and transcription factors. Cell penetrating peptides (CPPs), which due to their cationic nature are able to electrostatically bind to the phosphate backbone of nucleic acids, have been previously used for effective gene transfection *in vitro* and *in vivo* [1,2]. Despite their high transfection efficiencies and low cytotoxicity, CPPs-mediated gene delivery remains relatively unexplored for tissue engineering applications. The objective of this study was to use a novel amphipathic peptide composed of repeating RALA units [2] to develop gene-activated alginate hydrogels capable of supporting non-viral gene transfer and subsequent cell-mediated protein production to enhance chondrogenic differentiation of adult stem cells.

METHODS: To produce these gene-activated constructs, mesenchymal stem cells (MSCs) and RALA particles complexed with plasmid DNA (pDNA) encoding for the reporter genes green fluorescence protein (pGFP) and luciferase (pLuC) were encapsulated into alginate hydrogels. The hydrogels were allowed to form cylindrical discs through ionic crosslinking. Cellular transfection was assessed by fluorescent imaging of GFP expression. Luciferase expression was analysed over time. Semi-quantitative analysis of Cell viability was performed through live/dead assay.

RESULTS: Fluorescent imaging of the RALA-pGFP containing hydrogels confirmed transfection of the encapsulated MSCs at day 3 (Fig.1.A). Increasing luciferase gene expression was observed in the RALA-pLuc containing hydrogels over 14 days (Fig.1.B), suggesting continuous transfection of the encapsulated MSCs. No effects over cell viability were observed in the RALA-pLuc containing hydrogels at day 1 and 7 (Fig.1.C) in comparison to the untransfected control. In addition, we have also used this system to deliver pDNA encoding for TGF-β3 to MSCs within alginate hydrogels, leading to increases in cartilage-specific matrix production (data not shown).

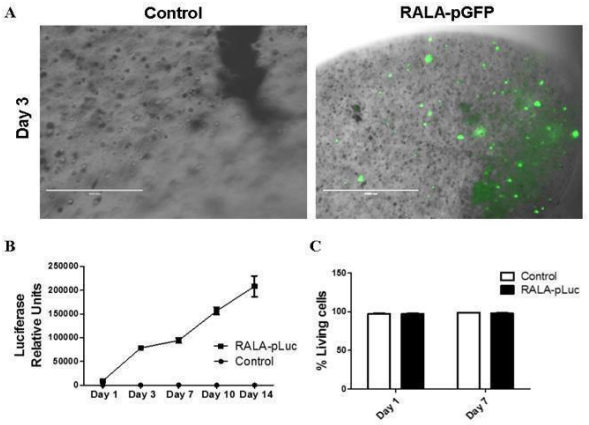

Fig. 1: (A) Fluorescent microscope images of the control and gene-activated hydrogels containing MSCs and RALA-pGFP complexes at day 3. (B) Luciferase expression in the RALA-pGFP containing hydrogels over 14 days. (C) Percentage of living cells in the RALA-pLuc containing gels and the control at day 1 and 7. Scale bar = 1mm.

DISCUSSION & CONCLUSIONS: As shown by fluorescent imaging and luciferase expression analysis, the developed hydrogels successfully supported the transfection of MSCs, facilitating continuous gene delivery over 14 days without cytotoxic effects. Gene delivery of TGF-β3 also resulted in effective expression of the protein and increased cartilaginous matrix deposition. These results show the potential of the incorporation of novel CPPs into 3D matrices for effective gene delivery for tissue engineering applications.

ACKNOWLEDGEMENTS: Funding from Science Foundation Ireland and the European Research Council.

The strength of the protein-material interaction determines cell fate

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INTRODUCTION: Cellular interactions with the ECM occurs in a highly dynamic way in nature [1,2]. Among ECM proteins, fibronectin (FN) is an important regulator of ECM remodeling [3]. Polyethyl acrylate (PEA) is a material able to induce the organization of FN into physiological-like networks, while on polymethyl acrylate (PMA), a material with similar chemistry, the protein adopts a globular conformation. In this work, FN adsorption on PMA, PEA and glass control is characterised in terms of distribution, surface density and strength of the protein-material interaction. Then, the influence on the cell-mediated FN remodeling at the micro and nanoscale in the absence of other serum proteins is investigated.

METHODS: Thin PMA and PEA films were prepared by spin coating of the corresponding polymer solution on glass coverslips. The surface density of fibronectin (FN) adsorbed onto the different substrates was quantified by BCA. FN conformation and distribution on the surfaces was analyzed by atomic force microcopy (AFM). Force spectroscopy measurements were performed in order to determine the strength of the FN-material interaction. MC3T3-E1 cells were seeded on the FN-coated substrates and cell-mediated FN reorganization was assessed via immunostaining for FN, and at the nanoscale via AFM.

RESULTS: Similar FN density is found on PMA and PEA surfaces, while the strength of the protein-material interaction is significantly higher on PEA (Fig.1a,b). In previous works, these materials were found supporting cell adhesion when growing in serum-containing media, with an enhanced cell response -in terms of cell adhesion and differentiation- on PEA surfaces, where FN is organised in physiological-like networks with enhanced biological activity [4]. However, in this study, an *imprint effect* on the adsorbed FN layer was observed, after the first initial adhesion, around cells growing on PEA when incubated in serum free conditions, i.e. with the sole influence of the adsorbed FN layer; which eventually led to cellular apoptosis (Fig.1c), while cells remain alive and with spread morphology on PMA and glass control

DISCUSSION & CONCLUSIONS: The results of this work suggest that the protein-PEA interaction is strong enough to limit the mobility of the protein layer and therefore the ability of cells to reorganise it, which ultimately leads to cell death when culture in absence of other serum proteins. We identify the strength of the protein-material interaction as a crucial parameter to determine cell fate.

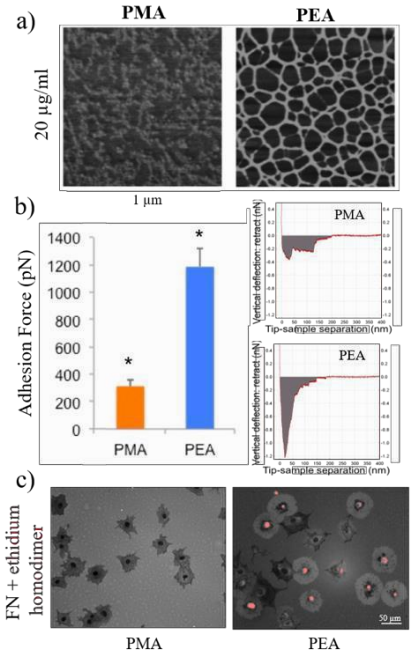

Fig. 1: FN activity at the cell-material interface determines cell viability. a) FN distribution on PMA and PEA surfaces, b) Adhesion force of the FN to the substrates evaluated from Force spectroscopy curves, c) Cell-mediated FN organization and cell viability in serum-free conditions.

ACKNOWLEDGEMENTS: *Support from the IOF-Marie Curie fellowship program (Protdel 331655) and the European Research Council (ERC) through HealInSynergy 306990 is acknowledged.*

Increase of radiopacity of PCL scaffolds for their *in vivo* monitoring using x-rays imaging

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INTRODUCTION: In vitro studies and mathematical modeling are very helpful in predicting behaviour of scaffolds after implantation. However, they cannot substitute in vivo monitoring. For this purpose, scaffolds should be visible in X-rays that enables to observe changes in morphology, detect cracks and defects, etc. [1] Contrast properties of polyesters can be improved by adding some radiopaque fillers. [2] The aim of the study was to investigate the influence of radiopaque filler barium sulfate (BaSO4) on widely used polycaprolactone–hydroxyapatite (PCL-HAp) composites.

METHODS: Materials used in following experiments were prepared by solvent casting technique, dried in air at room temperature and then in a vacuum dryer. The compositions of prepared materials are presented in Table 1. Rods made of pure polymer and composites were fabricated by Fused Deposition Modeling (FDM) method and then incubated in phosphate-buffered saline (PBS).

Table 1. Compositions of investigated materials.

	Composition [% wt]		
Material	PCL	HAp	$BaSO_4$
PCL	100	-	-
PCL0010	90	-	10
PCL1010	80	10	10
PCL2010	70	20	10
PCL3010	60	30	10
PCL4010	50	40	10

Calculations of mass loss and water absorption, micro computed tomography and cytotoxicity assay were used to characterise materials during degradation.

RESULTS: The obtained composite rods had different image intensity. BaSO4 caused strong increase of visibility using X-rays but not increase of degradation rate of polymeric matrix. Additionally, higher content of HAp caused higher degradation rate and also increase of radiopaque properties of composite. There was no cytotoxic effect of using a BaSO4 as a filler in contact with murine fibroblast cell line L929.

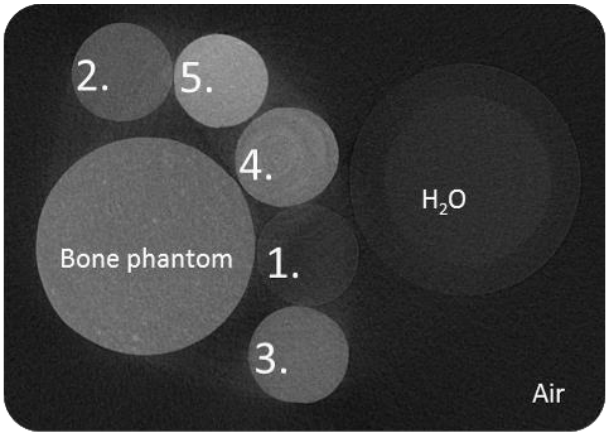

Fig. 1: CT scanned image: 1 – PCL, 2 – PCL0010, 3 – PCL1010, 4 – PCL2010, 5 – PCL4010.

DISCUSSION & CONCLUSIONS: The concept of using a BaSO4 as a filler in scaffold material allows for a non-invasive approach for in vivo imaging. Absence of serious hazards in presence of BaSO4 was also observed in other works. [3,4] The results confirmed there is a possibility to obtain non-cytotoxic scaffold composition which enables monitoring of its stability after implantation in surrounding soft tissue, even in bone tissue.

ACKNOWLEDGEMENTS: This study was supported by the National Center for Research and Developments in Poland (STRATEGMED1/ 233624/4/NCBR/2014, project MENTOREYE)

The role of charged synthetic homo-polymer coated surfaces on rat bone marrow derived mesenchymal stem cells; interaction of the cells with the charged surfaces

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INTRODUCTION: The reconstruction of tissues in-vitro is a big challenge in regenerative medicine. Tissue engineering is emerging as significant potential alternative solutions by using three-dimensional structured biomaterials. Biomaterials do not serve only as a mechanical support, but they also have the influence on the cell attachment and the proliferation [1]. Generally, stem cells are considered as a cell source in regenerative medicine and tissue engineering because of their potential to differentiate into desired tissue cells. The biophysical, mechanical and biochemical properties of the microenvironment influence the stem cells. Therefore surface modification is important for the stem cell fate with respect to cell maintenance, proliferation, self-renewal and differentiation [2]. In this study we used synthetic charged homo-polymers to promote cell adhesion by the electrostatic attraction between the positive charge of the polymer coated surface and negatively charged cells, and focused on the effect of charged synthetic homo-polymers coated surfaces on stem cell survival and their differentiation direction.

METHODS: Rat bone marrow stem cells were cultured (20,000 cells/well) on commercially available synthetic homo-polymers (0.1 mg/ml) for 48 hrs. Collagen type 1 was used as positive and uncoated glass surface is used as negative control. Glass surface was coated by poly-L-lysine, poly-D-lysine, poly-L-proline, poly-L-histidine, poly-L-leucine, poly-L-alanine, and poly-L-glutamate. The adhesion and proliferation of the cells were analyzed by WST-1. Released lactate dehydrogenase from lysed cells was measured by LDH assay. For the cell viability, FDA/PI staining, and for the cell morphology Phalloidin-FITC staining was used. The altered differentiation potential of the cells on each surfaces were compared.

RESULTS: By using various charged homo-polymers different results were obtained on cell adhesion, proliferation, toxicity, viability and cell morphology. We have found that poly-L-lysine, poly-D-lysine and poly-L-glutamate coated surfaces showed lowest support to the cell attachment and the viability of stem cells. The adhesion rate of the cells cultured on the surfaces coated with these three polymers was also very low compared to other surfaces, and their LDH values in the culture were moderately increased, but not significantly toxic compared to the cells on the collagen coated surface. The changes in the integrity of the actin microfilaments in the cytoplasm were only observed in the cells on the poly-L-lysine and poly-D-lysine polymers. The viability was estimated highest in the poly-L-histidine and poly-L-proline groups. The differentiation capacities of the cells on the different polymers showed significant differences. Poly-L-alanine, poly-L-leucine and poly-L-histidine support the adipogenic differentiation considerably.

DISCUSSION & CONCLUSIONS: The results showed that the homo-polymers based on the amino acids with cyclic structure, L-histidine and L-proline, are the prominent materials for the cell culture of the mesenchymal stem cells dissimilar to poly-L, D-lysine or poly-L-glutamate. In the case of the differentiation potency into adipocytes, poly-L-histidine is still an outstanding coating material compared to the other used materials. In the case of the polymeric forms of the aliphatic amino acids, poly-L-alanine and poly-L-leucine, the adipogenic differentiation was favored. With respect to the effects on the proliferation and differentiation characteristics of the mesenchymal stem cells, poly-L-histidine was a highly supportive coating material as well as type-1 collagen.

Biological evaluation of degradable phosphate-based glass microspheres for stem cell therapy

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INTRODUCTION: Bioactive glasses have been one of the major commercial successes in the dental and bone implant industry in the last few decades[1]. With better understanding of mechanisms leading to bone formation in human body and development of phosphate-based glasses (PBG), which offer tuneable chemistry and degradation rates depending upon the end application, the advent of next generation of bone implant materials is on the horizon [2, 3]. In this study, degradable PBG microspheres (MS) have been compared to non-degradable borosilicate MS (BSG) and tested for cell growth and osteogenic differentiation for end application of bone marrow derived mesenchymal stem cell therapy for bone regeneration.

METHODS: Bone marrow derived human mesenchymal stem cells (hMSC) were seeded at 2,500 cells/cm^2, on 100µm-sized MS (see Fig. 1 for formulations) and allowed to grow for 42 days in culture medium. Cell growth was assessed by DNA quantification. For the degradation study, MS were incubated in medium only. Scanning electron microscopy (SEM) was performed to assess the number of MS with damage on their surface at each time point. To study the effect of the MS degradation products, cells were grown in medium conditioned with MS for 25 days. For osteogenic differentiation, cells were seeded on MS and Alkaline Phosphatase (ALP) activity and osteocalcin (OCN) expression were investigated over after 3 weeks culture period.

RESULTS: The degradation study over 42 days showed that there were significantly higher number of damaged P40 MS compared to BSG and P45 MS. DNA measurements showed that there were more cells on PBG MS after 14 days in culture however, by day 31 there were significantly lower number of cells on P40 MS compared to BSG and P45 MS (Fig.1). Moreover, there was no effect of MS degradation products on cell growth compared to control media with no MS (Fig. 2). ALP activity was significantly higher for cells on P40 MS compared to other conditions after 7 days and positive OCN staining was detected in BSG and P40 samples but not in P45 samples (Fig. 3).

DISCUSSION & CONCLUSIONS: The results indicate that surface damage on P40 MS may negatively affect the cell growth, while the formulation of P40 promoted ALP activity at day 7 compared to slower degrading P45 [3] and the non-degradable BSG. These results demonstrate the potential of degradable PBG MS for MSC culture and the importance of their chemical composition for bone repair applications.

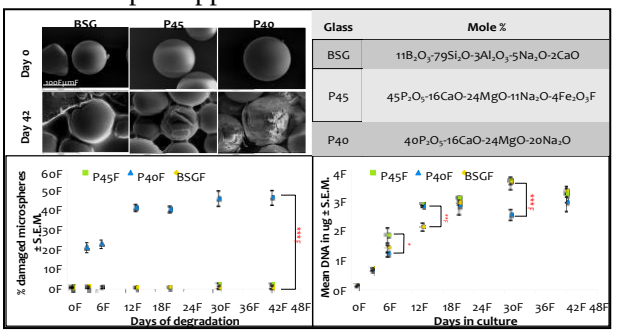

*Fig.1: Degradation behaviour and hMSC growth on MS over 42 days in culture medium. ***p<0.00001,**p<0.001, *p<0.05.*

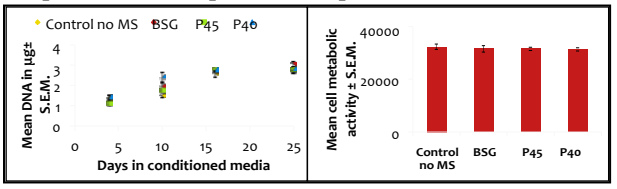

Fig.2: Cell growth over 25 days and cell metabolic activity on day 25 in medium conditioned with MS.

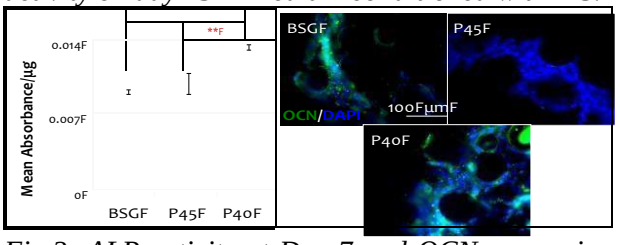

*Fig.3: ALP activity at Day 7 and OCN expression on Day 21 of culture. ***p<0.00001,**p<0.001.*

ACKNOWLEDGEMENTS: The authors are thankful for support from the University of Nottingham's Vice Chancellor's Scholarship for Excellence in Research for this study.

Capture of outgrowth endothelial cells by anti-CD146 antibody immobilization on silicone nanofilament-coated stent

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[2]*Dept. of Biomedical Engineering, Korea University of Science and Technology, Daejeon, Korea.*
[3]*Dept. of Bioengineering, Hanyang Univ., Seoul, Korea*

INTRODUCTION: Restoration of damaged endothelium might be a fascinating way to prevent late stent thrombosis and restenosis. Some reports demonstrate that outgrowth endothelial cells (OECs) can differentiate to endothelial cell directly and then facilitate re-endothelialization. In this study, we focused on CD146 as a target surface biomarker for OEC capture. CD146 is a transmembrane glycoprotein that is constitutively expressed in the whole human endothelium [1]. Silicone nanofilament (SiNf) was modified on metal surface for increasing the amount of antibody immobilized on surface [2]. We expected that anti-CD146 antibody (Ab)-immobilized stent can effectively capture circulating OECs and may restore a damaged endothelium.

METHODS: SiNf surface was prepared using a trichloromethylsilane (TCMS) and modified by 3-aminopropyl triethoxysilane (APTES) for introducing the amine group. Morphology of SiNf surface was confirmed by SEM. SiNf, anti-CD34 Ab (control), and anti-CD146 Ab-immobilized surfaces were evaluated by immunofluorescence staining, SEM, ATR-FTIR, water contact angle, and XPS. The effect of Anti-CD146 Ab-immobilized SiNf surface on cell capture was evaluated by OEC capture assay.

RESULTS: SiNf treatment changed a comparatively hydrophilic Co-Cr surface to a hydrophobic one and the SEM image showed tangled nanofilaments (Fig. 1). Fluorescence staining demonstrates that anti-CD146 Ab was homogeneously covered on the surface. Capture assay exhibited that OECs were more significantly captured on anti-CD146 Ab-immobilized surface than bare surface and anti-CD34 Ab-immobilized one (Fig. 2). Conversely, monocytes were captured non-specifically on all surfaces. This result suggested that anti-CD146 Ab-immobilized SiNf surface provided the specific binding to OEC and larger surface area of SiNf.

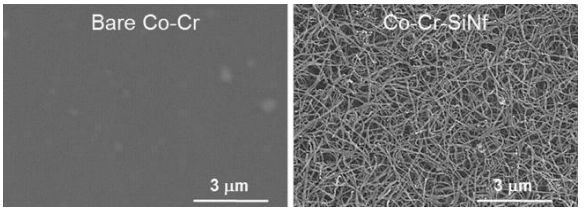

Fig. 1: SEM images of Co-Cr and SiNf coated Co-Cr surfaces.

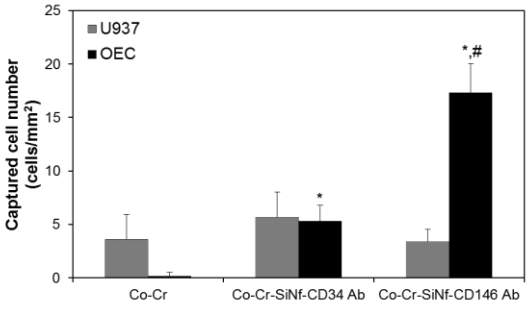

*Fig. 2: The number of cells captured on Co-Cr, CD34, or CD146 immobilized SiNf surfaces in circulation system. * or #: p<0.05 (* vs. Co-Cr, # vs. Co-Cr-SiNf-CD34 Ab).*

DISCUSSION & CONCLUSIONS: Structure of SiNf and immobilization of anti-CD146 Ab provided an effective surface for capturing specifically OECs. Further studies are ongoing to demonstrate re-endothelialization by captured OEC *in vitro* and *in vivo*. This approach will become a promising way for preventing existing stents from restenosis and thrombosis by accelerating re-endothelialization after stenting.

2

ACKNOWLEDGEMENT: This research was supported by the Bio & Medical Technology Development Program (NRF-2014M3A9D3033887) of the National Research Foundation (NRF) funded by the Ministry of Science, ICT & Future Planning, Korea.

Potential therapeutic applications of functional PLGA/ECM/$Mg(OH)_2$ scaffolds for kidney tissue regeneration

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INTRODUCTION: Recently, biodegradable scaffolds to help repairing injured tissue are emerging as a promising approach in the field of renal regeneration. Poly(lactide-co-glycolide) (PLGA) is a useful biomedical material, but its insufficient biocompatibility and acidic degradation products can reduce the cell function and physiological pH to cause inflammatory response in the surrounding tissue. In this study, renal extracellular matrix (ECM) and magnesium hydroxide ($Mg(OH)_2$) were employed to improve the biocompatibility of PLGA scaffold and to neutralize the acidic environment, respectively. The physico-mechanical properties and effect on kidney tissue regeneration of ECM and $Mg(OH)_2$ incorporated PLGA (PLGA/ECM/$Mg(OH)_2$) scaffolds were investigated.

METHODS: The PLGA/ECM/$Mg(OH)_2$ scaffolds were prepared by ice particle leaching method [1]. The structures, morphology, pH change, and thermal and mechanical properties of the scaffolds were characterized using various analytic tools. The cell adhesion and proliferation of human renal cortical epithelial cells on the scaffolds were studied. To evaluate the *in vivo* toxicity and renal tissue regeneration, the PLGA/ECM/$Mg(OH)_2$ scaffolds were implanted into nephrectomized area of mouse. The retrieved renal constructs were conformed using polymerase chain reaction, histological and immunohistochemical analysis.

RESULTS: 3D porous PLGA/ECM/$Mg(OH)_2$ scaffolds with different ECM concentrations (0.2, 1, 5, and 10%) and $Mg(OH)_2$ 15% were successfully fabricated. The scaffolds have interconnected pore structure with porosity of 80% and pore sizes up to 200 μm. In the degradation-dependent pH change study, the pH of PLGA control decreased to 2.7, whereas the PLGA/ECM/$Mg(OH)_2$ composites were retained around pH 7. The PLGA/$Mg(OH)_2$ scaffold containing 10% of ECM exhibited excellent cell proliferation as compared to PLGA control. Following murine *in vivo* implantation, the PLGA/ECM/$Mg(OH)_2$ scaffolds showed the successful reconstruction of glomerulus and very low inflammatory response, which means that this scaffold was enhanced biocompatibility, has effective neutralizing ability of acidic environment, and thus supports glomeruli-like structure formation and neovascularity.

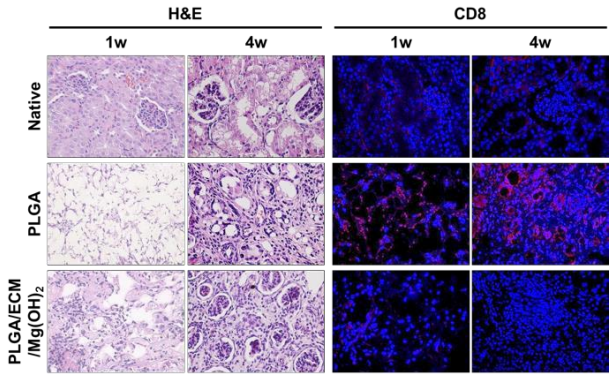

Fig. 1: Histological analysis of ECM and $Mg(OH)_2$ particles incorporated PLGA scaffold in vivo.

DISCUSSION & CONCLUSIONS: The obtained results demonstrated that incorporated ECM and $Mg(OH)_2$ into the PLGA scaffold could improve the biocompatibility, reduce inflammatory response, and ultimately enhance renal reconstruction. The functional PLGA/ECM/$Mg(OH)_2$ scaffolds could be one of the promising materials for biomedical applications including tissue engineered scaffolds and biodegradable implants.

ACKNOWLEDGEMENTS: This research was supported by the Bio & Medical Technology Development Program (2014M3A9D3033887) through the NRF funded by the Ministry of Science, ICT & Future Planning, South Korea.

A biomaterial approach for maintaining glide at tendon tissue interfaces

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INTRODUCTION: Acute tendon injuries are common and typically occur in the young, independent workforce. Tendon division following injury is reliant on surgical repair for restoration of function. Severely compromised outcomes with scar formation impeding tendon glide (adhesions, 4%) or rupture (4%) occur, however, most patients experience some functional impairement[1]. The flexor tendons of the hand are the typical site of injury, where any functional reduction can have a devastating effect on both the individual and socio-economically as a member of the workforce.

A novel scaffold, rationally designed to optimise outcomes following primary tendon repair, is presented. A spatially discrete antifouling surface controls cell and protein binding that can lead to scarring adhesions onto the tendon gliding surface. An opposing cell adhesive surface is prepared that may be further modified to support tendon healing. The additional ability to produce a modular design allows for the facile spatial localisation of a variety of interchangeable functional groups and gives a toolbox of options for adapting this design to other applications in the future.

METHODS Poly-ε-caprolactone, (PCL) was end-functionalised with a polymerization initiating group or a cyclical cell-adhesion peptide sequence Arg-Gly-Asp-Ser (cRGDS). These were then processed using a sequential electrospinning technique to produce a porous scaffold. Following which, a versatile controlled free radical polymerization technique was used to graft a poly(ethylene glycol) methyl ether methacrylate (pOEGMA) polymer brush from fibre surfaces in spatially defined locations to give an antifouling coating. Using this approach, a bilayer scaffold is produced with an antifouling, cell resistant surface, and an opposing cell adhesive surface. Functionalization of the polymer brush with biotinylated monomers is further achieved allowing for the addition of further functionality through the streptavidin-biotin interaction. Materials characterisation including XPS, fluorescence microscopy, cell and protein adhesion assays confirm the presence and function of the opposing surface functionalities.

RESULTS: Reproducible polymerization was confirmed and characterized. Fluorescent labelling of histological sections, water contact angle measurement and XPS confirmed pOEGMA functionalization scaffolds. A high performance antifouling effect was seen with biomolecules and tenocytes at 7 days in culture.

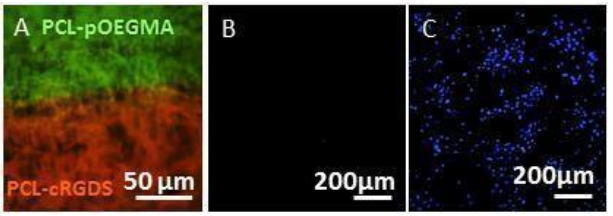

Fig. 1: The bilayer scaffold, adapted from[2]. (A) overlaid fluorescence microscopy demonstrating spatial control of antifouling (pOEGMA, green) and cell binding (cRGDS, red) groups. (B-C) Tenocyte adhesion to opposing scaffold surfaces at 7 days shown with DAPI (blue, cell nuclei) of the pOEGMA surface (B) and cRGDS surface (C).

DISCUSSION & CONCLUSIONS: Gliding tissues, such as tendons, are vulnerable to tethering scarring leading to compromised function. This scaffold could offer protection from these scars through the control of cellular migration. Furthermore, the versatility of the design and cell adhesive inner surface may improve tendon healing. Whilst not yet tested in an *in vivo* tendon model, this scaffold shows promise for tendon repair and reconstruction.

ACKNOWLEDGEMENTS: RHH gratefully acknowledges the Blond Research Fellowship from the Royal College of Surgeons of England and the Rosetrees Trust.

Investigation of cell adhesion mechanism by the distribution of adsorbed proteins on nano-scale phase-separated structures

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INTRODUCTION: In order to create suitable biocompatible materials for various tissue engineering applications, it is important to be able to understand protein adsorption and cell adhesion behaviours on the material surfaces. It is known that the nanoscale distribution of adhesive ligands affects cell adhesion behaviours [1]. However, how nanoscale distribution of adsorbed proteins affect cell adhesion behaviours is still unclear. Therefore, in this study, we investigate the effect of the distribution of adsorbed proteins by the phase reversal of amphiphilic block copolymers composed of protein-adsorptive poly(3-methacryloyloxy propyltris(trimethylsilyloxy) silane) (PMPTSSi) and non-adsorptive poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) on cell adhesion behaviours.

METHODS: Amphiphilic di- and tri- block copolymers with the same chemical compositions (molar ratio: MPC/MPTSSi = 7/3) were fabricated by the reversible addition-fragmentation chain transfer polymerization. The di-block copolymer was coated using the good solvent for PMPTSSi (BP1) and the tri-block copolymer was coated using the good solvent for PMPC (BP2). The nanostructures of BP1 and BP2 were observed by transmission electron microscopy (TEM). Then, the amount of adsorbed fibronectins (FNs) on BP1 and BP2 were measured. Finally, L929 fibroblast cells using the serum-free media were seeded on BP1- and BP2-coated surfaces immersed in the FN solution in advance. In 2 days after seeded, the cell adhesion behaviours were observed.

RESULTS: The nanodomain structures of phase-separated block copolymers were successfully confirmed using TEM. The BP1 surface had hydrophilic PMPC dot-like domains (23 ± 4 nm) and the BP2 surface had hydrophobic PMPTSSi dot-like domains (25 ± 6 nm) (Fig. 1a,b). From the protein adsorption test, it was found that the amounts of adsorbed FNs on BP1 and BP2 were not significantly different. Moreover, the density of adsorbed FNs on hydrophobic areas were calculated using the ratio of the hydrophobic areas from TEM images because the distributions of adsorbed FNs were expected to be like Fig. 1c,d. The FN density on BP1 was almost the same as that on BP2 (BP1: 580 ng/cm^2, BP2: 570 ng/cm^2). Therefore, cell adhesion behaviours were expected to be similar, however, the result was different. The fibroblast cells could adhere and spread on BP1 while they could not adhere on BP2 (Fig. 1e,f).

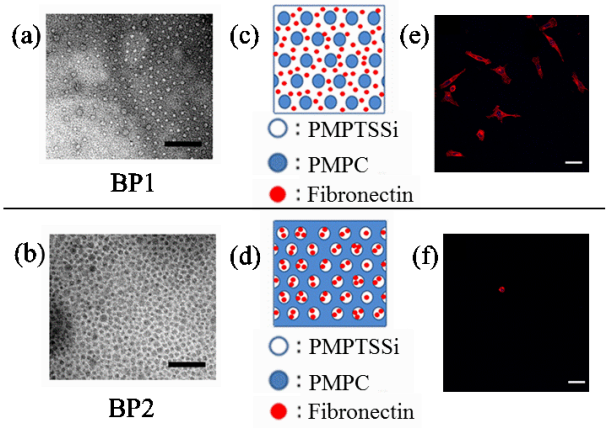

Fig. 1. The top row is the data of BP1, and the bottom row is the data of BP2. (a, b) TEM images stained with osmium solution. The black areas represent PMPTSSi, and the white areas represent PMPC. Scale bar = 200 nm. (c, d) The schematics of presumed protein adsorption on each surface. (e, d) Fluorescence microscope images of L929. Actin stress fibers were stained with Alexa Fluor 594 phalloidin. Scale bar = 20 μm.

DISCUSSION & CONCLUSION: The difference of cell adhesion behaviours between BP1 and BP2 may be caused by the distributions of adsorbed proteins. It was assumed that the connective hydrophobic areas on BP1 promoted cell adhesion and spreading. Thus, it was suggested that in addition to the density of adsorbed proteins, the distribution also greatly affects cell adhesion [2].

Green light initiated collagen hydrogels as cell-hydrogel constructs in regenerative medicine

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INTRODUCTION: The microenvironment design can possess features that induce appropriate gene expression and thereby direct cell fate as a result of cell-matrix interactions.[1] Collagen hydrogels can be compared to the natural hydrated extracellular matrix with a microenvironment largely dependent on the degree of functionalization, cross-linker type, chemical net-point reactivity, cross-link density and porosity.[2] Adjustment and regulation of these physical factors can result in hydrogels with tunable mechanical and biological properties with complex microstructures.[3] The aim is to create a scaffold capable of mimicking the mechanical and biological properties of pre-mineralized alveolar bone for use as a tissue engineering construct.

METHODS: A collagen (type I)- poly(ethylene glycol) diacrylate (PEGDA) precursor solution was prepared and hydrogels were formed using green and UV light. Green light-induced gelation was performed using eosin Y as the photo-catalyst and triethanolamine (TEOA) as a sacrificial reducing agent (539 nm). This was compared to UV light-induced gelation with Irgacure 2959 (365 nm) as the initiator. The physical and mechanical properties of the hydrogels were investigated by compression, swelling and degradation tests. Cell viability with response to different wavelengths of light was examined by comparing cells exposed to either green or UV (365 nm) light for 20 minutes using an ATP assay. The degradation experiments were performed in vitro under physiological conditions (T = 37°C, pH = 7.4), by measuring their residual weight. ATP assays were used to examine cell viability in gels after 1, 3 and 7 days and LIVE/DEAD stains with confocal microscopy examined cell encapsulation and cell spreading with dental pulp stem cells (DPSCs).

RESULTS: Cell viability in response to UV light resulted in a third less viable cells than when exposed to green light, fig. 1. The mechanical and physical properties were varied by changing the initiator type and the concentration of the prepolymer solution, table 1.

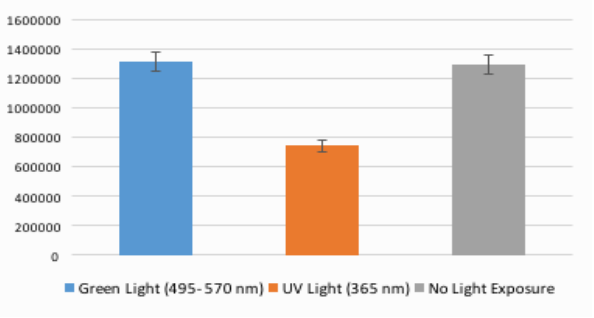

Fig. 1: ATP assay to demonstrate cell viability after 20 minutes' exposure to: UV light (365 nm), green light (496 – 570 nm) and no light.

Hydrogels were prepared using collagen type I, PEGDA and eosin Y/ TEOA or I2959 to produce gels of varied compressive stiffness (Table 1).

Table 1. Mechanical and physical properties

Initiator	Collagen: PEGdA (%)	Swelling Ratio (%)	Young's modulus (kPa)
Eosin Y/ TEOA	95:5	587	11.1
	92:8	445	17.0
	90:10	429	73.3
Irgacure 2959	95:5	646	44.4
	92:8	564	225
	90:10	479	277

DISCUSSION & CONCLUSIONS: Hydrogels formed through green light photopolymerization have great potential for cell encapsulation and 3D bioprinting, with increased cell viability compared to using traditional UV light. Further work needs to assess the gene expression in response to gelling.

Photosensitive gelatine-methacrylamide as engineered extracellular matrix for three-dimensional cell culture

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INTRODUCTION: In order to meet the need to repair or replace damaged tissue, the fabrication of functional tissues remains a major aim for tissue engineering and regenerative medicine. Methacrylamide-modified gelatin (gelMOD) [1], is an enzymatically degradable hydrogel derived from collagen. Due to the introduced methacrylamide groups it becomes photocrosslinkable with tunable mechanical properties. As it is already well known that cells respond to their environment [2], gelMOD might represent a good material to study the influence of substrate properties on cells.

METHODS: Human bone-marrow derived mesenchymal stem cells (hMSC) were encapsulated in 10 wt% of gelMOD with 0.6 mM of photoinitiator LiTPO-L. The cells were resuspended in the gelMOD solution at a cell density of 10^7 cells/mL. The hydrogel was then polymerized for 10 minutes using UV-A light (365 nm, 4 mW/cm^2). Afterwards, the cells were cultured in basal or osteogenic medium for 17 days. Metabolic activity and viability was monitored using a Presto Blue assay and live-dead staining.

RESULTS: The cells survived the process of encapsulation and remained viable after 17 days in culture. There was no significant difference in metabolic activity between the cells cultured in basal- and osteogenic medium. Furthermore, no significant difference in metabolic activity was present between day 0 and day 17. Live-dead staining indicated a round morphology of the cells after 0 and 7 days, but a more stretched appearance was present after 17 days. Moreover, the hydrogel pellets, which were stimulated with osteogenic medium were milky in contrast to the pellets cultured in basal medium. This indicates a strong presence of calcium deposits in the stimulated samples. Gene expression analysis will support our data to characterize the influence of gel properties on the cells.

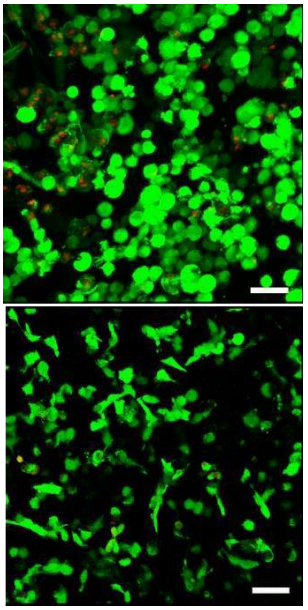

Fig. 1: live dead staining of hMSC cells inside the hydrogel: the day after encapsulation (left), after 17 days in basal medium (right); scale bar: 50 µm

DISCUSSION & CONCLUSIONS: As the cells showed consistent metabolic activity, the material is suitable for long-time cell culture for hMSC cells. As stretching of the cells and mineralization occurred, gelMOD might also be a good material to mimic the microenvironment of bone.

By varying the degree of crosslinking of gelMOD hydrogel, the mechanical properties can easily be tuned. As a consequence, gelMOD is a promising material for studying the effect of substrate stiffness on stem cell fate.

ACKNOWLEDGMENTS: The authors acknowledge the financial support of the European Research Council (Starting Grant-307701, A.O.). We would like to acknowledge support of Dr. Irina Druzhinina (Institute of Chemical Engineering, Technical University Vienna).

DISCLOSURES: We declare no potential conflicts of interest relevant to this work.

Influence of cell culture and detachment conditions on cell sheet release from thermoresponsive surfaces

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INTRODUCTION: Thermoresponsive surfaces allow for the detachment of confluent cell sheets by a temperature drop without the use of enzymatic or mechanical treatments. They are based on thermoresponsive polymers that switch at their lower critical solution temperature (LCST) between a rather hydrophobic state allowing for cell adhesion and a more hydrophilic state allowing cell detachment (fig. 1). Produced cell sheets can be arranged into 3D tissues. Besides the widely used polymer poly(N-isopropylacrylamide) (pNIPAM, LCST: 32 °C), further polymers are developed to enable a quick and reliable sheet detachment [1]. However, not only the polymer itself but also the culture and detachment conditions might influence detachment efficiency. Therefore, our aim was to optimize cell sheet release via variation of cell culture and detachment parameters.

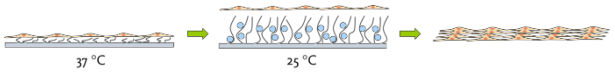

Fig. 1: Scheme of cell sheet engineering based on thermoresponsive polymers

METHODS: Surfaces of pNIPAM and a glycerol-based linear copolymer of glycidyl methyl ether and glycidyl ethyl ether (PG, 1:5) were prepared by the previously published spin-coating procedure [2], a relatively simple method compared to the usually used covalent coating procedures. Surfaces were characterized via contact angle and used for cell culture and detachment assays.

RESULTS: By contact angle measurements above and below the switching temperature, the switchability from a dehydrated, collapsed polymer that allows for cell adhesion to a more hydrophilic polymer enabling cell detachment was shown for pNIPAM. However, PG showed only a weak contact angle change. Cell sheets of NIH/3T3 cells on pNIPAM or PG were detached under different conditions. Detachment was more reliable and faster when cells were cultured with 1.0 g/l instead of 4.5 g/l glucose; further reduction of the detachment time could be achieved with a second washing step with PBS (37 °C) 10 minutes after detachment initiation with PBS (RT). Under these conditions a reliable and fast cell sheet detachment could be achieved on both, pNIPAM and PG surfaces.

Cell sheet detachment assays were also performed with human dermal fibroblasts since they are more relevant for tissue engineering. Complete cell sheet detachment could be achieved exclusively with the optimized conditions of the NIH/3T3 cells (fig. 2). Detachment time was multiple times longer than for the NIH/3T3 cells (~ 80 min).

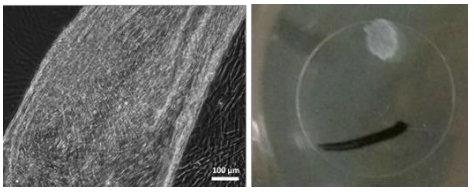

Fig. 2: Cell sheet detachment of human dermal fibroblasts (p3) from spin-coated pNIPAM

DISCUSSION & CONCLUSIONS: Culture and detachment conditions have a strong impact on cell sheet detachment from thermoresponsive surfaces. Evaluated conditions can now be transferred to surfaces with covalently linked thermoresponsive polymers. Furthermore, by the investigation of the cellular processes that are affected by the different conditions, cell release could be influenced more specifically. Detachment time strongly differed between NIH/3T3 cell line and primary fibroblasts. However, with the NIH/3T3 optimized method also primary fibroblast cell sheets could be produced but detachment time still needs to be optimized.

2

ACKNOWLEDGEMENTS: This project is funded by the German Federal Ministry of Education and Research in the NanoMatFutur programme.

The effect of fish collagen and fabrication of scaffold for hard tissue regeneration

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INTRODUCTION: In tissue engineering, polymeric and/or ceramic scaffolds have been developed to regenerate various tissues [1, 2]. When fabricate scaffolds, factors like biocompatibility, growth factor, and porosity are important since they affect various cellular activities. Among various polymers, collagen is a biocompatible material which is main component of ECM and has high initial cell attachment. However, conventional collagen from porcine or bovine has some disadvantages such as potential of disease transmission for human. In this study, to overcome those disadvantages, we extracted collagen from fish skin and fabricated scaffolds by fish collagen, and compare efficiency with porcine/bovine collagen. On the surface of PCL scaffold, we coated phlorotannin as a growth factor and collagen/alginate mixture to control the release of phlorotannin. Using this scaffold, we cultured osteoblast (MG63) cell for 14 days, and measured physical properties such as water absorption, water contact angle, and tensile strength. Also, we examined biological properties such as cellular morphology, differentiation, andviability.

METHODS: Preparation of materials: 2wt% of Type-I collagen (density = 1.3 g cm^{-1}, Matrixen-PSP; Bioland, Cheonan City, South Korea) from porcine tendon and 2wt% of extracted fish collagen from flatfish were mixed with alginate (FMC BioPolymer, Philadelphia, USA) solution. The ratio of collagen and alginate, fish collagen and alginate was 1:1. Phlorotannin powder was dissolved at 99% EtOH as 5 wt%. Osteoblast-like cells (MG63, CRL-1427; ATCC, Manassas, VA, USA) were used to assess the cell activities of the composite scaffold.

RESULTS: We fabricated PCL based scaffolds with four different fabricating conditions: (1) phlorotannin coated scaffold, (2) collagen/alginate coated scaffold, (3) phlorotannin/collagen/alginate coated scaffold, and (4) phlorotannin/fish collagen/alginate coated scaffold. Phlorotannin/collagen/alginate coated scaffolds have higher cell activity then pure phlorotannin or collagen/alginate coated scaffolds. As a result, MG63 cultured on fish collagen scaffolds showed similar cell activities as those cultured on conventional collagen coated scaffolds.

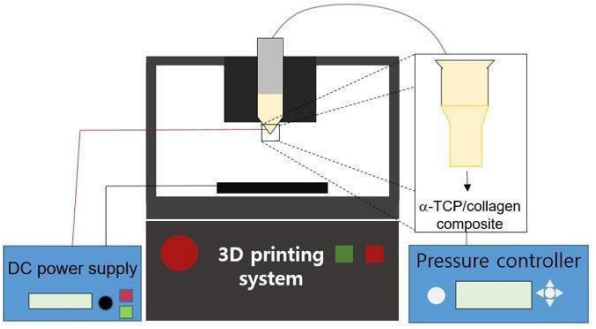

Fig. 1: Schematic of fabrication process of phlorotannin/collagen/alginate scaffolds.

DISCUSSION & CONCLUSIONS: We fabricated phlorotannin/fish collagen/alginate coated PCL scaffolds, and its influence on cell activities was as effective as conventional collagen coated scaffolds. Also, we were able to regulate the release of growth factor. We suggest that fish collagen can be applied to tissue engineering field including hard tissues regeneration.

ACKNOWLEDGEMENTS: This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and future Planning (NRF- 2015R1A2A1A15055305) and also was partially supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI15C3000).

Non-cartilage decellularized matrix in 3D-bioprinted hybrid constructs for cartilage tissue engineering

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INTRODUCTION: Decellularized extracellular matrix (DCM), which has intrinsic bioinductive constituents and low immunogenicity, has received increased attention as a scaffold material for tissue engineering (TE). Cartilage DCM, which is relatively hard to decellularize, has some biological advantages for cartilage TE [1], but it has not been tested whether the DCM has to be tissue-specific for cartilage regeneration. The most studied DCM sources, i.e. small intestine submocusa (SIS) and urinary bladder matrix (UBM), are readily available in large volumes and show promising bioinductivity and low host response for regenerating a variety of tissues [2]. Here, we test the hypothesis that non-cartilage DCM biomaterials can support chondrocyte differentiation in 3D-bioprinted hybrid constructs for cartilage TE.

METHODS: Our hybrid 3D-bioprinting method fabricates tissue constructs made of polycaprolactone (PCL) for mechanical support and cell-impregnated hydrogel for biological properties [3]. Primary chondrocytes are isolated from chick embryos, expanded in 2D culture for a week, and then mixed with hydrogel solutions prior to bioprinting. We biofabricate 3D hybrid constructs of five different cell-impregnated hydrogels, including SIS, UBM, alginate, and SIS-alginate and UBM-alginate composites. We evaluate biological and structural features, including cell viability, proliferation, chondrogenic differentiation, and scaffold integrity and degradation, during 28 days of *in vitro* culture

RESULTS: Our hybrid 3D-bioprinting method showed high cell viability and secretion of Alcian blue positive matrix in the cell-alginate hybrid constructs (Fig. 1). In non-cartilage DCM hydrogel, primary chondrocytes also showed good cell viability, increased cell number, and secretion of abundant Alcian blue positive matrix (Fig. 2).

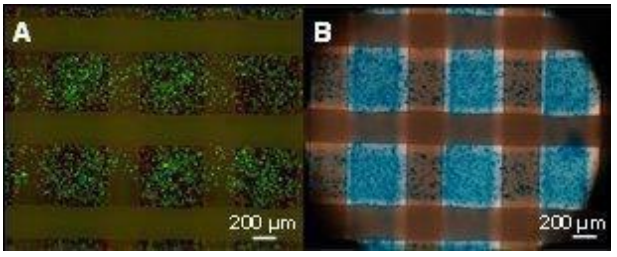

Fig. 1: 3D-bioprinted alginate-PCL hybrid constructs with impregnated chondrocytes stained with (A) green(live)/red(dead) for cell viability and (B) Alcian blue for cartilage matrix production after 7 days of culture.

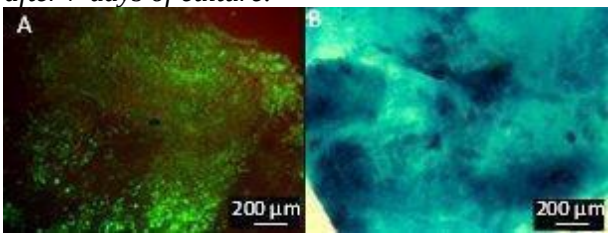

Fig. 2: Primary chondrocytes embedded in SIS hydrogel show (A) good cell viability and (B) Alcian blue-positive matrix secretion after 14 days of culture.

DISCUSSION & CONCLUSIONS: Biological performance of chondrocytes in hybrid constructs indicates the success of hybrid 3D-bioprinting technique for cartilage TE. Our preliminary results from non-cartilage DCM hydrogel suggested that non-tissue-specific DCM biomaterials can be used for cartilage TE, and we quantitate how the biological performance of DCM compares to alginate. We also predict that structural properties of hybrid constructs will be improved with alginate-DCM composite hydrogels.

ACKNOWLEDGEMENTS: Project funded by Canadian federal (CIHR and NSERC) and provincial (SHRF) grants.

Fabrication of mechanically improved PCL/M13-bacteriophage-conjugated alginate scaffold for hard tissue engineering

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INTRODUCTION: Alginate is commonly used biocompatible polysaccharide which has good biocompatibility, hydrophilicity, non-antigenicity, easy/rapid gelation using calcium ions, and controllable biodegradability.[1] However, as reported by several groups, it has no cell-activating sites and has low mechanical properties for hard tissue regeneration. To overcome these issues, chemically conjugated M13 bacteriophage/alginate was used as a bioactive component. Also to enhance mechanical properties, we use core/shell nozzle to set PCL in the core region.

METHODS:

Prepare for conjugated alginate: Alginate was dissolved in deionised water at a concentration of 2%. EDC (1-ethyl-3 3-dimethylaminopropyl carbodiimide) was added to the solution at a molar ratio of 1:20 to uronic acid monomers of alginate. NHS (N-Hydroxysuccinimide) was added at a molar ratio of 1:2 as a coreactant to stabilise the cross-linking process and incubated for 12 h. Phage solution at a concentration of 4 mg/mL was mixed at a 1:9 ratio with 3.5wt% alginate solution. After conjugation, alginate solution was purified through extensive dialysis against three shifts of distilled water and three shifts of 50 mM NaCl for three days. The phage-conjugated alginate was freeze dried and stored in a deep freeze. The RGD peptide also conjugated with the same protocol.

Scaffold fabrication: To generate the core (PCL)–shell (alginate/phage) struts, we use a core–shell nozzle. To set the PCL in the core region, the PCL was extruded at the core nozzle from a heat barrel, and for the shell region, the biocompatible material was extruded from the shell nozzle, and the micro-sized struts were immediately frozen on the cryogenic plate.

RESULTS: MG63 cells were used to evaluate cellular behaviour on four types of 3D structured scaffold which is alginate (Alg), RGD peptide conjugated alginate (RGD-A), physically mixed phage/alginate (RGD-PA) and chemically conjugated phage/alginate (CRGD-PA). As shown in Fig. 1, the CRGD-PA scaffold has relatively high cell activities such as cell proliferation and differentiation.

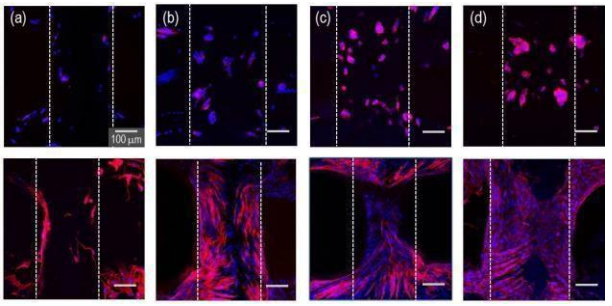

Fig. 1: DAPI (blue)/phalloidin (red) images after 1 and 7 days of cell culture for various biocomposites, such as (a) Alg, (b)RGD-A, (c)RGD-PA and (d)CRGD-PA

DISCUSSION & CONCLUSIONS: In summary, a new biocomposite scaffold consisting of core (PCL)-shell (RGD phage-based biocomponent) structure both enhances cellular activities and mechanical properties. This result showed that RGD-phage-conjugated alginate with core-shell method has a great potential for hard tissue regeneration.

ACKNOWLEDGEMENTS: This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and future Planning (NRF-2015R1A2A1A15055305) and also was partially supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI15C3000).

A novel biohybrid which mimics the (bio)mechanical behaviour of tubular organs

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INTRODUCTION: Reconstruction of tubular organs is still a challenge. There is a need to improve reconstruction methods and tissue engineering (TE) approaches especially in terms of tubular template manufacture. The ideal template should have specific dimensions to match recipient tissue, guarantee adequate cell (in)growth and cell distribution and should have adequate (bio)mechanical properties which match those of the native tissue. In this study we created and mechanically tested a hybrid tubular template composed of a tubular collagen sponge reinforced with a coupled helical coil -a promising tool inspired by nature- giving sufficient strength to this structurally weak biomaterial [1-3].

METHODS: A coupled helical coil (CHC) was prepared by winding a resorbable surgical suture filament (Maxon®) in a clockwise- and anticlockwise helix, resulting in tubular structure with a highly organized crosswise pattern. Fiber crossover points were fused together in a "joint-like'' structure by gentle heating in a laboratory oven. To create a biohybrid, CHC were embedded in 0.5% (w/v) type I collagen in tubular polystyrene molds and subsequently frozen, lyophilized and cross-linked [4]. Alternatively, tubular templates without CHC were prepared. Construct morphology was assessed using scanning electron microscopy (SEM, JEOL 6310). To evaluate the construct strength, tensile ring test was performed on a tensile tester (Z2.5 TN, Zwick/Roell) using a crosshead speed of 50mm/min. A uniaxial load was applied in radial direction until rupture of the construct. Ring specimen (width: 5mm) of native ureteral tissue and 0.5% (w/v) collagen alone were used as reference.

RESULTS: Fabrication of the biohybrid resulted in a seamless tubular structure (l: 70mm; Øi: 6mm). Wetted constructs containing CHC maintained an open lumen, whereas the collagen-only templates collapsed. Scanning electron microscopy revealed a porous structure with the CHC incorporated in the scaffold wall with collagen attached to the CHC. The hybrid scaffolds and native tissues showed a typical ''J'' shaped stress/strain curve beginning with an initial high elasticity (0.03MPa±0.01MPa, 0.05MPa±0.02MPa, respectively) up to an uprising and significantly stiffer region (1.23MPa±0.6MPa, 1.11MPa±0.34MPa respectively). The (bio)mechanical behavior of tubular templates prepared from collagen alone significantly differed in both initial- (0.01MPa±0.002MPa) and upswing modulus (0.03MPa±0.004MPa).

DISCUSSION & CONCLUSIONS: Tubular templates consisting of CHC embedded in collagen were prepared with mechanical characteristics comparable to the envisioned recipient tissue. Tubular templates of collagen only were much weaker and collapsed when wetted. The reinforcement of these templates with a CHC, a structure similar to the arrangement to the collagen fibers within the submucosa of various tubular organs like esophagus, small intestine or the human ureter, prevented template collapse. CHC can be produced from different (biodegradable) materials and different fiber arrangements can tune the mechanical properties. In combination with different densities of collagen and cross-linking methods, this procedure holds promise to match and tailor complex stress/strain behaviors of tubular templates to mimic soft tubulartissues.

ACKNOWLEDGEMENTS: The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement No. 607868 (iTERM).

Engineering 3D single stem cell microniches with dynamically tunable stiffness

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INTRODUCTION: The microelasticity of extracellular matrices is tissue-specific and varies significantly throughout the body. It ranges from 0.2 kPa in brain up to 45 kPa in bone, but also changes during development and wound healing [1]. Cells sense and respond to these mechanical cues via mechanotransduction pathways, which affect migration, proliferation and differentiation [2]. Typically, mechanobiology is studied using 2D cell cultures that offer straightforward read-outs, but also induce unnatural cell spreading that does not reflect 3D in vivo conditions [3]. We have developed a novel microfluidic platform for the production of single cell microgels to investigate the effects of matrix microelasticity on mesenchymal stem cell (MSC) differentiation under 3D conditions, while maintaining spherical cell shape. Moreover, sequential crosslinking of the biomaterial enabled in situ stiffening of the cell microenvironment, allowing us to investigate single cell responses to dynamically altering matrix stiffness in a manner that uncouples stiffness and cell shape.

METHODS: Single MSCs were encapsulated in horseradish peroxidase (HRP) containing dextran-tyramine gel precursor microdroplets at a rate of 2 kHz using a microfluidic droplet generator and subsequently crosslinked using a novel diffusion-based microfluidic platform that enabled precise control over the degree of microgel crosslinking (Fig. 1a). Specifically, we engineered microgels with a tunable stiffness ranging from 2.5 to 45 kPa by varying the H_2O_2 feed concentration. Soft microgels were cultured for 4 weeks in either adipogenic or osteogenic differentiation medium and were dynamically stiffened at predefined time points by post-curing using HRP and H_2O_2.

RESULTS: Encapsulated MSCs remained viable and metabolically active during long-term culture (4w), as determined by live/dead and MTT assays, respectively. Regardless of the differentiation medium, only MSCs in soft microgels differentiated into the adipogenic lineage (Fig.1b). Furthermore, only stiff microgels could induce the osteogenic phenotype (Fig.1c). Moreover, dynamic stiffening of the microgels on predefined time-points during the culture indicated that the stiffness-imposed differentiation was not reversible. Specifically, stiffening the microgels after more than 3 days of culture in osteogenic medium could not rescue the osteogenic differentiation potential of the MSCs.

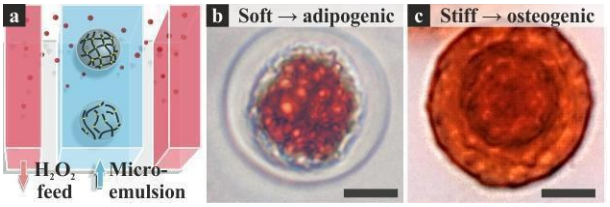

Fig. 1: (a) Schematic representation of our novel microfluidic platform for diffusion-based enzymatic crosslinking. Stiffness-imposed differentiation of individually encapsulated MSCs into the (b) adipogenic and (c) osteogenic lineage, indicated by Oil Red O and Alizarin Red S staining, respectively (scale bars: 10µm).

DISCUSSION & CONCLUSIONS: Here, we report high-throughput engineering of microniches with a dynamically tunable stiffness to control single stem cell fate. In contrast to previous monolayer-based studies, cells were forced to remain spherical throughout the culture as a result of the confining 3D nature of our microniches. Since cell response in terms of differentiation was comparable to monolayer-based studies, this work suggests that alterations in cell shape such as spreading are not crucial for stiffness-mediated differentiation.

ACKNOWLEDGEMENTS: Dutch Foundation (Grant #12-2-411) to JL and MK.

Osteogenic differentiation of human mesenchymal stem cells on nanofiber based microtubular scaffolds

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INTRODUCTION: Clinical needs for bone graft procedure are diverse and include applications arising from resection of metastatic tumors, bone loss after skeletal trauma or infection, failed fracture healing and congenital craniofacial deformities. One of the major challenges in reconstruction of large bone defects is the inadequate ability of designing biomimetic scaffolds that induces differentiation of osteoprogenitor cells by cell-matrix interaction at the nanoscale as well as mimicking micro assembly of compact bone while providing high mechanical strength. Although there have been many studies on building bone mimetic scaffold by using composite nanofibers (NF), there is still tremendous need for new approaches and strategies for the new osteon mimetic load bearing biomimetic scaffolds.

METHODS: In bone tissue, biomineralization is mediated by extracellular matrix (ECM) proteins with amino acid sequences rich in glutamic acid. Therefore, in order to mimic the biomineralization process in bone tissue, the EEGGC peptide (GLU) [1] conjugated low moleculer weight polylactic acid acrylate (PLLA) (4000 Da) with two glutamic acid residues will be used to modify the surface of poly(lactide-co-glycolide) (PLGA) NF in order to improve calcium-phosphate (CaP) crystals deposition. Engineered small-diameter microtubuler osteon-mimetic scaffolds will be fabricated by wrapping the perforated calcium-phosphate (CaP) deposited nanofiber (NF) layers around the stainless steel needle upon heating to shrink and keep the shape of the microtubes (shape memory effect). Microtubuler bundles will be fabricated by annealing multiple microtubes while having contact to each other. Afterwards, human mesenchmal stem cells (MSCs) will be seeded inside the microtubular bundles and differentiation of MSCs will be evaluated.

RESULTS: ALPase activity, calcium content and total collagen content of the MSC-seeded microtubes with incubation time were investigated. The results of ALPase activity, calcium content and total collagen content confirms the osteogenic differentiation of MSCs. However, the inner diameter of the microtubes (0.35mm and 0.8 mm) did not have a significant effect on osteogenic differentiation of MSCs. The results demonstrate that geometry supports differentiation and mineralization of MSCs.

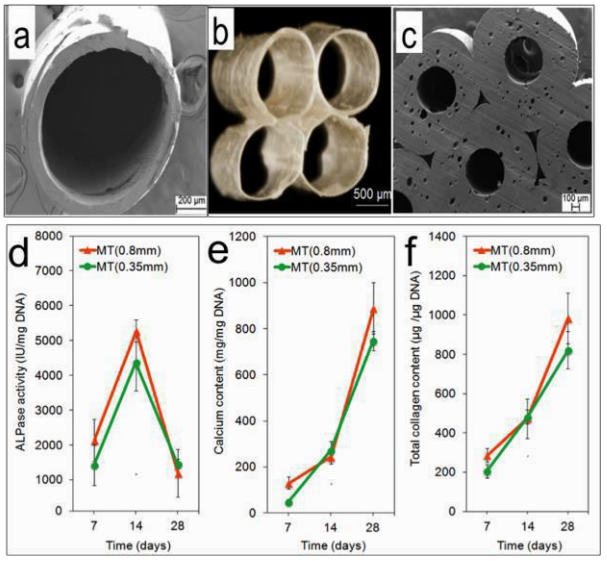

Fig. 1: Images of developed nanofibers based single microtubes (a), and microtubes bundles (b.c). Alkaline phosphatase activity (d), calcium content (e),and total collagen amount (f) of MSCs seeded inside GLU-NF/CaP50 microtubes (0.35 and 0.8 mm) cultured in osteogenic media.

DISCUSSION & CONCLUSIONS: Different inner sizes of microtubes (0.35 and 0.8 mm) and microtubalar bundles were fabricated succesfully. Our results indicate that 3D structure of the osteon-mimetic microtubes supported osteogenic differentiation of MSCs as evidenced by enhanced calcium content, ALPase activity, and total collagen secretion. The developed microtubular bundles could potentially be a promising scaffold for load bearing bone tissue engineering applications.

ACKNOWLEDGEMENTS: This study was financially supported by The Scientific and Technological Research Council of Turkey (TÜBİTAK, 214M268).

Impact of solubilized muscle-specific urethral extracellular matrix on skeletal muscle differentiation for sphincter reconstruction

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INTRODUCTION: Stress urinary incontinence is muscle and/or nerve damage resulting from natural childbirth, aging, obesity or cancer therapy. Besides surgical interventions, current therapeutic options include pelvic floor muscle training and pharmacological treatments [1,2]. The long-term efficacy of such approaches is often low and accompanied by possible side effects [3]. Alternatives rely on cell therapy. Here fore, we have cultured muscle precursor cells (MPC) on decellularized urethral tissue suitable for cell adhesion and myoblast fusion yielding skeletal muscle-like structures.

METHODS: We have isolated, characterized and expanded MPCs and cultured them on urethra extracellular matrix (ECM) coatings derived from transversal fragments of urethra bioscaffolds of the rhabdosphincter (RBS) layer containing either skeletal muscle ECM (skECM) or smooth muscle ECM (smECM). Tissue formation was analyzed via fiber formation assay, morphological analysis and protein / gene expression. Collagen, polystyrene respectively FBS coatings were used as negative controls.

RESULTS: MPCs showed a significant longer morphology on skECM coatings (109 ± 4.9 µm) compared to all others coatings (70 ± 3.6 µm (p-value = 0.002), 75 ± 3.7 µm (p-value = 0.002), 50 ± 3.1 µm (p-value = 0.0001) and 80 ± 4.3 µm (p-value = 0.003) for collagen coating, non-coated polystyrene, FBS coating and smECM, respectively. Regarding cell circularity, there were no significant differences (p-value = 0.532). MPCs cultured on skECM and smECM revealed a similar cell proliferation profile compared to controls. After 14 days of differentiation, the cells fused into fibers expressing skeletal muscle-associated markers.

DISCUSSION & CONCLUSIONS: The acellular urethra bioscaffolds were dissected (to separate muscle-specific ECMs – smooth and skeletal) and solubilized through partial digestion to formulate coating substrates. Albeit required improvements cells were able to adhere and proliferate *in vitro* on these substrates. The direct comparison of cell performance in both muscle-specific ECM substrates (i.e. smooth versus skeletal) explained the impact of biochemical cues in cell behavior. Together, these results show the great potential of the acellular urethra bioscaffolds to be used as a starting point for different Tissue Engineering strategies with the aim to enhance skeletal muscle formation.

ACKNOWLEDGEMENTS: We thank Souzan Salemi and Deana Haralampieva for all the assistance and guidance

Enhanced mineralization and vascularization in pitavastatin loaded calcium phosphate scaffolds

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INTRODUCTION: Calcium phosphate scaffolds (CaPs) have been incorporated with growth factors with views on bone regeneration[1] but they have been questioned due to their serious side effects. In parallel, studies have shown therapeutic benefits of some statins in neovascularization and bone formation[2]. In this study, the potential of Pitavastatin (PTV), an unexploited statin, on bone and vascular tissue formation, has been evaluated in vitro and it has subsequently been incorporated to CaP scaffolds.

METHODS: Rat mesenchymal stem cells (rMSCs) and rat endothelial progenitor cells (rEPCs), were cultured in cell culture media containing PTV (1 and 0.1 µM) and the expression of different genes involved in bone regeneration (Osteocalcin, collagen type 1, BMP-2, Alkaline phosphatase, β-actin) and vascularization (VEGFA, VEGFR1, VEGFR2) was monitored by Real Time - Polymerase Chain Reaction (RT-PCR) for 72h. Self-setting CaP foams were prepared by mechanical mixing of a solid phase (α- tricalcium phosphate with 2wt% hydroxyapatite) and a liquid phase (PTV (200, 100, 75 & 50 µM), 1% Tween and 10% Pluronic in MQ water) at 0.65 ml/g Liquid-to-Powder ratio at 1700rpm. Microstructural and textural properties were assessed by scanning electron microscopy, porosity by mercury intrusion porosimetry, density by He pycnometry and specific surface area (BET method). Drug release was evaluated in 5ml of PBS at 37ºC with shaking coupled with HPLC.

RESULTS: Cells incubated with PTV in the culture medium showed enhanced expression of genes involved in mineralization (Osteocalcin and Collagen type 1) and vascularization (VEGFA, VEGFRs), in a dose-dependent way over the course of 72h, with an over expression for 0.1µM (*Fig. 1).* Self-setting CaP foams loaded with PTV showed no differences in microstructural or textural properties such as porosity (~78%) and interconnected porosity, with dose-dependent drug release behavior (*Fig. 2*).

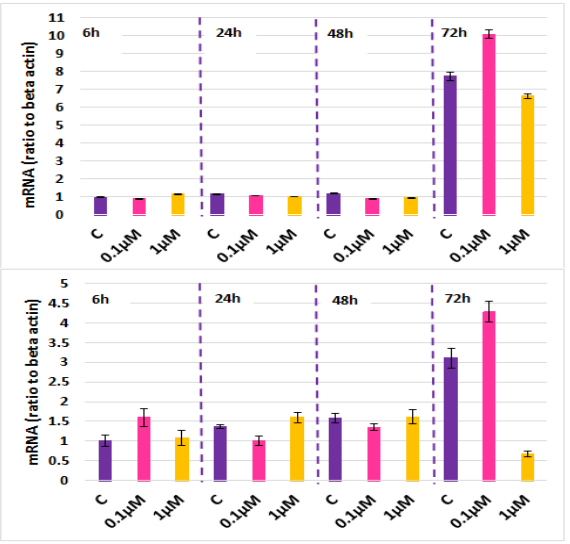

Fig. 1: Gene expression of collagen type 1 in rMSCs(a) and VEGFR1 in rEPCs (b) in growth media with PTV (0, 0.1 and 1 µM).

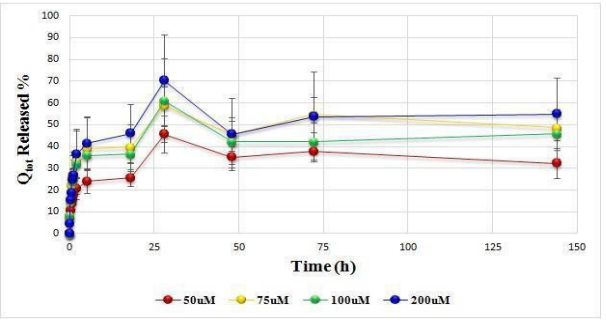

Fig. 2: PTV-loaded CaP scaffolds show a dose dependent release.

DISCUSSION & CONCLUSIONS: In vitro studies have provided new evidence on the potential of PTV-loaded CaP scaffolds being dose-dependent and without modification in mechanism allows the design of novel bone grafts with enhanced regeneration properties.

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ACKNOWLEDGEMENTS: Project MAT2015-65601-R, Ramon y Cajal of CC. and DocMASE of KK. MPG acknowledges the Generalitat de Catalunya for the ICREA Academia Award.

Low temperature fabrication of three-dimensional alginate scaffold coated with poly(ε-caprolactone) (PCL) for enhancing mechanical properties

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INTRODUCTION: In recent studies, alginate was widely used in various tissue engineering applications due to its high hydrophilicity and biocompatibility. However, this natural polymer has low mechanical properties which are crucial for hard tissue regeneration. [1] Therefore, synthetic polymers with high mechanical properties were employed on natural polymer to enhance the mechanical strength. [2] In this study, we fabricated an alginate scaffold *via* cryogenic direct-plotting system, and coated the scaffold with poly(ε-caprolactone) (PCL) to enhance the mechanical strength of the alginate scaffold sustaining the biocompatibility and porosity.

METHODS: To fabricate novel scaffold, 4 wt% alginate (purchased from FMC BioPolymer, Drammen, Norway) was plotted on cryogenic system. During this process, the alginate was printed by a 3-axis robot system (DTR3-2210-T-SG; DASA Robot, Bucheon, South Korea) using controlled pneumatic pressure generated by dispensing system (AD-3000C; Ugin-tech, Siheung, South Korea). To cross-link the alginate scaffold, it was immersed in 5 wt% calcium chloride ($CaCl_2$) solution for 15 minutes. Then, the scaffold was placed in a freeze-dryer (SFDSM06; Samwon, Busan, South Korea). The cross-linked alginate scaffolds were coated by immersion into 1, 5, and 10 wt% PCL solutions (M_w=90,000) (Sigma-Aldrich, St. Luis, Mo) for 1 h, and dried naturally. After the pure alginate scaffolds and the PCL-coated alginate scaffolds (APS-1, 5, and 10) were fabricated, the osteoblast-like cells (MG63, CRL-1427; ATCC, Manassas, VA, USA) were seeded on the scaffolds to evaluate the cellular activities.

RESULTS: As shown in Figure 1, the struts were partially covered by 1 wt% PCL solution. However, 5 wt% PCL solution covered most of the struts and 10 wt% covered the struts entirely. The mechanical properties of the scaffold were considerably improved compared to the pure alginate scaffold due to the reinforcing component, i.e. PCL. Evaluation of in vitro cellular activities using MG63 cells demonstrated that the viable cell proliferation and calcium mineralization levels on the scaffold were completely similar compared to those of the pure alginate scaffold.

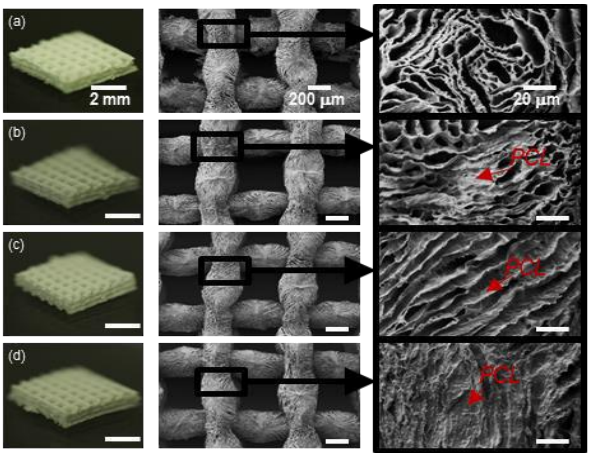

Fig. 1: Optical and SEM images of the scaffolds: (a) alginate scaffold, (b) APS-1, (c) APS-5 and (d) APS-10.

DISCUSSION & CONCLUSIONS: Here, a new biomedical scaffold consisting of alginate and PCL was proposed and fabricated using a low temperature direct-plotting and coating method. The APS-1, 5, and 10 showed higher mechanical properties compared to those of the alginate scaffold without any sacrifices of cellular responses. These newly fabricated scaffolds can be utilized for hard and soft tissue regeneration by high porosity and enhanced mechanical properties.

ACKNOWLEDGEMENTS: This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and future Planning (NRF-2015R1A2A1A15055305) and also was partially supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI15C3000).

Bio-engineering of neo-corneas using nature-derived aloe vera gel blended silk fibroin scaffolds

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INTRODUCTION: Corneal transplantation, a common surgical protocol for visual acuity improvement, is limited owing to shortage of high quality donor corneas and/or lack of accurate replication of structural and biochemical composition of native cornea in a scaffold.[1] Construction of neo-corneas utilizing novel, biocompatible and biodegradable scaffold/film source, could possibly address such formidable challenges. In this study, we have designed optically transparent, micro-structurally stable silk fibroin scaffold blended with nature-derived aloe vera gel (AV/SF) as an alternative template for neo-cornea regeneration. Aloe vera gel is nature-derived biomaterial, well-studied for its anti-inflammatory properties and used as medical herb for thousand years. Furthermore, the morphological, structural analysis and *in vitro* biological studies were assessed using primary rabbit corneal endothelial cells (rCEnCs) as models.

METHODS: Silk fibroin (SF) solution was prepared using our previously reported study.[1] The aloe vera gel was extracted by centrifuging aloe vera leaves at 10000 rpm for 30 min. Next, AV/SF scaffolds were fabricated by blending different proportions of AV (1, 3 & 5 wt %) with SF solution, dried and cross-linked using methanol for 1 h at RT.

RESULTS: The 3 wt.% AV/SF scaffolds showed significant increment in initial cell attachment and proliferation of cultured cells with well-maintained characteristic polygonal shape of rCEnCs (Fig 1). Although any remarkable changes regarding the morphology, expression of ZO-1 and Na+/K+-ATPase were absent, however the cells were found to be capable of well-expressing their functional proteins which regulates functions of corneal endothelium.

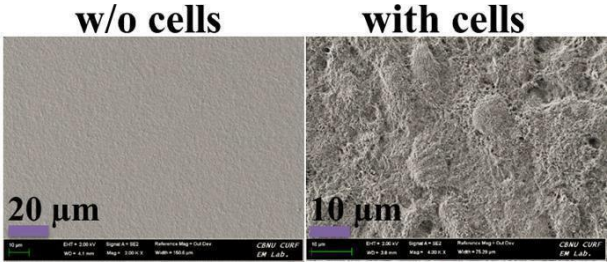

Fig 1: SEM images of 3 wt% aloe vera gel/silk fibroin scaffolds with and without rCEnCs cells.

DISCUSSION & CONCLUSIONS: We have successfully fabricated AV/SF scaffolds to achieve an enhanced rCEnCs adhesion and tissue specificity of SF without compromising SF mechanical strength for rCEnCs bio-regeneration. Morphological and structural analysis showed good transparency and hydrophilicity with required water permeability of the 3 wt.% AV/SF scaffolds, highly suitable for rCEnCs carriers. In vitro results further showed the 3 wt.% AV/SF scaffolds not only increases the initial rCEnCs adhesion but also can efficiently maintain the proliferative capacity, cell phenotype, and cell-junction formation of rCEnCs. Thus, this study was envisioned to provide useful avenues for enhancing the clinical efficacy of carriers designed for tissue engineering applications.

ACKNOWLEDGEMENTS: This research was supported by NRF-2012M3A9C6050204 and BK21 PLUS.

Immunomodulatory surface coatings based on crosslinkable hyaluronic acid derivatives

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INTRODUCTION: Macrophages are one of the first cells that react to any tissue injury, as well as to any introduced foreign body, including implants[1]. They are derived from monocytes and show high level of functional and phenotypic plasticity. Macrophages are versatile biochemical factories with a huge arsenal of molecules to attack the invading microorganisms or foreign bodies, which is effective as a defense system, but at the same time causes huge collateral damage to the surrounding tissue which needs to be later resolved. The final aim of this work is to significantly decrease the implant and medical device failure due to adverse immune reactions by developing an innovative immunomodulatory system[2]. Our current focus is to use the controlled release capacities for the delivery of immunomodulatory cytokines such as Interleukin 4 (IL-4) and quantification of its effect on macrophages (monocytes).

METHODS: Two substrates with modified hyaluronic acid (HA) were developed by Layer-by-Layer method (polylysine/HA-aldehyde) and by spin coating (gelatin/HA-tyramine). Biofunctionalization of those substrates were envisioned by introducing the IL-4 that is a key cytokine in type 2 immunity related responses, essential for wound healing and allergy. Macrophages were seeded on those films in 2D and 3D culture.

RESULTS: Monocytes cultured on 2D **PLL/HA-Aldehyde film** functionalized with IL-4 produce anti-inflammatory cytokines while significantly reduce production of pro-inflammatory cytokines. THP-1 cells 3D cultured on **Gelatin/HA-Tyramine film** either with or without functionalization with IL-4 proliferated up to 21 days. The IL-4 release resulted in distinct morphological differences as in IL-4 release samples, THP-1 cells formed large clusters whereas without IL-4, they were homgenously distributed within the hydrogel. Moreover, it is observed distinctly higher CD206 expression (M2 macrophage phenotype) by the encapsulated cells with IL-4 compared to without IL-4 samples; which is a well-known M2 phenotype marker (Fig.1).

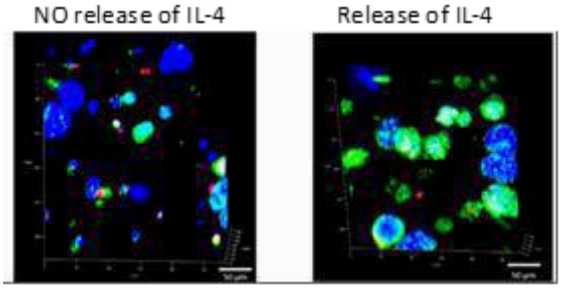

Fig. 1: Confocal images of THP-1 encapsulated in hydrogel after 21 days, with M1 marker: CD80 (red), M2 marker: CD206 (green) and DAPI (blue).

DISCUSSION & CONCLUSIONS: In summary, PLL/HA-Aldehyde and Gelatin/HA-tyramine based films are good candidates as surface coatings of implants of various architectures with controlled physicochemical properties and release capabilities. Furthermore, once functionalized with IL-4, they present a potential for a local prevention of inflammation and induction of healing via control of macrophage phenotype.

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ACKNOWLEDGEMENTS: "EU FP7 grant agreement no. 602694" (IMMODGEL).

Transparent silk fibroin nano-fibrous scaffold for corneal stromal regeneration

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INTRODUCTION: Silk fibroin, produced by the silkworm Bombyx mori, has been receiving full recognition as biomaterial, having biocompatibility such as non-cytotoxicity, robust mechanical properties etc. The excellent process ability into various shape, such as films, sponges, hydrogels, or nanofibers[1] also encourages the biomaterial use of silk fibroin[2]. In this context, recently we are trying to utilize silk fibroin nanofibers as a corneal stromal regeneration scaffolds[3]. The excellent histocompatibility and the efficacy of the silk fibroin nanofiber construct for the corneal stromal regeneration were shown in the preliminary implantation[4]. Even though, the transparency of the material itself does not achieve satisfactory level. Here we try to improve initial transparency of the fibroin nanofiber construct.

METHODS: Silkfibroin nanofibrous mats: For preparation of electrospinning solution, 8 wt% fibroin aqueous solution was gently mixed with a 5 wt% poly (ethylene oxide) aqueous solution in the volume ratio 4 to 1. An electrospinning system equipped with a rotation drum collector (NANON, MECC Co., Ltd., Japan) was used to fabricate aligned non-woven mats as following setting; applied voltage was 12 kV, a feeding rate was 0.3 ml/h, a spinning distance was 185 mm, a stainless needle was 25G, a diameter of the drum collector was 100 mm, rotation speed was 3000rpm and spinning time was 3 hours. Humidity in the NANON was kept below 25% by nitrogen gas perfusion. For insolubilization, electrospun aligned fibroin fiber mat was immersed into dehydrated ethanol for 24 hours. After the process, poly (ethylene oxide) was removed by immersing into 95% ethanol and DDW for 24 hours.

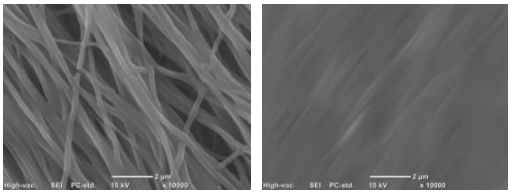
Treatments for the transparency improvement: to find out the suitable condition, after preparation of the electrospun nanofibrous silkfibroin mat, the samples were immersed into several concentration of calcium chloride solution ranging from 50mM to 500mM. The immersing time was fixed at 30min. After the treatment, the samples were dried up and then immersed into 120 mM sodium dihydrogenphosphate solution and 10% EDTA solution successively to remove the residual calcium. The light transmittances of the prepared samples were measured in distilled water. The relative transmittance values were obtained from the equation style: r.t.(%) = I/II x 100

I: brightness of control picture (without sample)

II: brightness of sample picture(with sample)

And the morphologies were evaluated by SEM observations. And liquid and protein permeability were evaluated by special camber apparatus.

RESULTS: The concentration range of 80 to 200 mM of calcium chloride solution treatment was improves the light transmittance of the nanofibrous silk fibroin mat significantly. The r.t. became 93.4 to 98.7%. From the SEM observation, suitable concentration of calcium chloride treatment induce surface flatting and improve the alignment of silkfibroin nanofiber(see *Fig.1*). After the treatment, liquid and protein permeability were still kept enough.



(1)Control (2) 80 mM $CaCl_2$trated mat

Fig. 1: *SEM observation of the samples*

DISCUSSION & CONCLUSIONS: There results suggest that the calcium chloride treatment can improve the light transmittance keeping with the liquid and protein permeability of the nanofibrous silkfibroin mat.

New designed hydrogels based on aldehyde-derivative of hyaluronic acid for application in tissue engineering and regenerative medicine

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[1] *Contipro Pharma a.s., Dolni Dobrouc, Czech Republic*

INTRODUCTION: One of promising application of hyaluronan (HA) is a development of hydrogel-based materials for use in biomedicine or tissue engineering. HA-aldehyde (HA-Ox), commonly prepared by selective oxidation of native HA, could be used for this purpose. Gelation is initiated by addition of diamine *O,O'*-1,3-propanediylbishydroxylamine dihydrochloride (POA) into HA-Ox solution [1].

The aim of this work was study of influence of derivative parameters and ratio of POA amino groups (NH_2) and aldehyde groups of HA-Ox (CHO) on hydrogels mechanical properties, kinetics of gelation and parameter of swelling which leads to optimization of hydrogels properties for usage in tissue engineering and regenerative medicine.

METHODS: The critical gel point (GP) was detect at 37 °C as cross-over point where the elastic and viscous moduli were equal (G'=G"). Young´s modulus (Y), compressive stress at breaking point and toughness (T) were evaluated in compression using device Instron 3343. The swelling ratio was chosen as parameter for determining of gels volume stability in buffer solution at 37 °C and was determined from hydrogels weight increase in their swollen state.

RESULTS: It has been found that mechanical properties of hydrogels ranged from soft to hard gels (Young´s modulus ranged from 0,5 up to 25 kPa) according to HA-Ox molecular weight and degree of substitution and ratio of amino and aldehyde groups.

Influence of NH_2:CHO ratio on mechanical properties is shown in *Fig. 1* where Young´s modulus is the highest at ratio of NH_2:CHO 1:1. Young´s modulus increases with increasing degree of substitution and molecular weight.

Kinetics of gelation ranged from tens of seconds to minutes. The fastest gelation occurred at ratio of NH_2:CHO 1:1. Speed of gelation increased with increasing derivative Mw and as well DS.

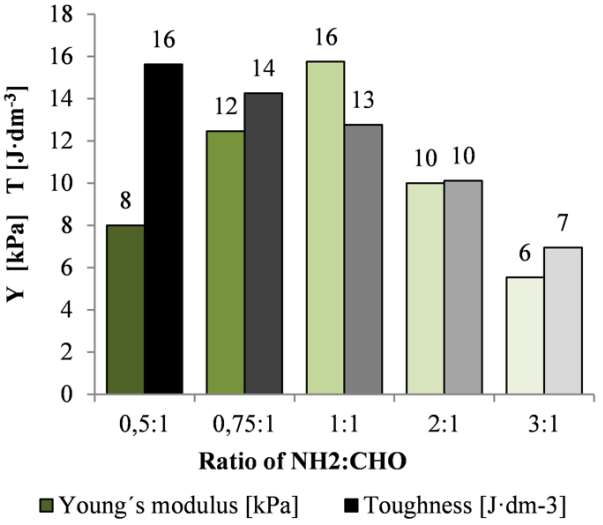

Fig. 1: Influence of ratio of NH_2:CHO groups (i.e. POA concentration)

The volume stability of prepared gels in buffer was measure for 8 weeks. It was possible to achieve low value of swelling ratio (increase less than 10 %) by choosing of suitable properties of gel.

DISCUSSION & CONCLUSIONS: Obtained results showed that it is possible to optimized hydrogel properties by changing of HA-Ox parameters or ratio of amino and aldehyde groups to achieve appropriate material for intended application. HA-Ox hydrogels are prepared by non-cytotoxic reaction that predisposed them as suitable matrix for cell incorporation. Moreover according to preliminary results the cells are viable after their incorporation into these hydrogels. All these aspects predispose these materials as suitable matrix for use in tissue engineering and regenerative medicine.

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ACKNOWLEDGEMENTS: This work has received funding from the European Union's Seventh Framework Programme for research and technological development and demonstration under Grant Agreement no. 604531 (AMCARE).

Interpenetrating network hydrogels based on gelatin and gellan gum for tissue engineering

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INTRODUCTION: Hydrogels produced by semi- or interpenetrating polymer networks (IPN) technology are cross-linked polymeric networks with another hydrophilic polymer chain or network inside their structure [1]. The formation of IPN can improve the biocompatibility, biodegradability and stability of the materials while ensuring the mechanical strength [1]. In this work, we are preparing and characterizing IPN hydrogels based on gelatin and gellan gum using the bioamine spermidine as a cross-linker.

Gelatin has excellent biofunctional and filmogenic properties, but poor mechanical strength while gellan gum (GG) is a biopolymer with good mechanical properties but need to improve the cell attachment [2]. Vicinal diols in the GG molecule were oxidized and gelatin was modified by amidation, allowing the generation of Schiff bases. Hydrogel was formed with ionitropic spermidine cross-linking of GG-gelatin IPN [3].

METHODS: Varying amidation degrees of gelatin (porcine, type A) were obtained by activation with EDC and linkage with ethylenediamine. Aldehyde groups in GG were generated by the oxidation of vicinal diols using the reaction with sodium periodate. For hydrogel formation GG, gelatin and spermidine were dissolved in sterile water with 10% sucrose to adjust the isotonicity. All reagents were purchased from Sigma Aldrich.

Success of modification was verified by FTIR and ^{1}H-NMR. The biocompatibility of modified gelatin as coating was tested in one week cell culture with WI-38 human fibroblast cell line followed by Live/Dead® assay. Gelation of IPN hydrogels was tested and visually inspected. Mechanical testing of cylindrical samples (h=7mm, Ø=12mm) was done by Bose Electroforce Biodynamic 5100 with WinTest 4.1 software in uniaxial unconfined compression in air with 10mm/min rate.

RESULTS: The chemical modifications were successful as shown by the FTIR spectra in Fig 1. The cytocompatibility test with WI-38 fibroblasts showed similar results with all control and all modified versions, as seen in Fig 2.

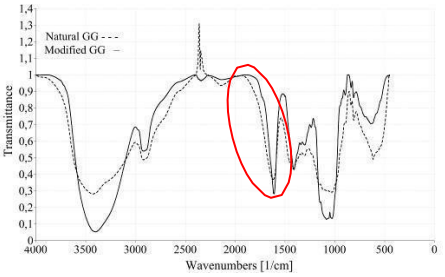

Fig1. FTIR spectra shows increase of aldehyde groups in modified GG compared to natural GG.

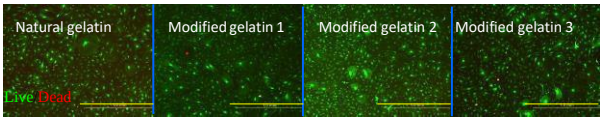

Fig2. Fluorescent micrograph of WI-38 cells on gelatin coating, scale bar length 1.0 mm.

DISCUSSION & CONCLUSIONS: Modified gellan gum and all three versions of gelatin were able to form IPN hydrogels via Schiff's base reaction and ionic cross-linking with the bioamine spermidine. The cationization of the gelatin does not affect the viability of human fibroblasts. The modified hydrogels had lower compressive modulus but were more elastic when compared with the non-modified polymers. Modified GG hydrogel behaved more similarly to native soft tissue. The results indicate that this hydrogel has potential as a tissue engineering scaffold material.

ACKNOWLEDGEMENTS: The authors would like to thank the Human Spare Parts project of TEKES – Finnish Funding Agency for Technology and Innovation for supporting this work.

DISCLOSURES: Authors have nothing to disclose.

Bioengineered silk vascular grafts for coronary artery bypass surgery

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INTRODUCTION: Cardiovascular malfunctioning is one of the leading causes of death globally. It is important to note that coronary artery occlusion incidences are more prominent [1]. Current surgical treatment methods require an adequate supply of native vascular constructs to replace the diseased vessels. Autologous vessels are most suitable target but nearly one-third of patients do not have their veins appropriate for grafting due to vascular disease and prior vein harvesting [2, 3]. In the current endeavor, we have developed a functional vascular conduit by combining the cell sheet engineering and patterned silk films.

METHODS: Both mulberry (*B. mori*) and non-mulberry (*A. Assama and P. ricini*) Indian endemic silk varieties were compared. Patterned silk films were characterized using FTIR, AFM, XRD, DSC and TGA. Vascular cells were co-cultured on to films and checked for maintenance of functional phenotype. Fabricated conduits were further characterized for blood compatability, burst strength and *in vivo* immunogenicity.

RESULTS: Non-mulberry silk films presented superior characteristics in terms of thermal stability, proteolytic degradation, cellular proliferation (~1.1-1.3 folds higher than mulberry silk) and alignment of vascular cells. Immunogenicity of films was assessed *in vitro* by looking into macrophage response in terms of TNF-α secretion. It was ~40.79-51.4% lesser as compared to positive control after 7 days. *In vivo* subcutaneous implantation of films in mice showed minimal fibrosis and inflammation after 28 days. Pattern induced alignment favored functional contractile phenotype of SMCs and they strongly expressed contractile markers calponin and α-SMA. Moreover, the expression of elastin in SMCs and punctuated pattern of vWF in ECs layer was observed. Burst strength of tubular construct ranged between 915-1260 mmHg, sufficiently higher than the physiological pressure.

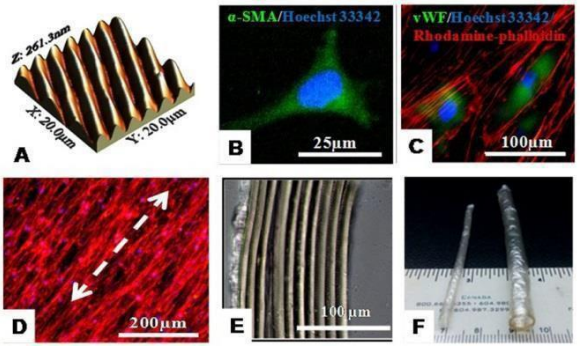

Fig. 1: A) AFM image of patterned silk film, B) Expression of α-SMA in SMCs, C) Expression of vWF in ECs, D) Alignment of vascular cells, E) Cross section of multilayered mature construct, F) Gross morphology of fabricated graft.

DISCUSSION & CONCLUSIONS: We demonstrated the formation of unidirectionally aligned monolayer of vascular cells on patterned silk films expressing their functional phenotype and were metabolically active. Constructs were mechanically suitable for bypass grafting since they withstood and did not fail even at significantly higher values than physiological arterial pressure. Non-mulberry silk varieties (*A.assama* and *P. ricini*) were superior to conventionally used *B. mori* silk in terms of stability, vascular cell compatibility and did not elicit any immunological response when implanted *in vivo* in mice. We envisage that future advancement in this direction would lead towards "off the shelf" clinical implementation of small diameter vascular graft.

ACKNOWLEDGEMENTS: We are thankful to Department of Atomic Energy (DAE), Department of Biotechnology (DBT), Government of India for their funding support.

Integrated experimental/computational approach of the nuclear pore complex

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INTRODUCTION: A basic recent understanding in stem cell differentiation is that the cell is able to translate its shape (e.g. roundish or deformed) into a fate decision. However, the mechanisms by which phenotype expression is regulated by cell shape are complex and poorly understood. Our hypothesis is that cell deformation induces nuclear deformation, which in turn causes strains in the nuclear envelope (NE). This induces a change in porosity and in permeability of the NE that affects the flux of transcription factors involved in stem cell differentiation [1]. To demonstrate this hypothesis, we set-up a numerical model of the interaction between the nuclear pore complex (NPC) and the NE.

METHODS: Firstly, we recreated the two extreme deformation conditions for the NE. We isolated mesenchymal stromal cells (MSC) from the bone marrow of adult rats. To recreate the deformed configuration, we processed the cells for microscopical analysis when they were in adhesion to a flat culture substrate. On the contrary, to recreate the roundish configuration, we fixed and processed the cells in suspension. In both configurations, we acquired images of the whole nuclei by scanning confocal microscopy, to measure the NE deformation. In addition, we reconstructed 3D portions of the NE by scanning transmission electron tomography (STEM), see Fig.1, to measure geometrical parameters of the NPC size/shape. Secondly, in order to couple a change in permeability of the NE at the microscale with a change in configuration of a single NPC at the nanoscale (in response to the deformation applied to the NE), we fed the measured data into a computational model of the NPC-NE mechanical interaction.

RESULTS: Our preliminary results show an increase in the NE surface by up to 50% together with a significant change of the local NE curvature in deformed cells, compared to roundish ones. However, we found non-significant differences in both shape and size of the reinforcement ring of single NPCs with NE deformation.

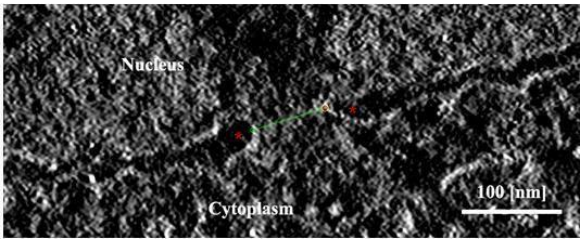

Fig. 1: Single tomographic slice through the nuclear envelope of a MSC cell grown in suspension. The highlighted distance (green) in the nuclear envelope (asterisks) corresponds to a nuclear pore complex

DISCUSSION & CONCLUSIONS: The computational simulations show that the highly different mechanical response of the NE structure, compared to the NPC structure, predominates in determining the change in porosity of the NE in response to the applied strains. This result is consistent with experimental published evidences showing a dramatic change in NPC density and shape in cells lacking lamin, an essential nuclear protein anchoring the NPCs to the NE [2].

ACKNOWLEDGEMENTS: This project has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement No. 646990 - NICHOID). This publication reflects only the author's view and the Agency is not responsible for any use that may be made of the information it contains.

Development of computational model using the *in vivo* subject-specific articular surface material properties: A probabilistic study

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INTRODUCTION: Finite element (FE) models of knee joints have been proven to be highly useful for understanding their functions in normal and injury states. No study has examined subject-specific *in vivo* material properties for knee joint using MRI under the various subjects' loading conditions and probabilistic FE models. The objectives of this study were to evaluate *in vivo* material properties of soft tissue in knee joint using subject-specific MRIs and FE models with the gold standard global sensitivity Monte Carlo (MC) approach.

METHODS: Sensitivity analysis, based on a MC method, was performed using a subject-specific FE model generated from *in vivo* computed CT and MRI data, subjected to two different loading conditions.[1,2] The MRI images were taken before and after axial compression, and when the flexion condition had been maintained at 30, 60, and 90 degree flexion in the subject-specific knee joint. The loading conditions of the probabilistic subject-specific FE model (axial compression and 90 degree flexion) were similar to the MRI acquisition setup. We were able to detect the influence of material parameters while maintaining the potential effect of parametric interactions. Throughout the in silico property optimization, a subject-specific FE model was used and less sensitive parameters were eliminated in the global sensitivity method. Soft tissue material properties were estimated using an optimization procedure that involved the minimization of the differences between the kinematics predicted by the subject-specific model and those obtained through *in vivo* subject-specific data.

RESULTS: Based on the probabilistic analysis, the elastic modulus of cartilage was the greatest in the vertical loading condition, following the elastic moduli of meniscus circumferential and radial directions, respectively, which are all sensitive factors. When overlaying the subject-specific FE model predictions for minisci movement with the

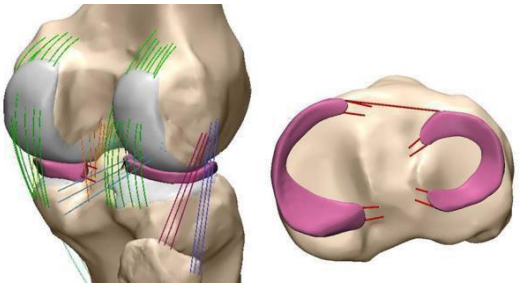

Fig. 1: FE models used in analysis

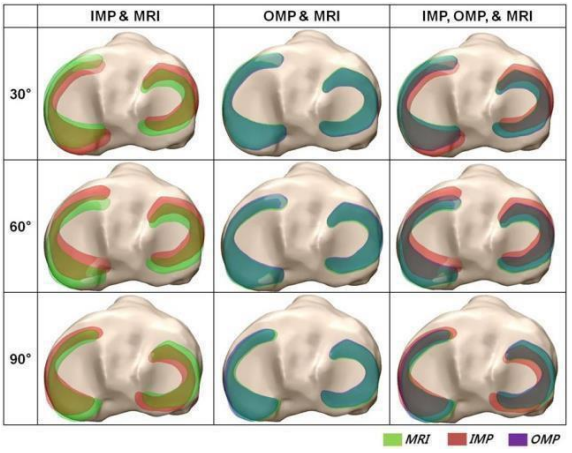

Fig. 2: Menisci movement for IMP model and OMP model at 30, 60, 90 degrees MRIs

MRI results, the optimized material properties (OMP) models were in better agreement with the MRI results compared to the initial material properties (IMP) model.

DISCUSSION & CONCLUSIONS: The results of this approach suggest that the articular surface mechanical properties could be found by using *in vivo* measurements, which clarifies the valuable tool for future subject-specific studies related to TF joint scaffolds, allografts andbiologics.

DISCLOSURES: Nothing to disclose.

Metabolic flux analysis of neural stem cells and astrocytes: metabolic alterations during neural differentiation

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INTRODUCTION: In vitro differentiation of neural stem cells (NSC) into neurons, astrocytes or oligodendrocytes provides a relevant context to study how these cell types are generated and evolve to acquire their mature phenotypes. In this respect, we have recently developed optimized protocols for differentiation of NSC, derived from murine embryonic stem cells, into pure cultures of mature astrocytes[1]. Herein, we took these cells to investigate what are the metabolic pathways that maintain and distinguish NSC and their differentiated astrocytes.

METHODS: To quantify the intracellular metabolic fluxes we applied isotopic transient metabolic flux analysis (^{13}C-MFA). The profiles of mass isotopomer distributions measured by GC-MS in NSC and astrocytes cultured with [1-^{13}C]Glucose, along with uptake/secretion fluxes (determined by HPLC and ^{1}H-NMR analysis of supernatant composition) were incorporated into a model of central carbon metabolism. The fluxes were estimated using the INCA software[2].

RESULTS: Significant metabolic differences between NSC and astrocytes were identified, with a general downregulation of central carbon metabolism during astrocytic differentiation. While glucose uptake was 2-fold higher in NSC, a high lactate-secreting phenotype was common to both cell types. Furthermore, NSC extracted glutamine from the medium: the highly active reductive carboxylation of alpha-ketoglutarate indicates that this was converted to citrate and used for biosynthetic purposes. In astrocytes, pyruvate entered the TCA cycle mostly through pyruvate carboxylase (81%). This pathway supported glutamine and citrate secretion, recapitulating well described metabolic features of these cells *in vivo*.

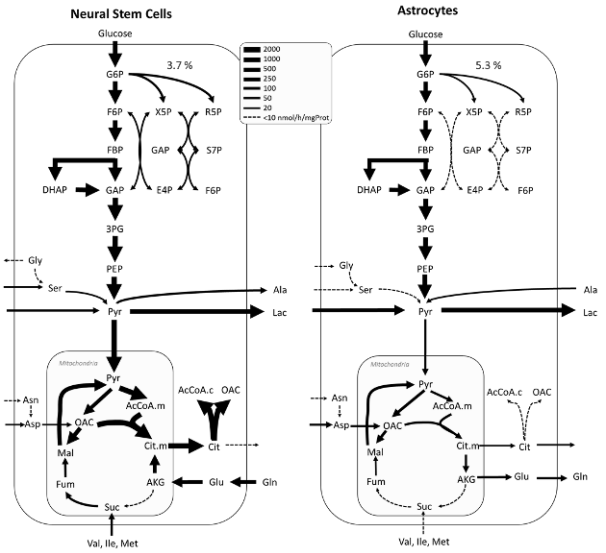

Fig. 1: Metabolic flux maps for NSC and astrocytes. Arrow thickness reflects absolute flux values in units of nmol/h/mg of protein.

DISCUSSION & CONCLUSIONS: In this work, we assessed the alterations in central carbon metabolism associated with the differentiation of NSC into astrocytes using non-stationary 13C-MFA. This is the first study in which a comparison of metabolic fluxes between these cell populations was performed, highlighting significant differences at the level of glycolysis, TCA cycle, and amino acids metabolism. Overall, this fluxomics study allowed us to quantify the metabolic rewiring accompanying astrocytic lineage specification from NSC.

Fluorescent labels PKH26 and Vibrant Dil do not influence the proliferation and differentiation of human mesenchymal stromal cells, but they interfere with their migration

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INTRODUCTION: Mesenchymal stromal cells (MSCs) are utilized manifold in quite distinct preclinical and clinical studies. For visualization cells can be labelled by incubation in fluorescent dyes to facilitate simple detection by microscopy. PKH26 or Vybrant Dil labelled cells result in a bright signal. But labelling cells with fluorescent dyes could bias their metabolism. We therefore investigated the viability, proliferation, differentiation, and migration of human MSCs after labelling in comparison to mock-treated controls.

METHODS: Human MSC were isolated from bone marrow of 6 patients undergoing surgery after informed and written consent. The MSCs were expanded in GMP-compliant media[1] and characterized to meet the ITSC consensus criteria[2]. Cells in the 2nd passage of *in vitro* culture were harvested, counted, and labelled by PKH26 or Vybrant Dil following the provider's instructions[3]. Viability, proliferation, migration and differentiation of MSCs were explored up to 14 days after labelling[4]. This study was approved by the Ethics Committee.

RESULTS:

The normalized viability and proliferation rate of MSC were not compromised by loading the cells with either PKH26 or Vybrant Dil (Tab. 1). Osteogenic and adipogenic differentiation was not altered after staining the cells with PKH26 or Vybrant Dil (Fig. 1). However, migration of human bmMSC was significantly reduced in cells after incubation with PKH26 ($p<0.05$) or Vybrant Dil ($p<0.05$) during a follow-up for 2 to 5 days (Fig. 2).

Table 1. Normalized viability and normalized proliferation rates of human bmMSCs after labelling with PKH26 (P) or Vybrant Dil (V) compared to mock-treated controls (ø).

Exp.	day1			day 7			day 14		
label	ø	P	V	ø	P	V	ø	P	V
viabil	89	88	83	84	86	84	88	84	90
prolif	99	99	99	81	72	90	52	53	41

DISCUSSION & CONCLUSIONS:

Labelling MSC with membrane-anchored dyes such as PKH26 or Vybrant Dil facilitate a simple detection of the cells in *in vitro* systems, in tissue explants or after application in organs *in vivo*. When the migration of the labelled cells plays a role an assay or in study, PKH26 or Vybrant Dil may bias the outcome of the experiment.

Fig. 1: Osteogenic (OD) and adipogenic (AD) differentiation of bmMSC compared to undifferentiated controls (mock-media, MM).

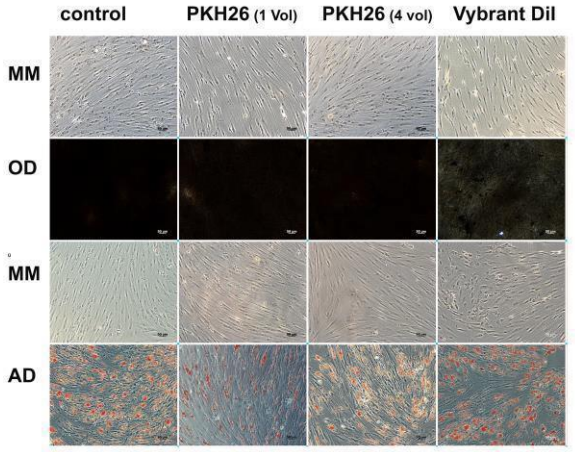
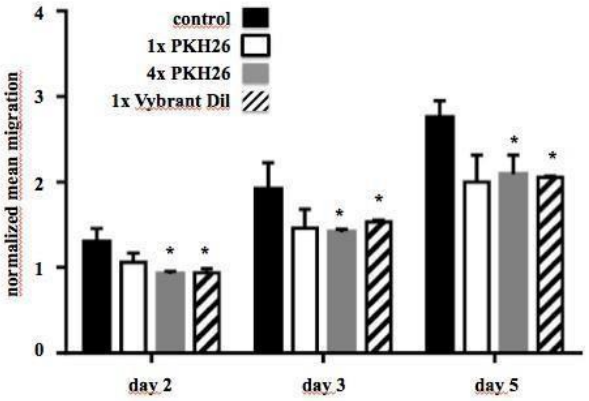


Fig. 2: Normalized migration of labelled MSC compared to mock-treated controls.



Fluorescent labelling of collagen production by cells for non-invasive imaging of extracellular matrix deposition

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INTRODUCTION: Monitoring of extra-cellular matrix (ECM) deposition within three-dimensional (3D) cellular constructs is highly reliant on destructive, end point techniques. Non-destructive monitoring of ECM deposition would allow for a reduction in samples required as well as reducing the reliance on end-point analysis. In this abstract, we present an online method for fluorescent labelling ECM production through cell labelling with incorporation of a modified azide-proline (AP).

METHODS: AP incorporation was performed using a method adapted from Amgarten *et al*. [1] MG63 were seeded on a salt leached PLGA scaffold (1 x 10^6/100 mm^3) and cultured with and without the addition of AP. At day 3, 5 and 10 of culture, constructs were washed with phosphatate buffered saline (PBS) and incubated with azide-reactive Click-IT Alexa Fluor488 DIBO Alkayne in serum-free media. The reactivity and specificity with the proline incorporated within the collagen was assessed using confocal microscopy, collagenase treatment of samples, assessment of non AP treated samples and correlation with destructive collagen assays. After treatment, MG63 metabolism was assessed using Alamar Blue™ to determine any detrimental effects of the staining process to the cells. The MG63 were then washed and placed back into culture with or without AP.

RESULTS: The addition of the AP itself did not detrimentally affect the metabolism or collagen production of the cells during the culture period. After treatment with the azide-reactive Click-IT Alexa Fluor488 DIBO Alkayne fluorescence was observed within the ECM of the cells. This fluorescence was shown to increase at all time-points and was shown have a positive correlation with the collagen content of the 3D cellular constructs (Pearson's r – 0.8434, $p<0.0005$). (*Fig 1*) Furthermore after treatment of cells with collagenase the fluorescent signal was diminished. This reduction in fluorescent signal was dependent on collagenase concentration and length of treatment. The staining and imaging process did not have a detrimental effect on the viability or metabolism of the cells throughout the experiment. With no significant changes observed in metabolism between treated and untreated samples. At day 10 there was also no significant difference in DNA content of the stained and un-stained samples.

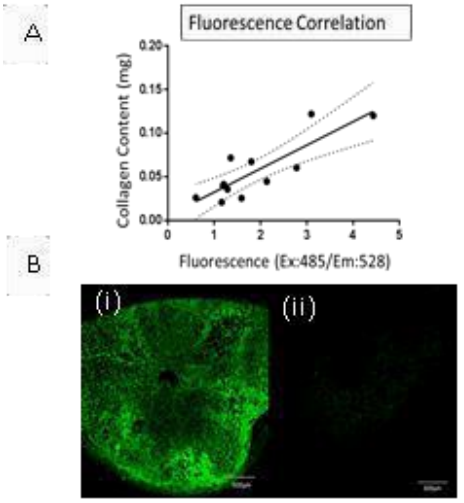

Fig. 1: (A) Correlation between collagen content and fluorescence within 3D MG63 constructs. (B) Visualisation of collagen deposition on PLGA cells cultured (i) with and (ii) without AP.

DISCUSSION & CONCLUSIONS: This research has highlighted the ability to monitor cell produced ECM to be monitored in real-time. This technique can be utilised to assess the effect of culture conditions and biomaterials on not only the quantity of ECM deposited but will also allow for the assessment of spatial deposition.

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ACKNOWLEDGEMENTS: The authors would like to thank the EU-FP7 BioDesign 'Rational Bioactive Materials Design for Tissue Regeneration' Project for funding (#262948).

Non-invasive tracking of oesophageal constructs engineered with smooth muscle precursor cells in a customised bioreactor

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INTRODUCTION: We have previously shown that decellularised scaffolds seeded with autologous cells are a promising option for an oesophageal replacement (Totonelli et al. 2013). Within this, we have developed an injection cell delivery method for introducing human mesangioblasts into the muscle layer of human oesophagi thereby successfully re-colonising the acellular oesophagi. The aim of this project was to examine the migration of these injected cells over a 7 day period using bioluminescence imaging. This innovative imaging system can be used to track the cells every 24 hours providing daily updates on cell viability and location.

METHODS: Human mesangioblasts (hMABs) were transduced with a luciferase ZS Green lentivirus using a 3rd generation packaging system. Transduction of the cells was confirmed using flow cytometry and a pure population of transduced cells was obtained using FACS sorting. The transduced cells were seeded by injection into the muscle layer of decellularised rat oesophagi and cultured both *in vitro* and in a bioreactor system for 7 days. Bioluminescence imaging was used to track the cells daily on the scaffold using an In Vivo Imaging System (IVIS), tracking both growth and migration of the cells. Non-transduced cell-seeded scaffolds were imaged as a control.

RESULTS: hMABs were successfully transduced with the lentivirus as confirmed with flow cyctometry, obtaining an optimal transduction with a multiplicity of infection of 10. The IVIS displayed high sensitivity to detect cell growth in vitro when compared with other invasive methods (MTT and CyQuant cell proliferation assay). Bioluminescence emanated by the cells was easily detected with maximal efficiency through the glass bioreactor and medium, giving clear insights into cell migration that could not be observed with other non-invasive techniques. The cells spread gradually from the point of injection from day 1 to the rest of the scaffold over the 7 days. End-point histology reflected the IVIS results with H&E sections displaying well re-populated oesophageal scaffolds.

DISCUSSION & CONCLUSIONS: In this study we show that using bioluminescence imaging we were able to track hMABs seeded and cultured in a decellularised oesophagus in a non-invasive fashion. Information on the location and viability of the cells was obtained daily, prior to experimental end-point analysis This is an invaluable tool to understand the cell fate post seeding on acellular scaffolds in tissue engineering.

REFERENCES: Totonelli, G. et al., 2013. Detergent enzymatic treatment for the development of a natural acellular matrix for oesophageal regeneration. *Pediatric surgery international*, 29(1), pp.87–95.

Microfluidic cell arrays for characterization of stem cell differentiation

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INTRODUCTION: Mesenchymal stem cells (MSCs) can be isolated from the bone marrow and may be used for regenerative medicine applications because they can differentiate into bone, cartilage and fat cells. However, MSCs constitute less than 1% of the human bone marrow mononuclear cell (BMMNC) population, which presents challenges for their isolation and characterization [1]. Moreover, they are heterogeneous and have variable potential for proliferation and differentiation. Cell surface markers and pathway activation (such as Wnt signalling) has been shown to mark putative stem cell or lineage progenitors, but methods such as flow cytometry limit analyses that can be performed on isolated cells.

Here we present a microfluidic device that is capable of arraying hundreds to thousands of BMMNCs, including MSCs. The device enables MSC microscopy studies without the requirement for a specific MSC purification step.

METHODS: We use a two-height microfluidic device that consists of eight chambers, each with a meandering main flow channel *(Fig. 1)*. Parallel segments of this main channel are connected by adjacent height-restricted cross-flow channels. Hydrodynamic flow patterns guide cells from the main channel into the cell trap pockets in front of these height restrictions (black arrow), sequentially filling the cell trap sites [2]. When the array is completely filled, excess cells can only follow the main channel flow (red arrow), preventing clogging of the microfluidic channels.

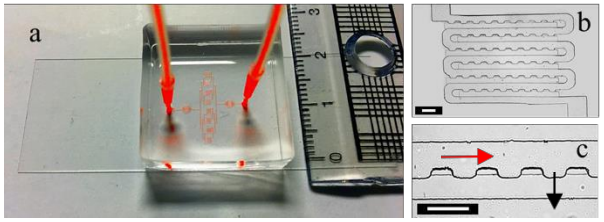

Fig. 1: a) microfluidic device; b) array with 50 µm wide side traps; c) magnification showing the working mechanism (scale bar: 100 µm).

BMMNCs are collected from the bone marrow of patients who underwent total or partial hip replacement. The bone marrow sample is treated with lysis reagents and subsequent density centrifugation results in BMMNC isolation. Following fixation and staining with suitable dyes, the cells are flown through the microfluidic device and trapped cells are imaged with brightfield and fluorescence microscopy.

RESULTS: As preliminary data, we show a single trap site that is occupied by six BMMNCs, identified by their DAPI-stained nucleus *(Fig. 2)*. These cells were also incubated with glycophorin A antibody (red fluorescence tag) that binds to erythroid progenitor cells. The imaged trap site contains six cells as seen in the brightfield image. One of these exhibits red fluorescence and is thus identified as an erythroid progenitor cell.

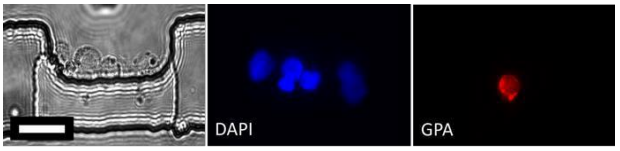

Fig. 2: Microscopy images of arrayed BMMNCs (scale bar: 15 µm). Six cells are retained in a 50 µm wide trap (DAPI). One of these is identified by immunostaining (GPA) as an erythroid progenitor.

DISCUSSION & CONCLUSIONS: These results indicate that the microfluidic cell trapping system can be utilized to array and identify different BMMNC sub-populations by immunostaining. Arraying of single cells is also possible in narrower – 10 µm wide – traps. Future work will address cell signalling properties of putative stem cell populations in a high-throughput manner. MSCs will be selected by specific fluorescent markers (e.g. STRO-1^{+}/GPA^{-}) and analysis on differentiation pathways and drug (differentiation factor) delivery studies will be performed on-chip.

Using non-invasive 3D imaging to monitor performance of 3D-bioprinted hybrid cartilage tissue constructs

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INTRODUCTION: The performance of tissue engineering (TE) constructs is rarely evaluated *in situ*, since current techniques cannot produce high-resolution images of soft tissues[1]. This limitation is especially prescient for cartilage TE, since cartilage has similar properties as surrounding fluid. To address this problem, we use synchrotron radiation-based phase contrast (SR-PC) imaging with computed tomography to monitor the *in vitro* and *in vivo* performance of 3D-bioprinted hybrid cartilage tissue constructs. Specifically, we aim to evaluate biomaterial degradation and structure, as well as secretion of extracellular matrix (ECM), using non-invasive 3D imaging.

METHODS: Our hybrid 3D-bioprinting method mimics the complex mechanical and biological features of cartilage, respectively, using alternating strands of polycaprolactone (PCL) and alginate impregnated with ATDC5 cells[2]. Constructs are cultured for 2 weeks, then either cultured for 4 more weeks (*in vitro*) or implanted subcutaneously into the back of nude mice for 3 weeks (*in vivo*). SR-PC imaging is performed at the BMIT beamline at sample-to-detector distance of 3m; 3D images are reconstructed using NRecon to analyze structural features of degradation. After imaging, constructs are subjected to biological assays to reveal cell viability and cartilage ECM.

RESULTS: Our hybrid 3D-bioprinting method showed high cell viability and secretion of cartilage ECM in constructs that were implanted *in vivo* (Fig. 1). SR-PC imaging revealed both PCL and alginate strands *in vitro* and *in vivo* (Fig.2).

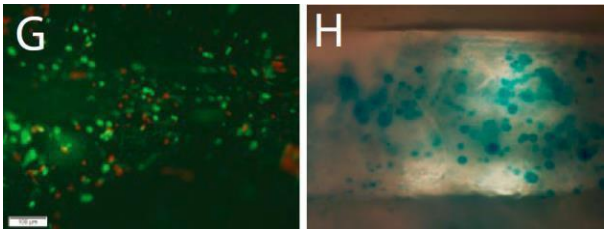

Fig. 1: 3D-bioprinted hybrid cartilage constructs showed good cell viability (G, green(live)/red(dead)), and cartilage matrix production (H, Alcian blue) after 3 weeks in vivo.

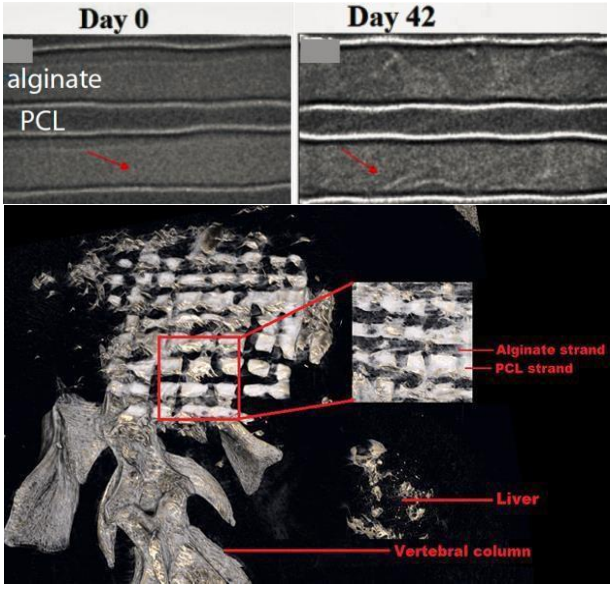

Fig. 2: SR-PC imaging of hybrid cartilage constructs reflects (top) structural changes to alginate (arrows) and PCL strands in vitro, *and (bottom) both materials after 3 weeks* in vivo.

DISCUSSION & CONCLUSIONS: Biological performance of chondrocytes in hybrid constructs indicates the success of the 3D-bioprinting technique for cartilage TE. Our preliminary imaging results demonstrate that SR-PC can visualize both PCL and alginate strands, which is remarkable, given the highly-hydrated nature of cell-impregnated alginate. Importantly, structural changes over time *in vitro* and *in vivo* were evident in these materials. In summary, our data suggest that non-invasive 3D SR-PC imaging can reveal degradation of biomaterials, and future applications might show secretion of cartilage ECM and integration of hybrid cartilage constructs with surrounding tissues.

Real-time and non-invasive measurements of cell mechanical behaviour with optical coherence phase microscopy

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INTRODUCTION: Stem cells play a major role in therapeutic and regenerative medicine due to their self-renewal properties, differentiation abilities and unlimited proliferation capacity. Mechanical stimuli have been shown to have a major role in regulating stem cell behaviour [1], with differentiation defined as a mechanosensitive process controlled by the stiffness of the stem cell substrate [2]. There is therefore a need to investigate the mechanical behaviour of the stem cell niche as well as its response to various mechanical stimuli. To address this need we have developed a novel non-invasive method to perform elastography on live cells at cellular resolution with an optical coherence phase microscopy.

METHODS: A commercial optical coherence tomography system (Callisto, Thorlabs) was coupled to the side port of an inverted fluorescent Leica DMIRE2 microscope. The superluminescent light source, centred at 930 nm with a FWHM of 90 nm provided an axial resolution of 5 μm in water, while the lateral resolution was determined by the objective. The custom scanning head was able to operate in an ad-hoc scanning mode allowing the collection of the varying phase over time [3], and in depth into the sample, with increase phase stability. Mesenchymal stem cells (MSCs), breast cancer cells (MCF-7) and mouse fibroblasts (3T3) were used in this study to provide a broad range of mechanical properties. Cells were cultured in Ibidi microchannels (microslide VI, Ibidi) in their respective medium supplemented with 10% foetal bovine serum, and exposed to cyclic mechanical stimuli inside the microchip ranging from 1,000 $N.m^{-2}$ to 20,000 $N.m^{-2}$ with a typical frequency of 300 mHz. A 4D data cube (256X256x512x36 in (X,Y,Z,T) pixels) was then captured with an acquisition frequency of 1,200 A scans per second to sample the varying phase over time which was converted to displacement through the equation:

$$d = \Delta\phi\lambda_0/(4\pi n) \quad (1)$$

Where; n is the refractive index, λ_0 is the central wavelength and $\Delta\phi$ *is the phase difference between adjacent B scans.*

RESULTS: Cyclic stress was successfully applied directly to cells within the microfluidic chip and the corresponding displacement was recorded in real-time at the nanometre scale for each pixel of the cell (*Fig. 1*). A change in amplitude and/or frequency of the stimuli was translated to a corresponding cell response. Differences were observed in relative strain rates between the cell lines under investigation.

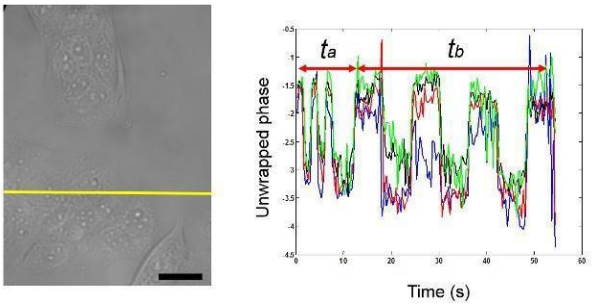

Fig. 1: a) Phase microscopy of MCF-7 monolayer, showing location (yellow line) of time-lapse OCPM B scans. b) Phase response recorded over time and corresponding to a sinusoidal stress with a 3s cycle (t_a) followed by a square wave stress with a 9sec cycle (t_b) and an amplitude of 4000 $N.m^{-2}$. Different colours represent different pixel locations within the cell.

DISCUSSION & CONCLUSIONS: We described a new method to monitor in real-time and non-destructively the mechanical properties of cells in monolayers and 3D gels that is directly translatable to the study of the mechanical behaviour of the stem cell niche.

[1]

ACKNOWLEDGEMENTS: We would like to thank the UKRMP hub for engineering and exploiting the stem cell niche for funding.

Measuring directional diffusion coefficients in native and in tissue engineered cartilage

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INTRODUCTION: The unique load-bearing properties of articular cartilage arise from its biochemical composition and structural organisation. Using appropriate culture conditions, the biochemical composition of cartilage tissue can be reproduced partially by *in vitro* culture. However, the high amount of collagenous fibres and their unique organisation are not achieved, even under loading conditions. Among the possible reasons for this lack of performance, limitations of mass transport of nutrients, growth factors and matrix components within the cultivated cartilage are considered. In this study, a photo-bleaching (FRAP) method is applied to determine directional diffusion coefficients of higher molecular weight substances in native and *in vitro* cultivated cartilage.

METHODS: FRAP measurements were carried out using a line bleaching method allowing for measuring anisotropic diffusion parallel and perpendicular to the joint surface according to Seiffert *et al*. (2005)[1]. Sequential post-bleach images were recorded and the intensity profiles averaged over the bleaching line. The diffusion coefficients were calculated by fitting Gaussian-like profiles to the intensity gradients using a one dimensional solution of Fick's second law. Articular cartilage was obtained from the knee joints of domestic pigs. Tissue slices were cut of full-thickness cartilage and stained for 36 h with FITC dextans (4-500 kDa). For the production of cartilage *in vitro*, the chondrocytes were isolated by sequential enzymatic digestion. TE cartilage was produced from P3 chondrocytes after 2 weeks of re-differentiation in alginate gel and subsequent tissue culture for up to 4 weeks. Diffusion co-efficients within native articular cartilage were measured near the surface, in the central part, and in the lower non-calcified cartilage tissue. The TE cartilage slices were subdivided into the surface area and the inner zone.

RESULTS: Native articular cartilage exhibited a distinct profile of diffusive properties related to the local matrix composition and organization as known from the literature[2,3]. Higher average diffusion coefficients for 70 kDa FITC dextran were measured in the central part (radial diffusion, 60,85 ± 15,67 μm^2*s^{-1}) compared to the surface zone (16,16 ± 10,47 μm^2*s^{-1}) and lateral diffusion coefficients (10,77 ± 6,10 μm^2*s^{-1}, central part) were lower compared to the radial diffusion coefficients. On the other hand, TE cartilage showed no significant differences of diffusion coefficients related to the localization and orientation of the bleaching lines (overall 38,35 ±10,75 μm^2*s^{-1}). These diffusion coefficients were lower compared to the surface zone of native articular cartilage but higher compared to the radial coefficients of the central part. However, biochemical analysis of TE cartilage confirmed lower glycosaminoglycan (GAG) and hydroxy-proline contents per wet weight compared to the native cartilage suggesting a lower matrix density and therefore lower diffusion resistance of TE cartilage vs. native cartilage tissue.

DISCUSSION & CONCLUSIONS: As expected, the diffusion coefficients of FITC dextran in native articular cartilage corresponded with the anisotropic structure of the tissue. However, the relatively low average diffusion coefficients measured for FITC dextran (70 kDa) within TE cartilage were not expected because of the lower matrix density compared to native cartilage. These results suggest that not only biochemical composition but also the micro-structure of the matrix contribute to mass transport properties of articular cartilage compared to TE cartilage.

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ACKNOWLEDGEMENTS: The Matlab code for FRAP evaluation was kindly provided by Seiffert *et al.*[1].

Imaging (tubular) organ templates with a smart polyisocyanopeptide hydrogel

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INTRODUCTION: Current follow-up of implanted templates is hampered by lack of non-invasive methods to longitudinally evaluate template and tissue remodelling, and resorption processes [1]. Non-invasive imaging methods may allow adequate follow-up, provided sufficient information at high resolution can be acquired. In this study we functionalized polyisocyanopeptide polymers (PIC) that can form thermoreversible hydrogels [2] with a contrast agent for magnetic resonance imaging (MRI) and incorporated the polymers into tubular organ templates to test its potential for non-invasive monitoring.

METHODS: Gd-DO3A was conjugated to the PIC polymer at different ratios via free copper strain-promoted click reaction. Afterwards, the functionalized PIC_Gd-DO3A hydrogel was loaded as a fluid to a tubular biohybrid composed of a coupled helical coil (CHC) embedded in a collagen sponge [3-4] via ‘’dip in’’ procedure at 4°C. At room temperature, the polymers gelified and was fully incorporated into the biohybrid forming a tubular organ template. Cell compatibility assays of PIC_Gd-DO3A extractions were performed on human adipose derived stem cells. To evaluate the construct strength, uniaxial compressive loads were applied till 50% of constructs origin diameter on a tensile tester (Z2.5 TN, Zwick/Roell) using a crosshead speed of 50mm/min. For non-invasive imaging, tubular organ templates were embedded in a tissue phantom composed of 1% (w/v) agarose. T1- and T2-weighted MRI of different samples were carried out in an 11.7T magnet resonance imaging scanner (BioSpec, Burker).

RESULTS: Formed templates (l: 20mm; Øi: 6mm) showed a seamless tubular structure which resisted compressive loads (0.09±0.03N/mm). Besides good shape-recovery, no distortion of the hydrogel during compression was observed. MR contrast increased with increasing Gd-DO3A functionalization. The best imaging results were achieved with a functionalization rate of 1:100 on T1-weighted images. However, a slight blooming effect started to occur on T2 weighted images. Furthermore, no cytotoxic effects of PIC_Gd-DO3A-1:100 extractions were observed when adipose-derived stem cells were exposed to PIC_Gd-DO3A.

DISCUSSION & CONCLUSIONS: We incorporated MR imageable PIC polymers in tubular templates and show that these can be adequately imaged compared to tubular templates without a contrast agent. Image quality was closely correlated to the PIC substitution rate. The PIC hydrogel was retained in the tubular templates after carrying external compressive loads which mimicked loads that can be exerted by adjacent tissues after template implantation. Addition of the MR imageable moiety (Gd-DO3A) did not lead to cytotoxic effects. Functionalized PIC hydrogels may be highly suitable for non-invasive monitoring of tissue engineered templates to localize and follow regeneration-, remodelling-, as well as resorption processes.

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ACKNOWLEDGEMENTS: The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement No. 607868 (iTERM).

Dose and particle size effects for SPIO-enhanced MRI as method to assess vascularization of 3D scaffolds planted on the chorioallantoic membrane of the chick embryo *in ovo*

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INTRODUCTION: We recently presented Magnetic Resonance Imaging (MRI) as a nondestructive *in vivo* readout to report perfusion capacity in biomaterials planted on the chorioallantoic membrane (CAM) in the living chick embryo *in ovo*. Perfusion capacity was assessed in various scaffold materials through changes in the longitudinal (T1) relaxation rates before and after injection of Gd-DOTA (Dotarem®, Guerbet S.A.). Hence local contrast agent concentration was dependent on perfusion, vascular permeability and extravascular compartment size. Here, we explore intravascular SPIO particles of 30-40 nm (M) size (Viscover™, Miltenyi Biotec, Germany) that stay in the vasculature to deliver a more direct measure of vascularization.

METHODS: Fertilized Lohman white LSL chick eggs were incubated at 37°C and 65% relative humidity. After 3.5 days a hole was excised into the eggshell and on incubation day (ID) 7 scaffolds were placed onto the CAM. MRI was conducted on ID 14. T1- and T2-weighted MR images were acquired from a sagittal slice through the scaffold at a spatial resolution 200 x 200 x 1000 um^3, with a RARE sequence of variable TR and TE pre and 8 and 140 min post i.v. injection of SPIOs at a dose of 80 umol/kg Fe.

RESULTS: MR images devoid of motion artifacts were obtained and the scaffold was clearly and reproducibly depicted in all MRI sessions (Fig. 1), indicating that our medetomidine anesthesia protocol optimized for this special application offered proper sedation of the chick embryo. No signal change was observed within the egg yolk, consistent with the SPIO remaining in the vasculature. Consequently, T1 positive signal enhancement (reduction in T1) and T2 negative contrast (reduction in T2) were observed only in the vasculature and hence were restricted mainly to the surface of the CAM (Fig 1, arrowheads). Effect upon T2 was stronger than in T1. Interestingly, no change in contrast was observed inside the scaffold, which might relate to our observation that the slightly viscous contrast agent did not distribute easily but rather slowly within the finer segments of the vasculature, like in the scaffold. Consistent with this notion is the observation that T1 as well as T2 reduction was more prominently seen at 140 min than 8 min post i.v. injection of the SPIO.

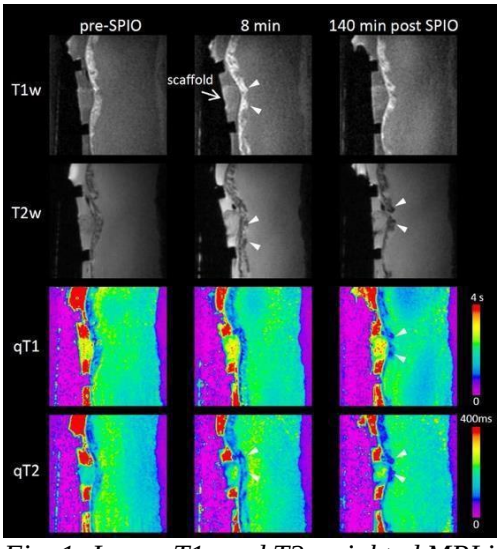

Fig. 1: In ovo T1- and T2-weighted MRI images and corresponding T1 and T2 quantitative maps from a sagittal slice through the collagen scaffold. Left to right: pre, 8 min and 140 min post SPIO injection. Arrowheads denote vessel structures on the CAM, which show contrast enhancement in T1 (positive) and T2 (negative enhancement).

DISCUSSION & CONCLUSIONS: This experiment demonstrates that SPIO-enhanced MRI is feasible in and well-tolerated by the chick embryo. Next we will explore smaller (10-20nm) SPIOs for their stronger (positive) T1 effect and XXL size (60-70nm) SPIOs for comparison.

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

Study of the involvement of seeded MSCs in bone repair using the model of transgenic mice

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INTRODUCTION: There are three key elements in tissue engineering to treat bone defects: cells, scaffolds and growth factors [1]. The cell approach consists of the preliminary seeding of cells onto scaffolds. Mesenchymal stem cells (MSCs) are thought to be the most attractive for making a bone repair [2,3]. However there is much that is unknown about MSCs and which needs to be established before this treatment can be widely applied in clinical situations. The purpose of the present work was to study the involvement of seeded allogeneic MSCs in bone formation, *in vivo*, using the model of transgenic mice and genetically labeled cells

METHODS: The scaffolds were sterilized, individually seeded with MSCs from the bone marrow of male 5-week-old GFP(+) transgenic C57/Bl6 or GFP(-) male C57/Bl6 mice. Critical-sized defects were created on the calvarial bone of each animal. Scaffolds with or without seeded cells were implanted into the injury sites. The cranial bones were harvested at either 6 or 12 weeks after implantation. All samples were stained with Hoechst and observed using fluorescence microscopes. GFP(+) transgenic mice having scaffolds with non-labeled MSCs were used for the observation of host cell migration into the scaffold. GFP(-) mice having scaffolds with GFP(+)MSCs were used to assess the functioning of the seeded MSCs. The control group comprised GFP(+) transgenic mice having scaffolds without any cells.

RESULTS: The data demonstrated that allogeneic MSCs were found on the scaffolds 6 and 12 weeks later. Moreover by week 12 there was newly formed bone tissue from the seeded cells without requiring osteogenic pre-differentiation. What is more important, host cells did not appear, and the control scaffolds without seeded cells remained empty. Also the possibility was shown for vessel formation from seeded MSCs without preliminary cell cultivation under controlled conditions.

DISCUSSION & CONCLUSIONS: There are many parameters that can affect the functions and behavior of MSCs. Cultures of MSCs are very heterogeneous, even within the same colony [4]. The heterogeneity and morpho-functional variability of MSCs may explain some of the conflicting data in the different research works. Among these pieces of data, the role of allogenic MSCs in bone formation should be highlighted. Although the exact mechanisms of the involvement of the seeded allogeneic MSCs in bone formation need further investigation, our data contributes to the understanding of the positive results of MSC transplantation.

ACKNOWLEDGEMENTS: This work has been financially supported by the Russian Science Foundation (grants No. 14-15-00536) and the Russian Foundation for Basic Research (№ 15-29-04851 ofi_m).

Quantitative evaluation of bone regeneration in a craniofacial implant using computed tomography images

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INTRODUCTION: Computed tomography (CT) is an important medical imaging technique to diagnose disease or trauma, plan therapeutic procedures and monitor the outcome of medical treatments (e.g. bone healing or bone ingrowth into implants). The technique also allows for three-dimensional visualisation and evaluation of implants in patients. Commonly, CT images are qualitatively evaluated. The image of for example an implant is visually examined to determine e.g. the level of tissue regeneration and the possible movement of the implant. Conversely, a quantitative approach is widely being used in the evaluation of micro-computed tomography (µCT) [1]. It would be of great interest to obtain also numerical data from patient CT images. However, the low resolution of the CT images might be a limiting factor.

The objective of the present study is to verify the feasibility of quantitative evaluation of bone regeneration using CT images.

METHODS: A patient was implanted with a bioactive calcium phosphate-based craniofacial implant (~18 cm^2 in size, manufactured by OssDsign). Previous studies have shown that this type of implant stimulates bone regeneration of cranial defects [2, 3]. The craniofacial implant and the method of implantation have been described in previous studies [2, 3]. The patient was CT scanned post operatively and followed up after 3, 8, 12 and 27 months. CT images were analysed using the software CTAn (Skyscan, Bruker, version 1.13). The images were binarised to separate the bone from the soft tissue and the titanium grid. Thereafter, a quantitative 3D analysis was conducted calculating the total bone volume in the region of interest (the same region was used for all scans). By comparing the total bone volume data from each image to the first scan, the bone regeneration could be evaluated for each clinical follow-up.

RESULTS: The quantitative analysis of the CT images did not show any bone regeneration at 3 or 8 months. One year after surgery approximately 1 % newly generated bone was observed. In the last clinical follow-up, 27 months after surgery, the bone regeneration had increased to nearly 8 %.

3D CT images and corresponding binarised images from three follow-ups can be seen in *Fig.1*. Note: the results cover only one patient and should be considered as preliminary.

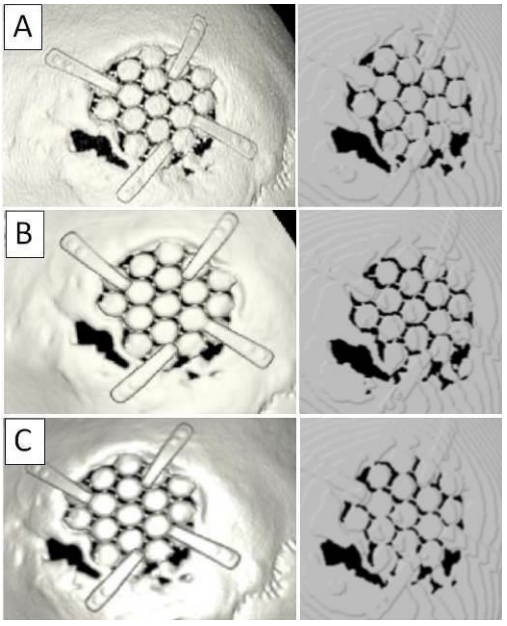

Fig. 1: Patient CT-scans from 3 months with no bone regeneration (A), 12 months with 1 % bone regeneration (B), and 27 months with 8 % bone regeneration (C).

DISCUSSION & CONCLUSIONS: This study demonstrates a potentially important use of quantitative evaluation of CT images in biomaterial implant follow-up. A quantitative evaluation method could increase the understanding of clinical results and provide valuable numerical data.

Characterization of metabolic changes of mesenchymal stem cell during adipogenic differentiation by two-photon FLIM

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INTRODUCTION: The non-invasive imaging of cell metabolism within tissues to assess the efficacy of stem cell therapy and understanding the tissue development is of great interest. In this study we investigated metabolic changes in mesenchymal stem cells (MSCs) during adipogenic differentiation, based on the fluorescence of the metabolic co-factors NADH, NADPH, and FAD. Cellular metabolism was examined by monitoring the optical redox ratio (FAD/NAD(P)H), the fluorescence lifetime contributions of the free and bound forms of NADH and the bound form of NADPH. Two-photon fluorescence microscopy combined with FLIM was used to analyze this fluorescence in living cells.

METHODS: Undifferentiated MSCs and MSCs on 5, 12, 19, 26 days of adipogenic differentiation were imaged with a Zeiss 710 microscope coupled to a FLIM system. The intrinsic fluorescence of NAD(P)H and FAD was excited at 750 nm and 900 nm respectively by a femtosecond Ti:sapphire laser. The data were analyzed with the commercially available SPCImage software. The fluorescence lifetimes and their contributions (free and protein-bound forms of NAD(P)H: a_{free} NADH, a_{bound} NADH, a_{bound} NADPH) for the areas of interest were calculated by finding the global minimum of the $\chi 2$ value for three-exponential fittings.

RESULTS: To estimate the general level of metabolic activity of the cells during adipogenic differentiation, the fluorescence intensities of NAD(P)H and FAD were measured and represented as their redox ratio (FAD/NAD(P)H). The optical redox ratio FAD/NAD(P)H decreased during adipogenic differentiation from 0.46±0.15 to 0.33±0.19 a.u. This was likely to be explained by the intensive biosynthesis of lipids and the enhanced NADPH production associated with this. To study cellular energy metabolism and fatty acid synthesis during adipogenic differentiation, we analyzed the fluorescence lifetime contributions of the free and protein-bound forms of NADH and NADPH. A statistically significant decrease in the contribution of free NADH (a_{free}NADH) was shown in differentiated cells on days 26 (53.2±10.7 %) compared to undifferentiated MSCs (60.7±2.8 %). The reverse trend was observed in the contribution of bound NADH (a_{bound}NADH) (from 29.5±1.8 % in undifferentiated MSCs to 34.6±8.2 in adipogenically undifferentiated MSCs on 26 day). The contribution of bound NADPH (a_{bound}NADPH) was higher also on 26 day of adipogenic differentiation (12.3±3.2 %) when compared with undifferentiated MSCs (9.7±1.7). The increase in the contribution of protein-bound NADH testify to the metabolic switch from glycolysis to oxidative phosphorylation during the differentiation of stem cells. The rise in the contribution of bound NADPH is probably associated with the biosynthesis of lipids and oxidative stress.

DISCUSSION & CONCLUSIONS: So we elucidate the metabolic changes in MSCs during adipogenic differentiation, based on the fluorescence of the metabolic co-factors NADH, NADPH, and FAD using the methods of two-photon fluorescence microscopy combined with FLIM. To estimate the contribution of energy metabolism and lipogenesis in the observed changes of the metabolic profile, a separate analysis of NADH and NADPH is required. In our study we demonstrated an increased contribution of protein-bound NADPH in adipocytes that is associated with lipogenesis. The optical redox ratio FAD/NAD(P)H decreased during adipogenic differentiation, and that this was likely to be explained by the intensive biosynthesis of lipids and the enhanced NADPH production associated with this [1]. Based on the data on the fluorescence lifetime contribution of protein-bound NAD(P)H, we registered a metabolic switch from glycolysis to oxidative phosphorylation in adipocytes.

ACKNOWLEDGEMENTS: This work has been financially supported by Russian Science Foundation (grants No. 14-15-00536).

Online markers as a potential tool for non-invasive monitoring of tissue development

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INTRODUCTION: The lack of techniques for non-destructive monitoring of tissue-engineered (TE) constructs is recognized as an issue which will complicate the translation of products from the lab to the clinic in the near future [1]. The aim of this study was to establish a non-invasive technique to evaluate the extracellular matrix (ECM) maturation of a TE implant during the in vitro maturation.

METHODS: Biomarkers of ECM components such procollagen I carboxyterminal peptide (PICP), procollagen III aminoterminal peptide (PIIINP), tropoelastin and sulphated glycosaminoglycans (sGAGs) present in the culture medium of a TE implant were quantified by commercially available assays and correlated to their amount in the ECM of the same construct. A TE heart valve developed for mitral replacement was used as a dynamic model and was evaluated after 7, 14, 21 and 25 days (n=3 per time point) of maturation performed into a dynamic bioreactor. Mechanical properties were evaluated by burst strength measurements. Presence of collagen, elastin and sGAGs was verified by immunohistochemistry.

RESULTS: PICP and PIIINP, cleavage products of collagen formation, correlated with the collagen content of the tissue-engineered valves (Pearson correlation coefficient =0,99) for both markers and with the mechanical properties. Tropoelastin, a precursor of elastin, was detected in the culture medium and correlated to the elastin found in the ECM of the tissue-engineered valves (Pearson correlation coefficient = 0,99). sGAG secreted into to the culture medium correlated to the amount present in the ECM of the tissue-engineered valves (Pearson correlation coefficient = 0,98). Immunohistochemistry confirmed the presence of collagen types I and III, elastin and sGAG, with a clear increase over the cultivation time.

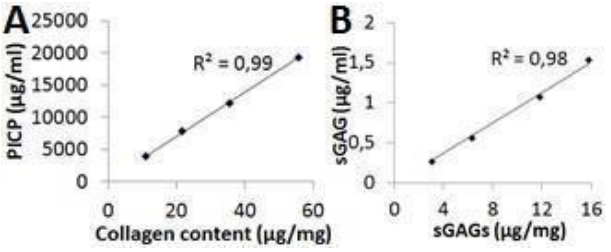

Fig. 1: Correlation of PICP in the medium culture with the collagen content (A) and correlation between sGAG detected in the medium with the sGAG amount in the tissue.

DISCUSSION & CONCLUSIONS: Non-invasive monitoring methods of tissue-engineered implants are crucial tools for quality management. This study demonstrated the feasibility to monitor non-invasively a complex cardiovascular TE implant by using a repertoire of commercially available assays. The method is applicable as it requires only small amounts which can be repeatable taken from the bioreactors, is easy to perform can be carried-out in a common tissue-engineering laboratory. The information acquired with this technique will be crucial to act with corrective actions during the in vitro maturation of tissue-engineered constructs or will prevent the implant of constructs with inadequate tissue composition and properties.

ACKNOWLEDGEMENTS: This research was funded by the People Program Marie Curie Actions of the European Union's Seventh Framework Program FP7/2007–2013 (REA grant agreement n°317512) and by the Integrated Interdisciplinary Institute of Technology for Medicine (I³TM) of RWTH Aachen University (Seed-Fund SF_14-4-08).

Non-destructive techniques to follow skeletal stem cell development for skeletal repair and regeneration

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INTRODUCTION: The use of skeletal stem cells (SSCs) in clinical applications is one of the most promising areas for skeletal tissue repair and disease treatment [1]. Currently, SSCs differentiation into the stromal lineages of bone, cartilage and fat, is evaluated through a number of assays which are invasive, require cell fixation or lysis, and/or are destructive. Current limitations in the characterisation of SSCs have led to the application of alternative strategies that aim to identify molecules at the subcellular level by using their inherent properties, without the use of any dye or label, *i.e.* "label-free" [2]. Label-free methods such as Raman spectroscopy, coherent anti-Stokes Raman scattering (CARS) and second harmonic generation (SHG) microscopy are minimally invasive (or non-invasive at low laser powers) and non-destructive, and are emerging as powerful alternatives to conventional techniques in biomedicine.

METHODS: Human adult bone marrow-derived SSCs and human foetal femur-derived SSCs were differentiated into chondrocytes and adipocytes for 14 days. Chondrogenic and adipogenic differentiation was assessed through quantitative real-time polymerase chain reaction (qPCR) analysis. SSCs were also characterised using Raman spectroscopy, CARS and SHG microscopy, to evaluate the potential of label-free techniques for skeletal regeneration.

RESULTS: Chondrogenic and adipogenic differentiation of adult and foetal SSCs was verified by gene expression analysis. Raman spectra provided chemical and molecular information of SSCs, before and after differentiation. By analysing the Raman spectra, it was possible to distinguish several biochemical changes in SSCs associated with the chondrogenic and adipogenic differentiation (Fig. 1). The formation of lipid droplets was assessed by CARS (Fig. 2a), and the presence of collagen fibres during the chondrogenic differentiation was detected using SHG (Fig. 2b), in the absence of any external dye/fluorophore.

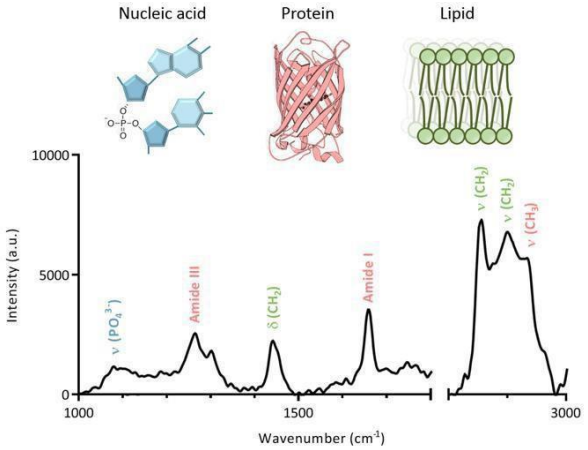

Fig. 1: Raman spectrum of SSCs cultured in adipogenic media for 14 days.

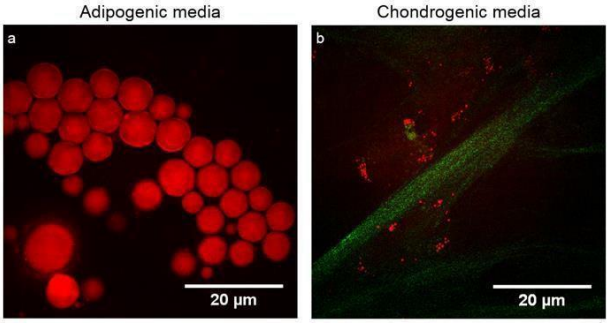

Fig. 2: (a) CARS image of SSCs cultured in adipogenic media (lipid droplets in red); (b) simultaneous imaging of lipids (red, CARS) and collagen fibres (green, SHG) of SSCs cultured in chondrogenic media.

DISCUSSION & CONCLUSIONS: The label-free and non-destructive nature of Raman spectroscopy, CARS and SHG microscopy present an effective alternative to characterise and monitor SSC development for skeletal repair and regeneration.

ACKNOWLEDGEMENTS: Financial support from the Institute for Life Sciences, University of Southampton, BBSRC (BB/L021072/1), ERC grant Nano-ChemBioVision 638258 and Wessex Medical Research is gratefully acknowledged.

Nonlinear microscopy to investigate brown and white adipocytes in three-dimensional tissue-mimicking environments in comparison with adipose tissue

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INTRODUCTION: With the rising global prevalence of obesity and the acknowledgement of the adipose tissue as an endocrine organ, it has become an important drug target. One strategy focuses on the understanding of brown adipose tissue (BAT), characterized by multilocular lipid stores, higher mitochondrial content and metabolic activity, and its relation to white adipose tissue (WAT) [1]. Current research relies on animal models or two-dimensional cell culture systems, lacking important properties of the human tissue. To meet this problem a variety of novel 3D cell culture systems have been reported in recent years, one of which is an elastin-like recombinant protein-engineered hydrogel (ELP) [2]. Here cell adhesion densities (RGD sequences) and mechanical properties can each be tuned at amino acid level and, hence, it is possible to influence cell differentiation, morphology and functionality. Toward the goal to form an adipose tissue model, which recapitulates the plasticity of adipose tissue, we investigate whether different microstructured ELP substrates can promote browning of adipose-derived stem cells. Additionally, we use microscopic methods to study the effect of metabolic cues *in vivo* and *in vitro*.

METHODS: We employ nonlinear microscopy techniques to study cells and cell matrix interactions with minimal sample preparation. Three different techniques are used at the same time: (i) Coherent Anti-Stokes Raman Scattering (CARS) for label-free imaging of lipids and proteins to monitor the number and sizes of lipid stores, as well as the microstructure of the ELP materials (ii) Second Harmonic Generation (SHG) of the structure of native ECM components like collagen and (iii) Multi-Photon Excited Fluorescence (MPEF) of rhodamine 123-labelled live mitochondria.

RESULTS: By systematically varying the morphological properties of the microstructured ELP materials, we were able to identify structures, which promoted cell adhesion and differentiation into adipocyte-like cells, forming promising platforms for studies of the browning of white adipose derived stem cells *in vitro*. Figure 1 displays an overlay of CARS, SHG and MPEF images of WAT and BAT from a mouse. By quantifying cellular lipid content and mitochondrial activity from the images we can show that our multimodal molecular microscopy approach is capable of distinguishing between WAT, BAT and even a β3-adrenergetic receptor agonist treated WAT.

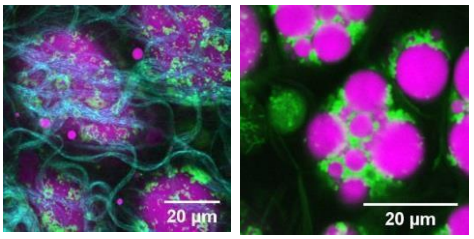

Fig. 1: WAT (left) and BAT (right): cellular lipid depots (pink, CARS), collagen (cyan, SHG) and live mitochondria (green, MPEF).

DISCUSSION & CONCLUSIONS: We have developed a non-invasive multimodal microscopy method, which is capable of following changes in lipid and mitochondrial content associated with the browning process of WAT. To further understand how the ECM influences this process, we are developing a 3D cell culturing system based on recombinant proteins.

ACKNOWLEDGEMENTS: The research leading to these results has received funding from the People Program (Marie Curie Actions) of the European Union's FP7/2007-2013/ Program under REA grant agreement nº[607842].

Optical measurement of oxygen concentration in melanoma spheroid and skin engineered models

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INTRODUCTION: Non-invasive quantitative measurement of oxygen concentration within cells and tissues is essential for understanding of normal and pathological processes, in particular tumourigenesis. One of the most promising optical methods for measuring oxygen concentration is by 2-photon laser scanning phosphorescence lifetime imaging microscopy (2PE-PLIM). We have used a phosphorescent dye (PtL^{s}Cl) that is ubiquitously but heterogeneously distributed within a whole cell and quenches in the presence of oxygen [1,2]. Phosphorescence lifetime imaging (PLIM) has many advantages over intensity measurement, for example it does not depend on fluorophore concentration and provides autofluorescence nullified imaging [3,4]. Furthermore, PLIM used with 2-photon laser excitation is useful as it allows deep imaging as the excitation light penetrates to greater depths than 1-photon excitation. The aim of this study was to investigate if a PtL^{s}Cl label could be combined with 2PE-PLIM for detection of a hypoxic microenvironment in a 3D *in vitro* melanoma spheroid within a human tissue engineered skin model.

METHODS: Human melanoma cell lines (HBL, A375-SM and C8161) were cultured in 2D monolayer or in to a multicellular tumour spheroid. In addition, a novel tissue engineered model was developed – this was comprised of the melanoma spheroids contained within a native human de-epidermized dermis, plus cultured human keratinocytes and fibroblasts in order to mimic developing melanoma tumours. Using the resultant model we then established a PLIM imaging method to detect the tumours by way of the oxygen concentration therein.

RESULTS: Our results showed a steep increase in phosphorescence lifetime in cells under hypoxic conditions compared to normoxic conditions. Similarly, measurements through the depth of the melanoma spheroids showed that oxygen concentrations were highest at the outer region and gradually decreased towards the core (*Fig. 1*). Lifetime measurements on melanoma tissue engineered models showed short, normoxic lifetime values in normal stroma and longer, hypoxic values in melanoma spheroid areas (*Fig. 2*).

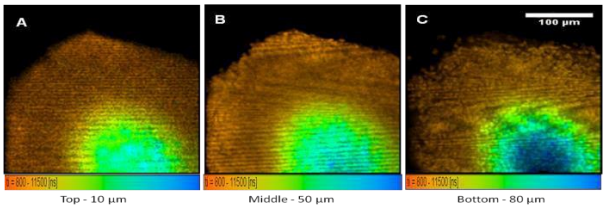

Fig. 1: Lifetime distribution of PtL^{s}Cl through a human melanoma spheroid using 1PE-PLIM.

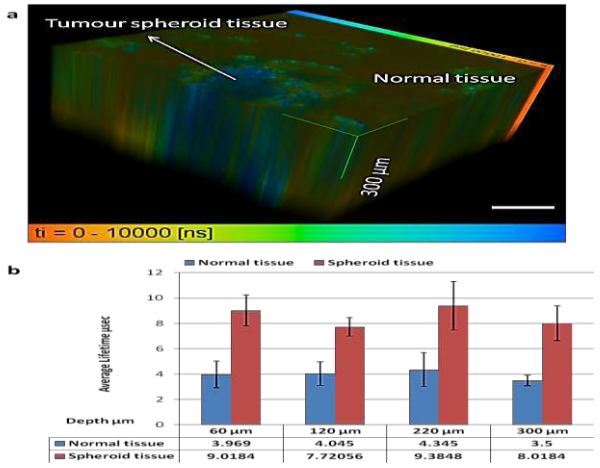

Fig. 2: Lifetime distribution of PtL^{s}Cl through a tissue engineered skin model containing melanoma spheroids using 2PE-PLIM.

DISCUSSION & CONCLUSIONS: The method provides a high-resolution, non-invasive, autofluorescence free measurement of oxygen concentration, which can be used to detect melanoma cells within a human tissue engineered skin construct.

ACKNOWLEDGEMENTS: We thank the BBSRC (U.K.) for funding AR with a PhD studentship, the EPSRC (U.K.) for funding and STFC for PLIM expertise.

A novel computational tool for 3D morphometric analysis of bone-resorbing osteoclast activity

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INTRODUCTION: Osteoclasts (OC) are multi-nucleated cells highly specialized in bone matrix resorption. Bone-resorbing OC acts in a concerted manner with bone-forming osteoblasts to achieve bone remodeling, which is tightly regulated by systemic hormones, autocrine/paracrine factors and neurotransmitters expressed by nerve fibers, such as neuropeptide Y (NPY). It is known that NPY Y_1 receptor knockout ($Y_1^{-/-}$) mice exhibit a high-bone mass phenotype [1-2], due to increased osteoblast activity. Nevertheless $Y_1^{-/-}$ OC resorption activity has never been assessed. Currently, there is a lack of available investigational tools to quantify specifically the osteoclastic resorption activity *in vitro*. Thus, in this work we developed a computational tool (BonePit) that allows detailed tridimensional (3D) quantitative analysis of resorption pits generated by OC.

METHODS: OC precursors derived from $Y_1^{-/-}$ and WT mice were seeded on top of dentine matrices and allowed to generate resorption pits (n=6 per condition). Dentines were then stained with calcein and volumetric image stacks were obtained on a laser-scanning confocal microscope system. The custom made program (BonePit) was written using MATLAB 2013a (MathWorks, USA) software. Briefly, resorption pit contours were manually defined in each layer of the image stacks. An automatic method combined all the contours, generating a mesh with 3D reconstruction (Fig. 1). This 3D reconstruction was used to calculate pit's volume, depth, top surface area and aspect ratio.

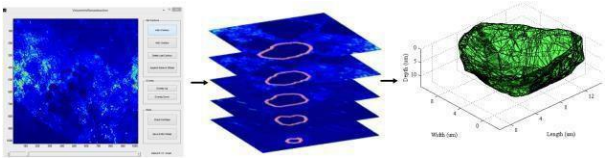

Fig. 1: Stacked images of calcein-stained dentine matrices are loaded into BonePit software. The program applies a Gaussian filter to reduce noise and changes the color representation to more easily identify pit regions. The contours of a single pit are then manually drawn in each stack and compiled into a mask. Every point in the mask is connected with the neighboring points creating a 3D reconstruction mesh of the resorption pit.

RESULTS: Resorption pits 3D reconstructions revealed striking morphological differences between WT and $Y_1^{-/-}$ genotypes. Quantitative analysis demonstrated that $Y_1^{-/-}$ resorptive pits have a significantly smaller top section surface area ($p<0.001$). To analyze these differences in detail, resorption pits were divided into three categories accordingly with their surface area: (a) small (<500 μm^2); b) medium (500-1000 μm^2) and (c) large (>1000 μm^2). The percentage of resorption pits calculated per each category revealed that $Y_1^{-/-}$ resorption pits are significantly small and medium sized, whereas WT pits are evenly distributed throughout all categories ($p<0.0001$, Fisher's exact test). Furthermore, 3D analysis showed that $Y_1^{-/-}$ resorption pits displayed a drastic reduction in volume and in depth, when compared to WT resorption pits ($p<0.001$). As an attempt to measure pit's circularity, the maximum length was divided to maximum perpendicular size, allowing conveying the "aspect ratio", where 1 corresponds to an approximately circular pit. $Y_1^{-/-}$ resorption pits are distributed far from the 1 value, in opposition to WT.

DISCUSSION & CONCLUSIONS: This comprehensive 3D analysis revealed that $Y_1^{-/-}$ OC exhibit a diminished resorbing capacity that results in a substantial reduction in resorption pit formation and eroded area, thus highlighting the therapeutic potential of targeting Y_1 receptor signaling pathway in osteoclastic bone diseases. It is recognized that the geometry of resorptive cavities (pits *versus* trenches) affects bone structure and its mechanical properties, thus taking advantage on the program developed future work are warranted to analyze this aspect in $Y^{-/-}$ mice.

ACKNOWLEDGEMENTS: Funding through National Funds and FEDER via the PT2020 Partnership Agreement under the 4293 Unit I&D.

A method to quantify MSC extracellular matrix secretion in hyaluronic acid-based hydrogels

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INTRODUCTION: The extracellular matrix (ECM), which is composed of cell-secreted molecules including proteins, such as collagens, and glycosaminoglycans (amongst others), can direct cell behaviours such as signalling, proliferation, and differentiation. [1] Cell-laden hydrogels are widely used in cell biology to study cell-matrix interactions due to their ability to recapitulate many aspects of the native 3D tissue niche. [2, 3] Characterising the secreted matrix in 3D is critical for understanding these processes, and may guide the design and evaluation of tissue engineering-based therapies. Here we used a tunable 3D hydrogel system based on thiolated HA (S-HA), which we crosslinked with various concentrations of poly (ethylene glycol) diacrylate (PEGDA). We encapsulated human bone marrow-derived mesenchymal stem cells (hBM-MSCs) to study their secreted matrix and describe a method for characterizing and carrying out a semi-quantitative analysis of their secreted proteins.

METHODS: hBM-MSCs were cultured in growth medium and utilized prior to passage 5. Hydrogels with varying concentrations of PEGDA were prepared. After 28 days of culture, hydrogels were fixed in 4% w/v PFA and embedded in a solution of 7.5% w/v gelatin, 15% w/v sucrose and 0.05% v/v sodium azide in PBS. Samples were then sectioned at 10 µm on a cryostat and stained using Avidin-Biotin enhanced immunohistochemistical methods to visualize types I and II collagen (col I/II) secretion. Sections were imaged on an A1R Si Confocal Microscope. NIS-Elements Advanced Research software was used for semi-quantitative image analysis. After background subtraction, the signal intensity and the volume of cell secreted proteins were measured and calculated. A minimum of 10 cells per condition were analyzed.

RESULTS: hBM-MSCs encapsulated within HA-PEGDA hydrogels were successfully cryo-sectioned after embedding in a solution that matched the hydrogels' mechanical properties. A two-step immunohistochemistry staining protocol was developed and used for signal amplification. The majority of hBM-MSCs showed positive staining for col I and II. Matrix protein density and the mean quantity of secreted proteins per cell were semi-quantitatively assessed using equations 1 and 2, respectively. 'Sum intensity' was calculated by summing all the intensity values of all pixels on the appropriate channel. Our results show that more extracellular matrix protein per cell was secreted by hBM-MSCs within clusters compared to by isolated cells.

$$\text{Protein density} = \frac{\text{Sum intensity}}{\text{Volume of ROI}} \quad (1)$$

$$\text{Mean protein per cell} = \frac{\text{Sum intensity}}{\text{cell number}} \quad (2)$$

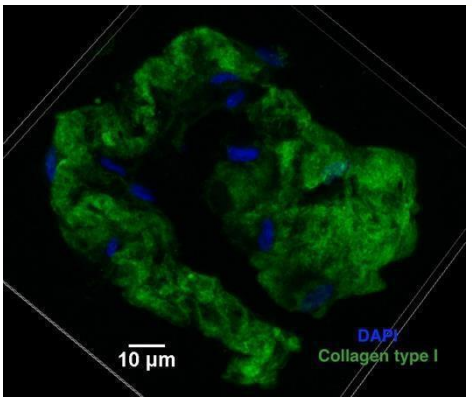

Fig. 1: hBM-MSCs (5 million cells/ml) encapsulated in a HA-PEGDA hydrogel surrounded by secreted matrix protein (collagen type I).

DISCUSSION & CONCLUSIONS: hBM-MSCs secreted col I/II, but its quantity and localisation in the intra and extracellular spaces were dependent on the proximity of nearby cells. This suggests that ECM protein secretion and hierarchical assembly are dependent not only on cell-matrix interactions, as others have reported [4], but also paracrine effects. Moreover, this observation highlights the complexity of cell-cell, cell-secreted matrix and cell-hydrogel interactions in the 3D environment.

ACKNOWLEDGEMENTS: This work was supported by a Wellcome Trust Research Career Development Fellowship (E.G.) and a fellowship from the Whitaker International Program (A.S.).

Generation of a complete substitute of the hard palate by tissue engineering

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INTRODUCTION: Cleft palate repair often requires surgical grafting of remaining native tissues to restore palate function [1]. This approach may influence the development of midface and dentition. Current tissue engineering protocols are typically focused on the development of partial palate substitutes consisting in oral mucosa or bone. For the first time, in this work we have developed a full-thickness palate substitute that recapitulates the anatomical structure of the palate consisting of an oral mucosa layer with epithelium and lamina propria and an underlying bone tissue to determine the possible clinical potential of this osteomucosal multilayered structure for cleft palate regeneration.

METHODS: First, primary cell cultures of rabbit adipose tissue-derived stem cells (ADSC) and oral mucosa fibroblasts and keratinocytes were cultured and expanded using specific culture media. First, a bone-like substitute was generated using osteogenic conditioning medium on ADSC immersed in nanostructured fibrin-agarose hydrogels as previously reported [2]. Then, a multilayered palate substitute (MPS) was developed using the same biomaterial, and MPS were grafted in vivo in New Zeland rabbits. Histological, histochemical and immunofluorescent analyses were performed in order to evaluate the three compartments of the MPS (epithelium, lamina propria and bone-like tissue) ex vivo and in vivo.

RESULTS: In general, MPS revealed the presence of a trilaminar structure consisting of a stratified epithelium, a cell-rich lamina propria and an underlying bone-like tissue. Moreover, MPS samples implanted in vivo on rabbits showed a well stratified epithelium with rete ridges and chorial papillae, although these structures did not reach the development state found in control rabbit oral mucosa tissues. Our immunofluorescence analyses revealed high expression of oral mucosa cytokeratin CK13 in the epithelium of the bioengineered tissues, especially after in vivo grafting. In addition, the analysis of the lamina propria showed negative expression of type-I collagen in ex vivo samples, but this component was very abundant after in vivo implantation, although the expression levels were lower than control tissues. Finally, alizarin red staining revealed the presence of mineralized tissue at the bone-like layer of MPS after in vivo grafting (similar to control bone).

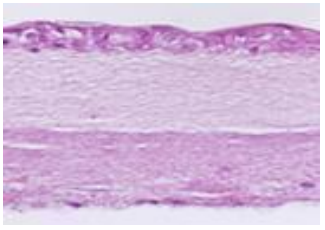

Fig. 1: Histological analysis of ex vivo generated MPS tissues showing the epithelial layer on top, the lamina propria in the middle and the bone-like layer below. H&E staining.

DISCUSSION & CONCLUSIONS: These results suggest that current biofabrication methods allow the generation of complex multi-layered tissues by tissue engineering. Development of a highly biomimetic palate substitute could contribute to the clinical treatment of patients with severe palate defects. Our results point out the clinical relevance of this novel method of multilayered tissue based on tissue engineering techniques as a new strategy for craneofacial regeneration.

2

ACKNOWLEDGEMENTS: This study was supported by the Spanish Plan Estatal de Investigación Científica, Desarrollo e Innovación Tecnológica (I+D+I) 2013-2016 from the Spanish Ministry of Economy and Competitiveness (Instituto de Salud Carlos III), grant FIS PI14/2110 and grant FIS PI15-2048 (co-financed by FEDER funds, European Union), and by grant SAS PI- 0386-2014 from Consejería de Salud, Junta de Andalucia, Spain.

Axonal regeneration and myelination trough fibrin-agarose/mesenchymal stem cell based nerve guides

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INTRODUCTION: Peripheral nerve tissue engineering is focused on the generation of bio-artificial substitutes to bridge severe nerve defects. Nowadays, several biomaterials were successfully used for the generation of different kinds of nerve guides or conduits, which showed promising experimental and even clinical results [1]. These conduits were efficient for the reconstruction of short nerve gaps, but they were not able to support peripheral nerve regeneration in large nerve gaps. In this sense, we described here a peripheral nerve reconstruction model based on the use of collagen type I conduits filled with fibrin-agarose hydrogels (FAH) and FAH containing adipose derived mesenchymal stem cells (ADMSC).

METHODS: 36 adult male Wistar rats were deeply anaesthetized and 1 cm of the left sciatic nerve was removed. The nerve defect was repaired by using collagen type I conduits (P-CTR) or collagen type I conduits filled with FAH (A-FAH) or with FAH containing ADMSC (C-FAH) (n=12 in each). After 12 weeks, animals were subjected to electromyography and euthanized for quantitative histochemical and immunohistochemical analysis of peripheral nerve regeneration.

RESULTS: Electromyography showed clear signs of re-innervation in both FAH-based groups as compared to hollow collagen conduits. These results were significantly higher when ADMSC were used as compared to the other experimental groups. Histological analyses revealed an advanced process of peripheral nerve regeneration in all groups, which was characterized by the presence of newly-formed nerve fascicles with axonal regeneration and myelination. When we compared all the experimental groups, histology confirmed the electrophysiological findings and both FAH-based groups showed better histology than hollow conduits. Quantitative histological analyses revealed that the use of FAH containing ADMSC as intraluminal filler trigger a significant increase of the peripheral nerve regeneration as compared to the other experimental groups. The regeneration profile of this group was characterized by a significantly higher axonal regeneration, myelination and extracellular matrix synthesis (fibrillar collagens and laminin) (Fig. 1 a, b).

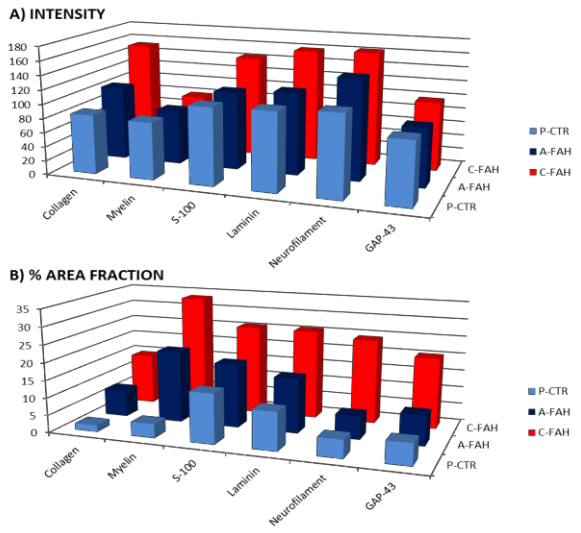

Fig. 1. Quantitative histochemical and immunohistochemical analysis of peripheral nerve regeneration.

DISCUSSION & CONCLUSIONS: In this study, we were able to promote a significant increase of peripheral nerve regeneration by using FAH as intraluminal filler of collagen type I conduits. These results suggest that FAH containing ADMSC is a promising therapeutic alternative to improve the regenerative potential of hollow conduits.

ACKNOWLEDGEMENTS: This study was supported by the Spanish Plan Estatal de Investigación Científica, Desarrollo e Innovación Tecnológica (I+D+I) 2013-2016 from the Spanish Ministry of Economy and Competitiveness (Instituto de Salud Carlos III), grant FIS PI14/1343 (co-financed by FEDER funds, European Union).

Bone organoids formed by devitalized hypertrophic cartilage through addition of fat-derived progenitors

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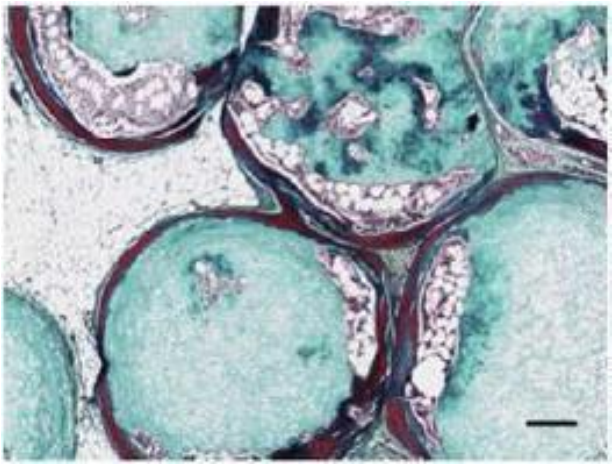
INTRODUCTION: Engineered and devitalized hypertrophic cartilage (HC) has been proposed as an off-the-shelf bone substitute material potentially combining the features of osteoinductivity, resistance to hypoxia, capacity to attract blood vessels and potential to be customized for specific indications.[1] However, as compared to the vital tissues, devitalized HC grafts have reduced efficiency of bone formation and longer remodeling times.[2] To address these limitations, here we tested the possibility to 'activate' devitalized HC by stromal vascular fraction (SVF) cells from human adipose tissue, which include mesenchymal, endothelial and osteoclastic progenitors.[3] We hypothesized that SVF-activation of devitalized off-the-shelf HC would result in enhanced bone formation at ectopic and orthotopic sites

METHODS: HC pellets generated by human stromal cells from bone marrow were devitalized by freeze/thaw and implanted ectopically in nude mice for 12 weeks or in calvarial defects in nude rats for 4 weeks, after embedding in fibrin gel with or without (control group) different amounts (6, 12 or 24 million) of SVF cells. Explants were assessed histologically and by micro-CT.

RESULTS: Ectopically, SVF cells added to devitalized HC increased the amount of bone formation in a dose-dependent fashion (up to 3-fold for 12 million SVF cells seeded) and directly contributed to endothelial, osteoblastic and osteoclastic populations in the explanted grafts. Bone volume correlated with the number of implanted CD31+/CD34+/CD146+ endothelial progenitors, which varied in different preparations. Also in the calvarial model, SVF activation of HC strongly enhanced bone tissue formation as compared to the unseeded constructs and resulted in a up to 7-fold increased percentage of defect area filled with bone matrix.

Fig. 1: Ectopic bone formation in nude mice. Devitalized hypertrophic cartilage pellets were



embedded in fibrin gel with SVF cells from adipose tissue. Masson-Tri-Chrome staining after 12 weeks in vivo.

DISCUSSION & CONCLUSIONS: SVF activation of the devitalized HC enhanced its bone formation efficiency, tested in ectopic and orthotopic models. In particular, the density of SVF cells was correlated with that of osteoclasts in the grafts, and the percentage of SVF-derived endothelial lineage cells was correlated with the amount of deposited mineralized matrix. Our findings outline a bone augmentation strategy based on devitalized allogeneic HC, intraoperatively activated with autologous SVF cells.

Thermo-responsive nanospheres with independent dual drug release profiles for combined cryotherapy of osteoarthritis

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INTRODUCTION: Dual drug delivery of drugs with different therapeutic effects in a single system is an effective way to treat a disease. One of the main challenges in dual drug delivery is to control the release behavior of each drug independently. In this study, we devised thermo-responsive polymeric nanospheres that can provide simultaneous and independent dual drug delivery in the response to temperature change. The nanospheres based on chitosan oligosaccharide (COS) conjugated pluronic F127 grafting carboxyl group (F127-COOH) were synthesized to deliver kartogenin (KGN) and diclofenac (DCF) in a single system. To achieve the dual drug release, KGN was covalently cross-linked to the outer part of the nanospheres, and DCF was loaded into the inner core of the nanospheres. The aims of this study were to (1) synthesize and characterize the F127/COS/KGN_{DCF} nanospheres with respect to independent dual release, and (2) to evaluate the F127/COS/KGN_{DCF} nanospheres as an independent dual drug delivery system for combined OA therapy *in vitro* and *in vivo*.

METHODS: 1) Preparation of F127/COS/KGN_{DCF}: Dicarboxylated F127 (F127-COOH) was cross-linked with COS of KGN conjugated COS during the nanospheres synthesis by emulsification/solvent evaporation. DCF was loaded in the nanospheres by change of wall-permeability according to temperature control. The nanospheres were characterized by SEM, TEM and DLS.

3) Controlled dual release by temperature change

4) *In vivo* thermo-responsiveness: The nanospheres labeled with fluorescence dye were injected into the joint of OA rats. After IA injection, cold temperature was treated to the joint for 10 min with cryotherapy device. Fluorescence spectrum was scanned using an IVIS.

5) *In vivo* cyclooxygenase inhibition: Serum and synovium were collected in OA rat after IA injection of the F127/COS/KGN_{DCF}. Cyclooxygenase-2 inhibition by DCF released from the nanospheres after cold temperature treatment was evaluated by RT-qPCR and ELISA.

6) *In vivo* cartilage regeneration: The OA rats were treated with the nanospheres by IA injection at weeks 6 and 9 after OA induction. The distal femora in each group were dissected at 14 weeks after OA induction and evaluated the OA regions by Safranin-O staining and OARSI scoring. Immunohistochemistry of COL2 and aggrecan was also carried out.

RESULTS: Swelling and shrinking of the nanospheres by thermal responsiveness was controllable by the composition ratio of F127 or KGN to COS. The nanospheres were ~125 nm in size at 37°C and expanded to ~442 nm when cooled to 4°C in aqueous solutions. The nanospheres demonstrated immediate release of DCF and sustained release of KGN, which were independently controlled by temperature change. The fluorescence-labelled nanospheres showed retention time longer than 14 days in the OA-induced knee joint. Bioluminescence of the nanospheres was significantly increased after cold treatment in the joint of OA rat. The nanospheres suppressed the progression of OA in treated rats, which was further enhanced by cold treatment. The nanospheres also reduced cyclooxygenase-2 expression in the serum and synovial membrane of treated rats, which were further decreased with cold treatment.

DISCUSSION & CONCLUSIONS: These results suggest that the F127/COS/KGN_{DCF} nanospheres provide dual-function therapeutics possessing anti-inflammatory and chondroprotective effects which can be enhanced by cold treatment.

ACKNOWLEDGEMENTS: This research was supported by the National Research Foundation of Korea (Grant no: 2013R1A1A2062978).

Application of body as a novel bioreactor for *in vivo* pancreas tissue engineering

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INTRODUCTION: Type 1 diabetes is characterized by autoimmune destruction of pancreatic cells [1]. Insulin replacement therapy represents the current gold standard treatment but exogenous insulin cannot imitate the physiology of insulin secretion [2]. Organ transplantation is an acceptable treatment for native organ failure. However due to many reasons, such as lack of donor and immunosuppression, it is associated with many problems . We present a novel model for in vivo recellularization of acellular pancreas by implanting between the host pancreas and adjacent omental flap.

METHODS: Twelve pancreases were harvested and cannulated via common bile duct. The scaffolds were decellularized by detergent based protocol and infused with methylene blue in order to confirm vascular integrity prior to implantation. Twelve rats were anesthetized and opened with midline incision. The decellularized scaffold was stretched over the host pancreas and the omentum was wrapped around it to make a sandwich like structure, then fixed with chromic sutures 6-0 and marked with prolene 4-0 at four borders.

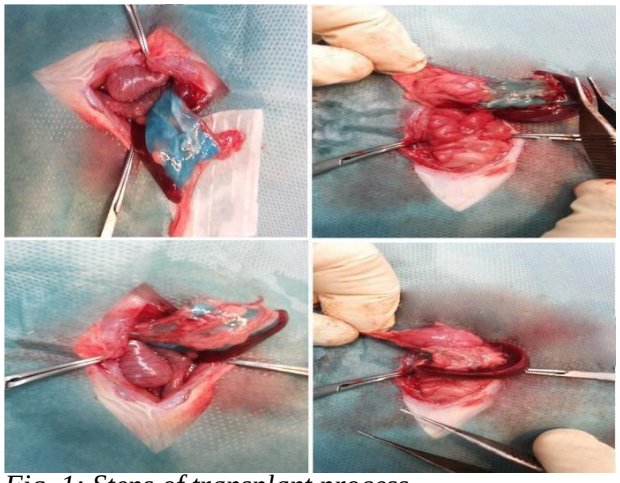

Fig. 1: Steps of transplant process.

RESULTS:

All implanted decellularized pancreases were removed at 2 and 4 weeks post transplantation. The samples were recognized by prolene sutures and the omentum can easily be separated from the tissue but distinction between host and implanted tissue was performed under the surgical microscope. The decellularized implanted tissue was illustrious from the native organ and was easily dissected free from the omentum and adjacent tissues.

The Histopathological analysis showed marked recellularization of acellular pancreas with marked nneovascularization and neoβ-cells with minimal inflammatory response were observed.

DISCUSSION & CONCLUSIONS: The preliminary results of this novel technique promising for pancreas tissue engineering. We observed that in vivo graft of decellularized pancreas can promote insitu recellularization, proliferation and differentiation possibly by circulating stem cells. These findings could pave the road for management of diabetic rat model.

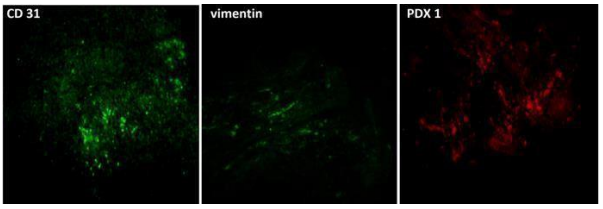

Fig.2: Immunohistochemical staining of recellularized pancreas

DISCLOSURES: The authors declare that there is no conflict of interest.

Whole organ decellularization of human testis by perfusion based method; introduction of novel technique for testis tissue engineering

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INTRODUCTION: Decellularized human testis could be helpful to treat different kinds of male infertility. The acellular testis can provide further clinical approaches to male sterility. Also, extra cellular matrix of testis is considered as a tool to study the basis of spermatogenesis.[1] Tissue engineering employs various methods such as cell transplantation, materials science, and bioengineering.[2] These methods provide opportunities for restoring normal function of damaged or abnormal tissue and organs.[3] Here in we present an optimal method for decellularization of human testis by perfusion-based methods.

METHODS: Eight human testes with spermatic cord were preserved from highly selected adult male patients who legally underwent orchiectomy following complex ethical, permission from Justices of the Supreme Court. Spermatic artery was dissected and cannulated. The samples were decellularized by detergent based methods and preserved in phosphate buffered saline (PBS) for further evaluation. After decellularization the samples were stained by movat pentachrome, red Sirius and mason trichrome for extracellular matrix component. For ultrastructure analysis SEM was performed with the DNA quantification test to confirm the decellularization. Magnetic resonance angiography was performed to investigate the preservation of vascular structure.

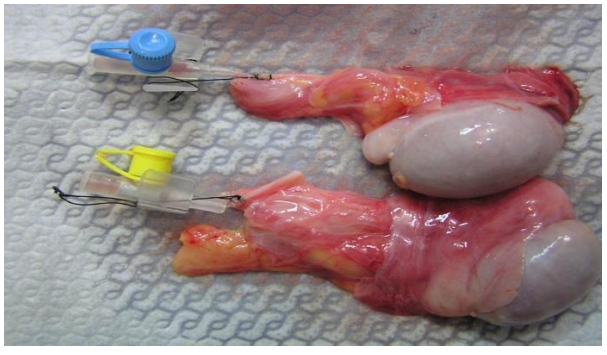

Fig. 1: Cannulation of human testis by testicular artery

RESULTS: In order to assess decellularization, macroscopic, microscopic and structural evaluations were performed. Macroscopically tissues looked translucent. Histological analysis and DNA quantification proved complete decellularization. The tissue's ultrastructure was intact in electron microscopy (SEM). Based on Movat pentachrome, Sirius red and trichrome mason staining, extracellular matrix was retained its integrity compared to control samples. MR angiography showed intact vasculature distribution.

DISCUSSION & CONCLUSIONS: In human testis, the intrestitium, blood vessels and tubular wall are rich in ECM, And accessing to a purifeid acellular ECM is the basis for tissue engineering and regenerative medicine. This study introduces perfusion–based methods for reaching an optimal acellular human testis without structural distortion .In other words it creates a platform to make the whole organ decellularized with no damage to the organ structure and ECM components, using detergents through testis vessels or vas deferent. Such methods which allow us to have the whole organ acellular are very practical in organ transplantation to create organs that have been recellularized by autologous cells.

DISCLOSURES: Authors declare no conflict of interest

Development of perfusion based decellularization of human-sized heart as a natural scaffold for organ engineering

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INTRODUCTION: Cardiovascular disease is still one of the prominent causes of mortality, and the definitive treatment for end-stage heart failure is a heart transplant[1]. The major limitations of heart transplantation are the shortage of organ donors, lifelong immunosuppressive therapy for the transplant recipient, and possible organ rejection[2]. Whole heart engineering can overcome these obstacles by replacing a functional heart. In the present study, we produce a whole heart scaffold by using detergent based coronary perfusion.

METHODS: In the present study, we decellularized ovine heart by antegrade coronary perfusion using a peristaltic pump. Efficiency of the decellularization and preservation of extracellular matrix (ECM) components of auricle, left ventricular myocardium, right ventricular myocardium, aortic valve and chordae tendineae were assessed using following evaluations: hematoxiline and eosine, Masson's trichrome, 4′,6-diamidino-2-phenylindole, Picrosirius red, and Movat's pentachrome staining; scanning electron microscopy; biochemical assay of sulfated glycosaminoglycans, and collagen. Coronary Magnetic resonance angiography (CMRA) and computed tomography angiography (CTA) were utilized to prove the preservation of vascular network integrity. Moreover the mechanical properties of the native and decellularized right and left myocardium were examined by performing two tests: tensile strength and compressive-strength tests. Human umbilical cord derived mesenchymal stem cells (hUC-MSCs) were seeded on a decellularized myocardial slice to demonstrate that the scaffold is ready for cell seeding. Statistical analysis was performed using SPSS, version 20. Biochemical analysis was performed using paired *t* test (mean ± SD). *P* value less than 0.05 was considered statistically significant.

RESULTS: The resultant five sections ECM retained collagen, glycosaminoglycans (GAG_s), and mechanical integrity whereas all cellular material effectively removes (Fig.1). CMRA, CTA did not detect destruction of the 3D architecture of vascular network (Fig.2). The results also showed that hUC-MSCs rapidly attached to the margin of the scaffold. Cell viability for approximately 14 days showed no signs of cytotoxicity of the scaffold.

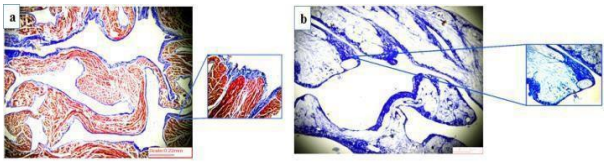

Fig. 1: Images of Masson's trichrome staining of auricle tissue before (a) and after perfusion decellularization (b).

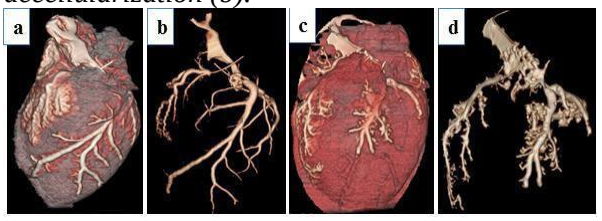

Fig. 2: Images of CTA. Three-dimensional volume-rendered (VR) reconstruction shows the coronary system (a), coronary tree (b) of native and coronary system (c), coronary tree (d) of decellular heart.

DISCUSSION & CONCLUSIONS: To the best of our knowledge, this is the first detailed report of separate evaluation of decellularization in five regions of a single human-size heart. We described successful decellularization of a whole ovine heart for the development of a scaffold with intact architecture and stable perfusable vasculature, which can be recellularized with hUC-MSCs. Our method seems to be necessary for development of the techniques allowing researchers to create a functional tissue-engineered large scale heart.

DISCLOSURES: The authores indicate no potential conflict of interest.

Application of decellularized colon *in situ* position versus mesenteric patch used as *in vivo* bioreactor with long-term patency; rat model of study

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INTRODUCTION: Intestinal failure is associated with high morbidity and mortality and its treatment remains a clinical challenge. Tissue engineering using biological scaffolds produced from mammalian tissues offers new hopes for small bowel bioengineering [1]. To assess the feasibility of generation of colon tissues from the decellularized bioscaffold by means of tissue engineering.

METHODS: Acellular scaffolds were produced from the rat colons using a detergent based protocol. Hematoxylin & Eosin staining and scanning electron microscopy were performed to evaluate the success of decellularization. Twenty rats were selected and randomly allocated into two groups. Acellular scaffolds were implanted into the mesentery in one group, while in the second group colon was interposed in situ position. Biopsies were taken after 9 months and immunostaining was performed to assess the cell seeding.

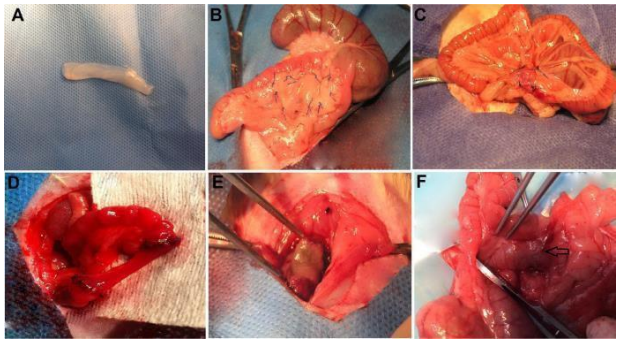

Fig.1: Surgical procedures for mesenteric and in situ implantation

RESULTS: Colon was successfully decellularized as confirmed by Hematoxylin& Eosin staining and scanning electron microscopy (SEM) and Immunohistochemistry showed the regeneration of epithelial and muscular layer in engineered tissue Neural elements were also found in histologic evaluation. Interestingly, the scaffold implanted in small intestinal mesentery showed the histologic features of small bowel, while the acellular segments grafted to the large bowel developed into colon. Intestinal stem cells were present in engineered tissues produced by implanting acellular segments into the mesentery but these cells were not detected in interposed colon group.

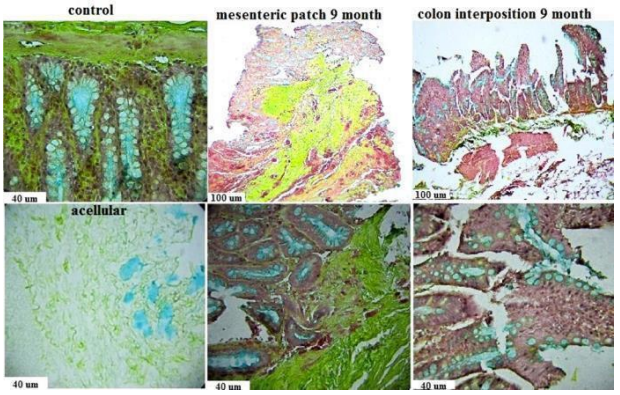

Fig.2: Movat's pentachrome staining shows the presence of collagen and mucin in grafts

DISCUSSION & CONCLUSIONS: This study highlights the role of micro-environment in tissue engineering; small intestine and colon were generated from the same scaffold merely by placing the scaffold in the suitable environment. Although we have been successful in recellularization of short segments of bioscaffold, the feasibility of this technique for longer segments- necessary for clinical applications- is still unclear and should be sought in future studies.

DISCLOSURES: Authors declare no conflict of interests

In-vivo human corpus cavernosum regeneration: fabrication of tissue-engineered corpus cavernosum in rat using the body as a natural bioreactor

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INTRODUCTION: Few researches have been conducted to develop an ideal method for tissue-engineering of corpus covernosum [1-2]. We produce a decellularized human corpus cavernosum scaffold and investigate the in-vivo cell seeding of the scaffold after transplantation into the rat omentum.

METHODS: Eight adult human male corpus cavernosum were obtained in sterile condition after obtaining ethical committee approval. After dissecting the urethra, corpus cavernosum was decellularized by inserting an 18 gauge needle into the body of the tissue. The gauge was connected to a peristaltic pump to circulate the detergents in the corpus. Histopathological examinations, DNA quantification, scanning electron microscopy (SEM), and tensile test were performed to evaluate the efficacy of decellularization and extracellular matrix (ECM) preservation. A section of decellularized scaffold was washed several times and transplanted into the omentum of 4 male healthy Sprague Dawley rats and located into the scrotum. Biopsies were taken 1, 3 and 6 months after transplantation. Histological examination, SEM, DNA quantification, and immunohistochemical (IHC) staining were performed to assess the efficacy of natural recellularization

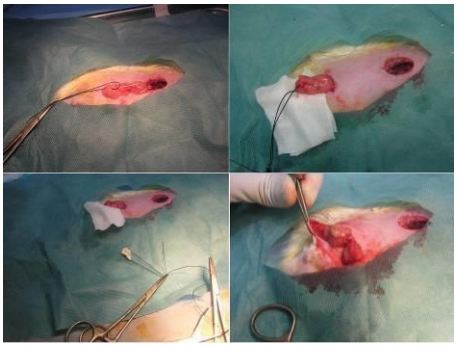

Fig 1: Surgical technique of decellularized human corpus covernosum transplantation

RESULTS: The results of the examinations performed prior to transplantation, revealed a decellularized ECM resembling to the native tissue with normal pits that may be appropriate for further in-vivo cell seeding. Histopathology examination of the biopsies after transplantations confirmed successful cell seeding with endothelium-like cells in different time-points. CD 34 staining was dominant in the short-time biopsies, while CD31 staining was higher than CD34 in long-term specimens.

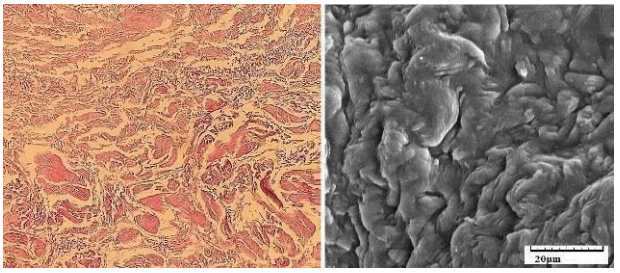

Fig 2: Histological evaluations and SEM analysis of decellularized scaffolds

DISCUSSION & CONCLUSIONS: This study theoretically may pave the road for corpus covernosum regeneration by transplantation of decellularized scaffolds into the scrotum. The feasibility of natural bioreactor in recellularizing corpus covernosum was confirmed. This technique may have the potential to facilitate homologous transplantation for repair of corpus defects.

DISCLOSURE: Authors have nothing to disclose.

Extra-luminal demucosalisation of intestine for bladder tissue engineering

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INTRODUCTION: Urinary incontinence affects more than 5 % of the world's population. The current 'gold standard' to manage end-stage bladder diseases remains enterocystoplasty, in which the patient's bladder is augmented with a segment of vascularised detubularised autologous bowel. Although enterocystoplasty provides a continent reservoir for storing urine at kidney-sparing pressures, it is associated with serious complications that occur because of the long-term exposure of bowel epithelium to urine. "Composite cystoplasty" is a promising tissue engineering approach where the pedicled bowel segment used for reconstruction is demucosalised and lined by autologous urothelium generated by ex vivo propagation [1]. The aim of this study was to provide evidence that the extra-luminal demucosalisation of porcine and human colon is effective at removing all bowel epithelial cell remnants, in support of the clinical translation of composite cystoplasty.

METHODS: Fresh porcine (female, ~33kg, n=2) and human (n=1) colons (1-3 cm in length) were subject to extra-luminal demucosalisation as described [2]. Human colon samples were obtained with informed consent and NHS research ethics committee approval. Histology was performed to evaluate the success of the extra-luminal demucosalisation procedure.

Subsequently, 16 porcine tissue samples (~0.5cm^2 each) were implanted subcutaneously in the abdomen of 4 SCID mice (4 samples each). In total two mice were implanted with demucosalised porcine colon samples and the other two were implanted with samples of full thickness porcine colon. Two mice (one from each group) were sacrificed at 4 weeks and the other two at 3 months. The porcine colon (demucosalised and full thickness) explants were processed for H&E staining and immunohistochemistry for CK18.

RESULTS: In both pig and human, histology confirmed that the extra-luminal demucosalised muscular segment was clear of residual crypts, and the mucosa showed complete, clean separation from the plane of the muscle with no ingress into the crypts (Fig. 1).

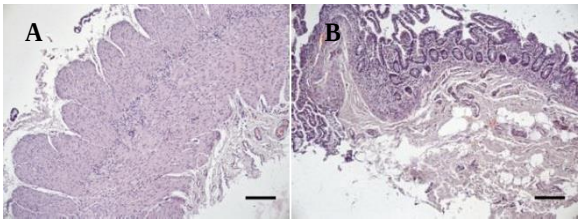

Fig. 1. H&E staining of (A) demucosalised smooth muscle and (B) epithelial mucosa of human colon. (Scale bar: 150 µm)

No epithelial tissue was observed in the demucosalised porcine implants at either the 4 week or 3 month time-points. Some crypt-like structures were evident in the recovered full-thickness colon implants, particularly at 3 months (Fig. 2).

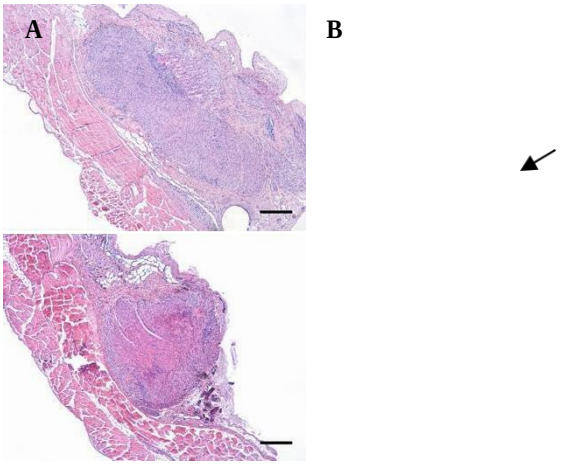

Fig. 2. H&E staining of porcine colon implants after 3 months. (A) Demucosalised smooth muscle; (B) full thickness. (Arrow: crypt-like tissue) (Scale bar: 300 µm)

DISCUSSION & CONCLUSIONS: Extra-luminal demucosalisation of porcine colon was effective in complete removal and prevention of the re-growth of porcine colonic crypts. For the first time, we have demonstrated this procedure is feasible and effective in eliminating all epithelial cells in human colon for composite cystoplasty.

ACKNOWLEDGEMENTS: This work was supported by the Medical Research Council, UK.

DISCLOSURES: The authors have nothing to disclose.

Platelet rich plasma drives cartilage regenerative response by activating chondroprogenitor cells

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INTRODUCTION: Regeneration of diseased hyaline cartilage continues to be a tremendous clinical challenge, mainly due to uncontrolled knee degeneration process and intrinsic poor reparative potential of articular chondrocytes. Platelet derivatives (PRP or PL) act on human articular chondrocytes as a proliferative stimulus maintaining their somatic differentiation potential with transient pro-inflammatory outputs (1). Despite this the most important challenge remains to identify and isolate tissue-specific stem cells within articular cartilage.

Recent data shown that platelet derivatives treatment of human cartilage biopsy increase the number of Nestin-positive cells resident in the tissue (2). Nestin is an intermediate filament protein expressed in dividing cells during the early stage of development, is used as a marker of proliferating and migrating adult stem cell in multiple tissues. Nestin is up regulated in response to injury and pathological processes. So, the aim of this study was to determine if PL is able to activate and recruit tissue specific nestin adult stemcells.

METHODS: The human articular chondrocytes (HAC) were isolated and expanded in two different culture conditions: Coon's F12 medium plus a) 5% of platelet derived lysate and b) Coon's F12 medium plus 10% FBS. We also performed an organ-culture study to isolate chondroprogenitor cells (CPC) migrating from pieces of cartilage tissue, maintain in 5% of platelet lysate and 10% FCS for 10 days in culture. Using the CPC we performed colony-forming units-fibroblast assay (CFU), multilineage differentiation, immunophenotypic characterization , migration assay and we tested the " in vitro" and "in vivo" capability to formcartilage.

RESULTS: Data have shown that PL treatment of human adult cartilage biopsies increases the number of Nestin-positive cells resident in cartilage. These cells isolated and cultured with PL at low density were able to generate CFU-F. Moreover, when we test the differentiation capability, CPC exhibited a potential to differentiate along the osteogenic and chondrogenic lineages.

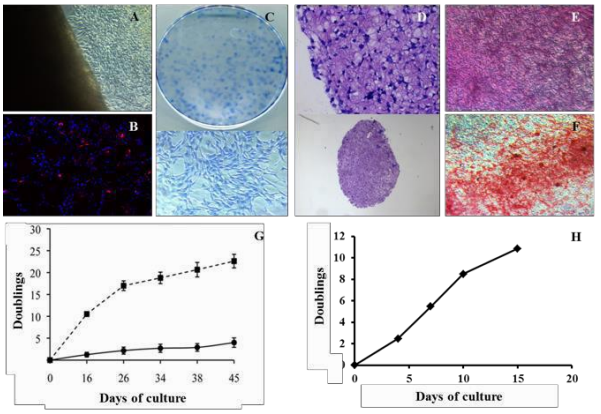

Fig. 1: (A-B) Chondroprogenitor cells growth from cartilage chips analyzed for nestin expression after treatment with PL; (C) Clonogenic potential: CFU assay of the colony formation by migrating CPC; (D) Chondrogenic differentiation in micromass pellet culture of CPC ; (E-F)Osteogenic differentiation of CPC: ALP staining and alizarin staining respectively; (G) Growth kinetics of HAC in presence of FBS (black line) and PL5% (dashed line);(H) Growth kinetics of CPC.

DISCUSSION & CONCLUSIONS: Our data shows that PL treatment trigger MSC-like chondroprogenitor cells resident in adult articular cartilage that can be directly involved in the maintenance of cartilage homeostasis by a self-repair mechanism.

In this perspective, our findings claim a key function role of PL in adult cartilage with active different regenerative mechanisms.

Umbilical cord tissue as a source of cell-based material for dermal restoration

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INTRODUCTION: Umbilical cord tissue is a fruitful source of different products for cell therapy. The most investigated one is mesenchymal stem/stromal cells (MSC) [1]. However the cord tissue itself and the cell-derived substances such as condition medium and exosomes [2] are in a focus of research. Skin aging and hair loss are problems that will always be on top of demand for the cure. Our team chose this area of interest to perform pilot studies.

METHODS: Human umbilical cords from both sexes were collected from full-term births after normal vaginal delivery with informed consent using the guidelines approved by the Institutional Ethics Committee (IEC). Three types of products were obtained from each cord: minced and homogenized fragments of umbilical cord tissue, suspension of MSCs, and conditioned medium. MSCs were isolated and cultivated using previously described approaches [3], the suspension was prepared at 2nd passage. Conditioned medium was collected at 2nd passage and the last passaging stage was performed on serum-free media for 48 hours. The medium was centrifuged and filtered. Hairless mice and mice with induced skin aging were used for testing the effects of abovementioned products on dermal repair and skin rejuvenation. Hair growth enchasing was also investigated. Parameters that were evaluated: skin morphology, telomere length, antioxidant enzymes system activity [4].

RESULTS: It was shown that the use of all three umbilical tissue-derived products can improve the state of skin in mice. Topical application of homogenized cord tissue, cultures of MSC and conditioned medium showed different levels of effectiveness.

DISCUSSION & CONCLUSIONS: It is quite ambiguous situation with understanding what exactly is a source of effects – is it the whole Wharton jelly that is rich in hyaluronic acid or cells with their stemness and homing abilities or the cocktail of substances that can act on triggers in skin. Our work shows the promise for using topical applications of umbilical cord-derived products as a skin restoration tool.

[1]

Liver organoid formation in-vitro: what triggers cell self-assembly?

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INTRODUCTION: Liver organoids (LOs) are of interest in tissue replacement, hepatotoxicity and pathophysiological studies. Takebe et al recently showed that induced-pluripotent stem cells, mesenchymal stem cells (MSC) and human endothelial cells (ECs), self-organize in-vitro into 3D LOs that became vascularized and produce hepatic factors (e.g. albumin) when transplanted in mice [1]. LO formation was observed seeding cells onto substrates made of Matrigel diluted 1:1 with endothelial growth medium. Increasing Matrigel dilution hampers LO formation, while changing substrate composition (laminin, entactin, collagen I or 1.5% agarose) prevents LO formation [1]. It is still unclear what triggers LO self-assembly, e.g. substrate properties and/or culture medium. Hypothesizing that LO formation occurs as a result of a fine balance between cell-substrate adhesion and cell-cell cohesion, we used upcyte® hepatocytes, upcyte® liver sinusoidal ECs (LSECs) and upcyte® MSCs from Medicyte GmbH to investigate LO self-assembly on different substrates keeping the culture parameters (e.g. culture media, cell types/number) constant.

METHODS: Matrigel diluted 1:1 with LSEC Growth Medium (Medicyte) substrates (Matrigel-LSEC) in 24 well plates were used as positive controls for LO formation. Since the LSEC medium contains foetal bovine serum and growth factors possibly affecting LO formation, Matrigel diluted 1:1 with PBS (Matrigel-PBS) was also investigated. Agarose gels at 0.1% and 1.5% w/v concentrations were prepared dissolving agarose powder in boiling deionised water (1/5 of final solution volume), then cooling to 40 °C and adding LSEC medium to reach the final volume. The solution was then cast in 24 well plates and cooled at 4 °C for 1h. Decellularised porcine liver [2] was pepsin digested to obtain 20 mg/mL liver ECM-gels rich with adhesive ligands [3]. Substrates were used either as obtained or after 1:1 dilution in LSEC medium (ECM-LSEC). The stiffness of all substrates is similar (0.3÷0.8 kPa), except 1.5% w/v agarose (~50 kPa). Upcyte® hepatocytes (10^6 cells), upcyte® LSECs (10^6) and upcyte® MSCs (0.2×10^6) were mixed in 1mL of medium, seeded on the different substrates and cultured for 48h. LO formation was visually assessed and adhesive (Integrin-ß1) and cohesive (Connexin-32) markers investigated using confocal microscopy.

RESULTS: Good LO formation was obtained on Matrigel-LSEC (positive control) and agarose gels. Cells on Matrigel-PBS formed an unstable and partially disrupted LO, while no LO formation was observed on ECM and ECM-LSEC gels (Fig. 1).

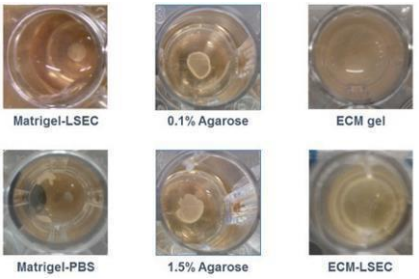

Fig. 1: LO formation on various substrates at 48h.

Cells on ECM and ECM-LSEC substrates express only Integrin-ß1, while those on LO forming substrates also express Connexin-32 (Fig. 2).

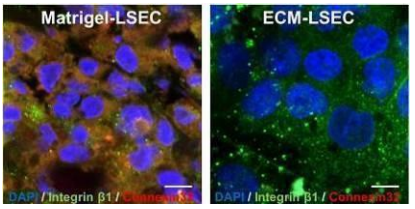

Fig. 2: Integrin-ß1 (green) and Connexin-32 (red) expression on Matrigel-LSEC and ECM-LSEC. Blue: cell nuclei (DAPI). Scale bar: 10 µm.

DISCUSSION & CONCLUSIONS: No LO formation occurs on highly adhesive ECM-based substrates, but a fine balance between cell-substrate adhesion and cell-cell cohesion is required. Thus, in addition to substrate mechanics (similar for most of materials studied), physicochemical properties which are also critical for cell adhesion play a role in LO self-assembly.

ACKNOWLEDGEMENTS: EU FP7, Grant no. 304961, ReLiver.

Rapid fabrication of mechanically adequate and hemocompatible fibrin-based vascular grafts

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INTRODUCTION: The clinical translation of tissue-engineered vascular grafts remains a challenge. For cell-based approaches, one of the issues is the long maturation time in bioreactor systems with the aim of reaching mechanical properties similar to those of the native arteries. This results, however, in a collagen-rich disorganized matrix mostly missing elastin. We present a cell seeded fibrin-based vascular graft with a fabrication time reduced to 4 days. While mechanically adequate for implantation, the graft exhibits a low-density extracellular matrix which offers the potential of a rapid remodeling in vivo into a native-like tissue.

METHODS: Small-caliber vascular composite grafts (n=5, internal diameter=3mm) were fabricated using a fibrin scaffold embedding human umbilical artery smooth muscle cells and a polyvinylidene fluoride warp-knitted macroporous mesh. Immediately after molding, the tubular constructs were seeded with human umbilical artery endothelial cells to create a hemocompatible surface. The constructs were kept under low flow and pressure conditions overnight. The flow and the pressure were gradually increased on day 1 and 2 to reach arterial shear stress (10 dynes/cm²) and pressure (120/80 mmHg), which were maintained for two more days. Tissue analysis included burst strength, collagen quantification and immunohistochemical stainings.

RESULTS: Fabrication and pre-conditioning under arterial pressure and shear stress conditions resulted in a robust and hemocompatible tissue-engineered vascular graft. The textile reinforcement was fully integrated into the cell/fibrin matrix. Immunohistology revealed extracellular matrix protein synthesis, including collagen I and III, as well as fibrillin-1 and low amounts of elastin. Collagen quantification by hydroxyproline assay revealed 12.0 ± 3.4 % of the collagen present in human umbilical arteries. The vascular grafts exhibited a burst pressure of 584.1 ± 45.0 mmHg. The luminal surface of the vascular grafts was lined by a confluent endothelial cell layer.

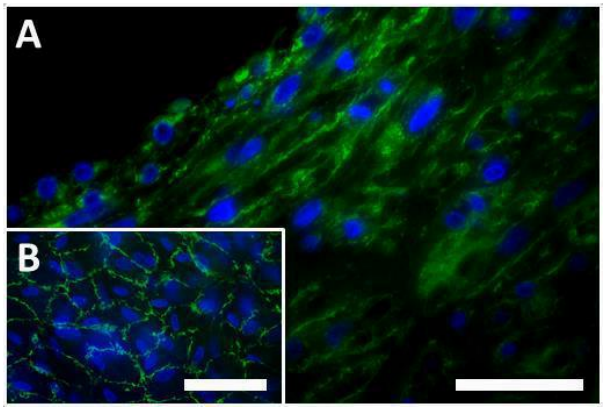

Fig. 1: Tissue analysis. A) Immunohistochemical staining of collagen (green) and B) en-face image of the endothelial luminal layer stained for CD31 (green). Nuclei were counterstained with DAPI (blue). Scale bars: A) 50µm, B) 100µm

DISCUSSION & CONCLUSIONS: Fibrin is a readily available autologous scaffold material. Degradation products are non-toxic and enhance ECM production[1]. Furthermore it supports cell adhesion and enables homogenous cell immobilization. All these advantages offered by fibrin have been historically counterbalanced by the belief that a long in vitro cultivation time is needed because of the poor initial mechanical properties. We present a fabrication and conditioning process, which enabled the production of textile-reinforced fibrin-based vascular grafts ready for implantation in just four days. This approach, when combined with readily available cell sources, e.g. adipose-derived stem cells, could result in on-demand, autologous vascular grafts, to be prepared shortly before implantation.

ACKNOWLEDGEMENTS: This research was funded by the Integrated Interdisciplinary Institute of Technology for Medicine (I3TM) of RWTH Aachen University Seed-Fund (SF_15-5-04).

Development of a humanized model of bone regeneration - harnessing the Chorioallantoic membrane model as a replacement model in animal research

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INTRODUCTION: New regenerative materials and approaches need to be assessed through reliable and comparable methods to facilitate rapid translation to the clinic. Typically, *in vivo* studies are routinely used to test novel biomaterials for bone tissue engineering. While it is generally accepted these studies are critical prior to any translation into the clinic, animal models are not always predictive of the human response[1]. Here we propose the use of the chorioallantoic membrane (CAM) assay to culture human living bone and the application of this system as a model to test biomaterials.

METHODS: Human bone cylinders were extracted from freshly isolated human femoral heads and devised to have an inner empty core to mimic the injury area. Bone cylinders were cultured for *in vitro* (organotypic) or CAM-implanted for 7 days. Micro computed tomography (μCT; Skyscan 1176) was conducted before and after culture to quantify the relative changes within the bone cylinders. Histological examination was conducted using the regular stainings (Alcian Blue and Sirius Red) and immunohistochemistry.

RESULTS: Histological analysis demonstrated the presence of chick vasculature within the bone cylinder and interdigitated within the marrow space of the human bone tissue. Alcian Blue and Sirius Red staining highlighted the presence of extracellular matrix deposition rich in collagen content evidenced by polarized light (Figure 1). Immunohistochemical detection for collagen type II and collagen type X further validated the presence of newly deposited collagenous matrix which was observed to co-localize with avian cells on the CAM-implanted cylinders. Furthermore, cell condensations, highly positive for Sox9 were observed in the junction of the CAM blood vessels and the human trabecular bone. Finally, the μCT analysis indicated a significant increase in bone volume formed on the CAM-implanted bone cylinders compared to the *in vitro* bone control and non-cultured control groups ($p<0.001$).

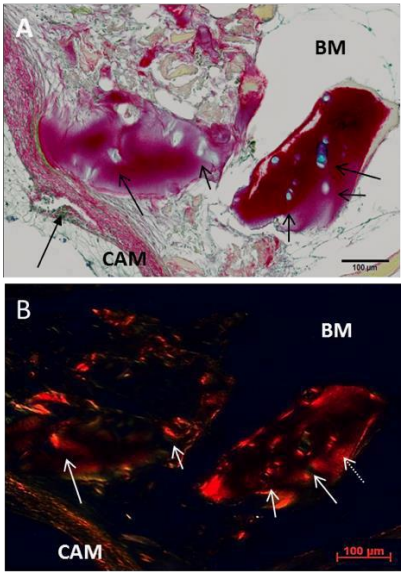

Fig. 1: Deposition of collagenous matrix in the junction of the avian chorioallantoic membrane (CAM) and the human bone marrow (BM). (A) Alcian Blue and Sirius Red staining demonstrating new collagenous matrix (pink) (B) Identical image under polarized light. Arrows indicate cells within the matrix. Solid arrow indicate avian blood vessel. Scale bar 100μm.

DISCUSSION & CONCLUSIONS: The current studies demonstrate the potential of the CAM to integrate human bone tissue and to provide a surrogate blood supply to aid tissue regeneration. This avian human *ex vivo* system in combination with the histological and μCT analysis offers a simple alternative preclinical model for the screening of biomaterials and biomaterial constructs for hard and soft tissue engineering.

ACKNOWLEDGEMENTS: The authors would like to thank the NCR3s for supporting this study.

Functional characterisation of a three-dimensional tissue engineered *in vitro* model of the oral mucosa using TERT2 immortalised oral keratinocytes

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INTRODUCTION: Tissue engineered oral mucosal equivalents are being increasingly used to model oral diseases and to measure drug toxicity. Current mucosal models are usually constructed of normal oral keratinocytes (NOK) cultured on top of a hydrogel matrix containing normal oral fibroblasts (NOF). However, the commercial supply of NOK is limited therefore restricting widespread use of these models. In addition, NOK suffer from poor longevity and donor-to-donor variability. Therefore, the aim of this study was to construct and functionally characterise oral mucosal equivalents using TERT2 immortalised oral keratinocyte (FNB6) cells in order to produce a more readily available alternative to NOK-based models.

METHODS: Oral mucosal models were prepared by culturing TERT2 immortalised (FNB6) oral keratinocytes on top of a collagen type 1 hydrogel containing NOF at an air-to-liquid interface for 14 days. These mucosal equivalents were characterised by immunohistochemical analysis for differentiation & proliferation markers and compared to normal oral mucosa and NOK-based models (MatTek). FNB6 mucosal models were stimulated with cytokines (TNF&IL-1), LPS or bacterial biofilm and their gene expression (Affymetrix array, qPCR) and section of inflammatory cytokines (CXCL8, IL-6) measured by ELISA and compared to those expressed by NOK-based mucosal models.

RESULTS: Co-culture of immortalised FNB6 and NOF cells resulted in models consisting of a NOF-containing connective tissue topped with a multi-layered epithelium containing basal epithelial cells, layers of spinous epithelium covered with differentiated non-keratinised epithelium. The FNB6 mucosal models exhibited expression of differentiation markers cytokeratin 13 in the upper, suprabasal layers and cytokeratin 14 in basal layer of the epithelium. Proliferating cells were restricted to the basal epithelial cells and there was expression of desmoglein and E-cadherin confirming the presence of cell-to-cell contacts. The histology and expression of these structural markers paralleled those observed in the normal oral mucosa and NOK-based models (Fig. 1).

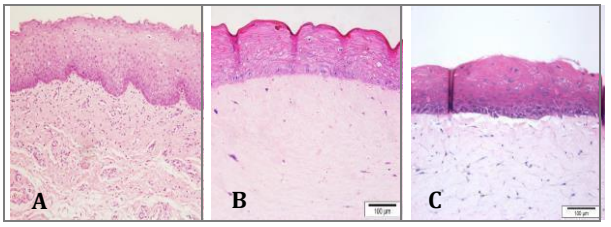

Fig. 1: Histology images of (A) normal oral mucosa, (B) NOK-Based and (C) FNB6 oral mucosal models.

Upon stimulation of with TNF&IL-1, FNB6 models displayed a similar global gene expression profile to NOK-based models with increased expression of many common pro-inflammatory molecules such as CXCL chemokines and IL-6 when analysed by gene array (Fig. 2A) and qPCR. Pathway analysis showed that both FNB6 and NOK models initiated similar signalling pathways upon stimulation. Interestingly, gene expression in FNB6 models was more consistent than NOK-based models that suffered from donor variation (Fig 2B). Both FNB6 and NOK models were less responsive to stimulation with LPS. These bacterial stimuli produced similar gene expression profiles in both models but were of a different signalling pathway to that instigated by cytokines.

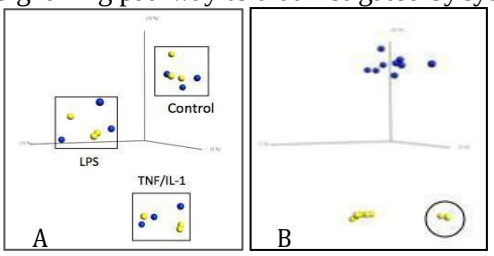

Fig. 2: Principal Component Analysis of gene array data showing (A) similarity of global gene expression and (B) model to model variation between FNB6 (Blue) and NOK-based (Yellow) oral mucosal models.

DISCUSSION & CONCLUSIONS: Oral mucosal models based on immortalised FNB6 cells can mimic native oral mucosa structure and replicate tissue responses observed with NOK-based models. FNB6 models may be used to replace those based on NOK where donor variation is important or access to primary cells limited.

ACKNOWLEDGEMENTS: This study was funded by Colgate-Palmolive.

Bone burrings harvested during spinal surgery offer an osteoinductive alternative bone graft for spinal fusion

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INTRODUCTION: Despite advances in modern surgical techniques, up to 35% of spinal fusion procedures fail to adequately fuse. As such, bone grafting is routinely performed to encourage osseous union. Autologous iliac crest bone is the 'gold standard' graft choice. However, significant surgical complications and limited harvest yields have highlighted the need for alternative grafts.

Much research is being conducted into synthetic biomaterial alternatives, yet bone burrings, created during spinal fusion surgery, could offer a simple graft alternative to iliac crest bone. These burrings are usually lost via suction, but using a simple suction trap can be collected for clinical use.

Bone burrings are made up of a mixture of bone, blood and fibrous tissue. Previous studies have demonstrated that bone burrings are a source of viable osteoblasts, yet little is known about how the blood and fibrous tissue may contribute to the healing process. We hypothesized that bone burrings offer a source of factors and cytokines that will encourage bone growth and regeneration.

The objective of this study was to assess the osteoinductive potential of factors released from bone burrings harvested during spinal fusion surgeries.

METHODS: Bone burrings, generated from facet resection or posterior osteotomies during spinal fusions were collected using an in-line suction trap in accordance with the local institutional review board.

Bone burrings were placed in tissue culture inserts (1μm pore size) and suspended over primary human osteoblasts cultured in 24-well tissue culture plates (Figure 1). The effect of factors released from the bone burrings on osteoblast proliferation, matrix production and gene expression was assessed over 7 days by ^{3}H-thymidine incorporation, pro-collagen Iα1 ELISA and real-time PCR, respectively.

The concentration of alkaline phosphatase, VEGF, IL-1β, IL-6, bFGF, IGF-1, BMP-2 and TGFβ1 released from bone burrings alone, over a seven day period, was quantified by p-NPP hydrolysis assay and ELISA.

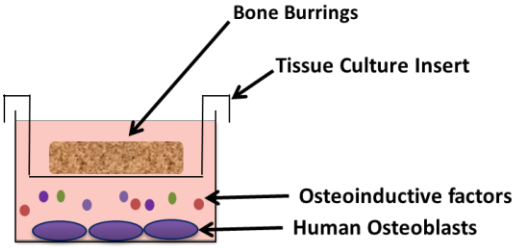

Figure 1: Experimental in vitro system

RESULTS: Osteoblast proliferation increased 5-fold in the presence of bone burrings, compared to control. Pro-collagen production remained unchanged, with negligible amounts released from the burrings themselves. Across four patient samples exposed to bone burrings, we had consistent increases in the expression of bone marker genes IBSP, RANKL and OPG, the cytokines IL-6, IL-11 and MCP-1, and the angiogenic factor VEGF.

Increasing amounts of alkaline phosphatase were released from the bone burrings over the 7 days, with high concentrations of VEGF and IL-6 also released. No BMP-2 or IL-1β was detected.

DISCUSSION & CONCLUSIONS: This study demonstrated that bone burrings enhanced osteoblast growth and the expression of bone cell differentiation markers.

The findings from this study, in conjunction with existing literature, suggest that bone burrings have the potential to be used as an effective autograft by acting as a source of viable osteoblasts and a reservoir of factors that induce local osteoblast growth and differentiation.

The autologous nature of this graft also means that there are none of the immune concerns associated with allografts and other natural and synthetic bone graft substitutes.

While 'gold standard' autografts harvested from the iliac crest require multiple surgical sites, bone burrings would not necessitate this, suggesting at least one benefit to bone burrings. Further studies are required to compare bone burrings with iliac crest autografts, yet the results from this study suggest that bone burrings have therapeutic potential to be used as a simple and effective autograft.

Dynamic simulation of hydrodynamics in reactor as bioartificial liver through a compartment model for liquid level control

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INTRODUCTION: Isolated hepatocytes represent a good model of liver metabolism. An important challenge in engineering devices for culturing hepatocytes is the development of bioartificial systems. Bioreactors are able to favour the liver reconstruction and to modulate liver cell behaviour. However, the increasing complexity of fluid dynamics and transport phenomena in present and future bioreactors requires advanced steady-state models and control strategies.

METHODS: A bioreactor model composed of an inlet region, a cell culturing environment and an outlet region was considered. They were modelled as lumped parameter compartments. In hydrodynamics, the inlet and outlet regions were assumed of fixed volume, while the cell culturing environment is fixed pressure, in order to limit fluid stresses on the cells. On the other hand, such an arrangement can lead to operational problems in keeping the desired liquid volume in the compartment. In terms of reaction engineering, the problem can be formulated as liquid level dynamics, for which a simulation study for level control purposed is proposed. A four-input/seven-state model of the bioreactor was developed in non-linear and linearized forms. The model is applied to an existing bioreactor prototype [1-2] which has shown irregular level behavior. Because the shape of the bioreactor is complex, the relation between volume and height of the liquid level in the bioreactor was calculated geometrically. The model has been developed within the MATLAB/Simulink environment.

RESULTS: Model results vs. experimental data were studied. Figure 1 shows the bioreactor level changed from 39 mm to 55 mm after 25 min. Both the linear and non-linear model predictions appear in reasonable agreement with the experimental data. Since the origin of the level variations is uncertain (fouling, unreliable distribution system etc.) external level control, based on the developed model, is proposed. A comparison between the simulated responses of system to change in the outlet flowrate (disturbance) with and without a simple level controller is shown in Figure 2.

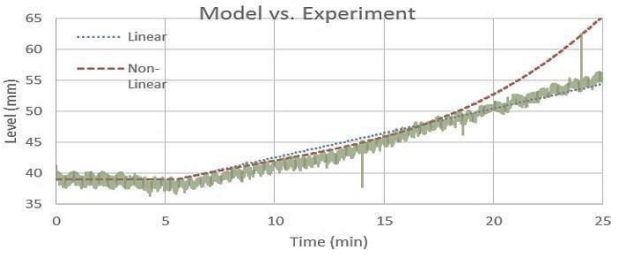

Fig. 1: Response of linear, non-linear and experimental data to a step change in outlet flowrate

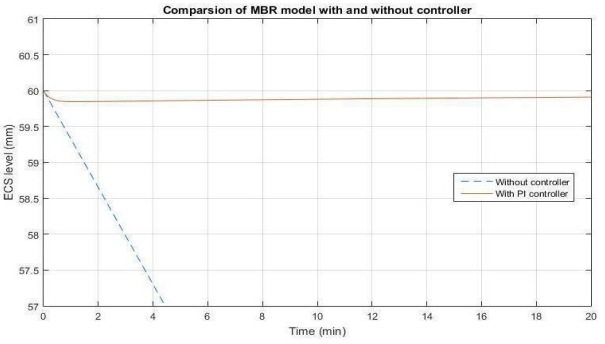

Fig. 2: A comparison of MBR model with and without controller

DISCUSSION & CONCLUSIONS: The necessity of using controller is clear in Figure 2. With 10% increasing of outlet flowrate, the bioreactor liquid level shows a dramatic decrease in the system without controller. On the other hand, with a simple PI controller the level will return to the level set point in few minutes. Ultimately, the present work allows improving the understanding of the dynamic behaviour of fixed pressure bioreactors as well as gaining the ability to proactively increase the performances by means of specific automation and control techniques.

ACKNOWLEDGEMENTS: The financial support of the European Union through the Project FP7-PEOPLE-2012-ITN "Training network for developing innovative (bio)artificial devices for treatment of kidney and liver Disease" (G.A. no. 316690) in gratefully acknowledged. The authors declare that they have no conflict of interest.

Dynamic regulation of TWIST1 expression during chondrogenic differentiation of human bone-marrow derived mesenchymal stem cells

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INTRODUCTION: Mesenchymal stem cells (MSCs) are clinically promising for repair and regeneration of damaged articular cartilage. In this study we investigated the role of *TWIST1*, an important transcriptional regulator in mesenchymal lineages [1], in adult human bone marrow-derived MSC (BMSC) chondrogenesis. We hypothesized that downregulation of *TWIST1* expression is required for *in vitro* chondrogenic differentiation.

METHODS: Expanded BMSCs (10 donors) were treated with or without small interfering RNA (siRNA) against *TWIST1* and then chondrogenically differentiated in a 3D pellet culture system supplemented with TGFβ1. TWIST1 mRNA and protein levels were determined by real time PCR and western blot analysis. Chondrogenic differentiation was determined with histological staining and real-time RT-PCR. Proliferation during 3D pellet culture was assessed by ki67 staining. Expanded and chondrogenically differentiated human articular chondrocytes (3 donors) were also investigated for *TWIST1* expression levels.

RESULTS: Significant downregulation of *TWIST1* was observed during chondrogenic differentiation of adult human BMSCs and culture expanded human articular chondrocytes, consistent with an inhibitory effect of *TWIST1* expression on chondrogenic differentiation. Silencing of *TWIST1* expression in BMSCs by siRNA, however, did not improve chondrogenic differentiation potential. Interestingly, additional investigation of *TWIST1* expression in BMSCs revealed that downregulation of *TWIST1* in chondrogenic BMSCs is preceded by initial upregulation at day 1 of pellet culture, associated with proliferation (increased number of Ki67-positive cells). Similar upregulation of *TWIST1* expression at day 1 is observed in non-chondrogenic BMSCs, however, in this case, these cells failed to downregulate *TWIST1* expression thereafter (Fig. 1).

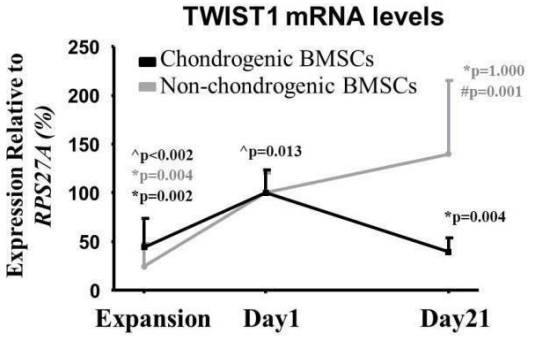

*Fig. 1: TWIST1 gene expression in expanded BMSCs and after 1/21 days of chondrogenic differentiation. Mean+95% CI; N=4-6 donors; *vs. Day1, #vs. Expansion and ^comparison of absolute TWIST1 expression levels by timepoint*

DISCUSSION & CONCLUSIONS: This study describes for the first time endogenous TWIST1 expression during *in vitro* chondrogenic differentiation of human BMSCs. By demonstrating dynamic regulation of TWIST1, we identified TWIST1 as a potential target for improvement of in vitro BMSC chondrogenic differentiation. Moreover, we established differing regulation of TWIST1 in chondrogenic and non-chondrogenic BMSCs, therefore providing a novel factor and signaling pathway for future investigations to understand and overcome the problem of heterogeneity in chondrogenic potential of BMSCs from different donors

ACKNOWLEDGEMENTS: **F**financially supported by NIRM (FES0908), SFI (11/RFP/BMT/3150) and ZonMw (116005009).

Smart platelet-rich plasma-based bioactive membranes as a new advanced wound care tool

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INTRODUCTION: Chronic skin ulcers, due to different pathological conditions among which diabetes, can heavily compromise the patients' life quality and are a high and constantly growing cost for National Health Services. Autologous platelet derivatives, and more specifically Platelet Rich Plasma (PRP), have been proposed for the treatment of these skin lesions. The absence of guidelines for the PRP production and the need of a fresh preparation for each treatment lead us to develop an allogeneic platelet derivative in form of a bioactive membrane, standardized for platelet concentrations and growth factors release. The present work compares two bioactive membranes (BAM) obtained starting from different platelet concentrations, to identify the best therapeutic dose for wound repair. BAMs were characterized for their growth factor content, their release of factors over time and for their in vivo efficiency.

METHODS: PRP was produced starting from buffy coat (BC) samples obtained from the whole blood of healthy donors at the Blood Transfusion Centre of the San Martino-IST Hospital, Genoa. BC was treated following a standardized procedure as recently described [1]. BAMs were prepared with a low or high platelet concentration (L and H). The biological activity was tested in terms of proliferation and cell migration using MTT and in vitro wound healing assay respectively. The angiogenic potential of the BAMs was evaluated by Luminex Angiogenesis assay. A diabetic mouse chronic ulcer model was used to define *in vivo* therapeutic dose of PRP.

RESULTS: In vitro experiments showed a different release of growth factors from both BAM and by Luminex assay a strong release of pro-angiogenic factors was revealed. Both BAM maintained a similar biological activity. Quantitative differences were observed in the time release of selected growth factors (PDGF and VEGF), able to support wound healing processes in vitro and in vivo (Fig.1). High platelet concentration provoked a slowdown in the BAM resorption, with a prolonged time for skin healing.

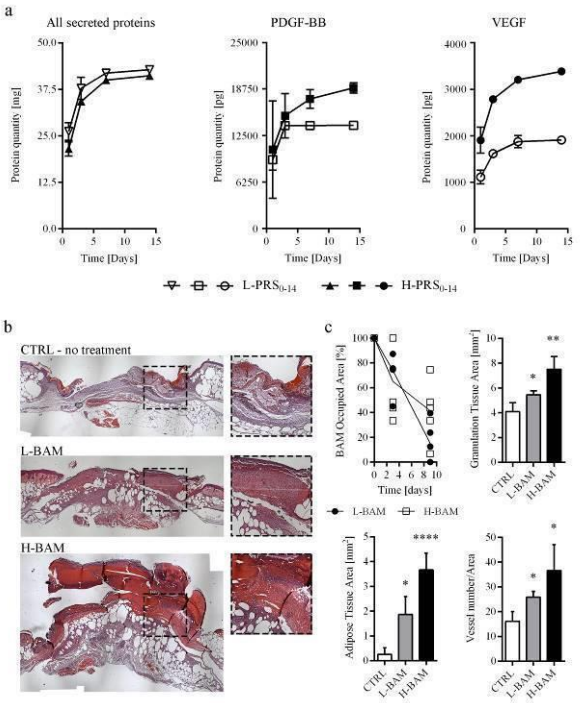

Fig.1: ***(a)*** *Proteins in released supernatant from BAM (PRS) at low (L) and high (H) platelet concentration.* ***(b)*** *Hystological analysis of in vivo treated tissues with L-H BAM after HE staining and* ***(c)*** *respective BAM speed resorption, granulation tissue and adipose tissue formation and blood vessel number evaluations.*

DISCUSSION & CONCLUSIONS: In our work, we show that our BAM have a gradually extended factor release over time. We can conclude that PRP-based bioactive membranes could be considered a good tool for the treatment of skin ulcer. Evaluating the critical role of platelet concentration and growth factor release in skin repair, we suggest a different application of both BAM for chronic or acute disease.

Directed-nanopatterning promotes earlier liver-specific maturation/ differentiation in human hepatic HepaRG progenitor cells

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INTRODUCTION: Drug-induced liver injury (DILI) accounts for the high attrition rate of drugs. In part, this is due to rapid loss of polarity and differentiated phenotype in conventional hepatic culture[1]. Cells respond to the entire context of their niche driven by nano- cell- and tissue-scale processes that integrate genetic programs with mechanical and chemical stimuli from the local microenvironment[2]. We previously demonstrated mesenchymal stem cells (MSCs) cultured on nano-displaced topography, significantly increased osteospecific differentiation[2]. Here, we aimed to assess which surface engineered nanopatterned topographies may promote earlier maturation /lineage commitment and differentiation of HepaRG cells for preclinical drug screening.

METHODS: HepaRG101 progenitor cells [Biopredic Inc: BPI] were seeded (>95% viable; 30000 cells cm^{-2}) and cultured for ≤28 days [BPI Growth Medium] on prototype 2D-nanopatterned polymer substrates [NPS: 24-well dish format/ 23 nanopatterns; with planar control; [0.32cm^2/well], fabricated using high-resolution electron beam lithography); or standard Corning plastic culture dishes (SCPs). Industry-standard morphological criteria, to assess formation of liver-like features on NPS *vs* controls, was used to delineate the optimal nanopattern(s). Metabolic competence (ATP content/ Prestoblue assays) of cells was assessed in parallel with phenotypic profiling (immunocytoflourecence staining) using liver-specific/ maturation-differentiation markers (Transferrin; HNF4a; Sox9; and the tight junction-associated protein ZO-1, critical for cell polarity).

RESULTS: We identified two NPS (designated NPS-1/2) that maintain metabolic competence and significantly enhanced liver-like morphological features (granular hepatocytes; with round nuclei), which appeared earlier (d7 *vs* d14 on SCPs), and compared with all other topographies tested, as well as d7 planar, or SCP controls. In addition, formation of the (intrinsic) HepaRG differentiated co-culture (hepatocyte:cholangiocytes) was evident earlier (d7-d14) on NPS-1/2 *vs* SCP control (morphology which normally appears d14-d28 on SCPs). This occurred even in the absence of liver differentiation supplements (ie -DMSO induction). Moreover, HepaRG progenitors had strong ZO-1 expression (Fig. 1) in NPS-1/2; indicative of diffusion barrier formation – essential in morphogenesis and hepatic differentiation/ functional cell-surface polarity. To further assess lineage-specific maturation of HepaRG progenitors, we observed reciprocal, temporal expression of transcription factors (TFs) HNF4a (hepatic differentiation) and Sox9 (*commitment* to cholangiocyte lineage), with early Transferrin (mature hepatocyte marker) expression - examined initially on SCPs. Work in progress on NPS-1/2 includes expression analysis of TFs and an extensive lineage-specific marker panel; as well as confirmatory protein and gene expression assays.

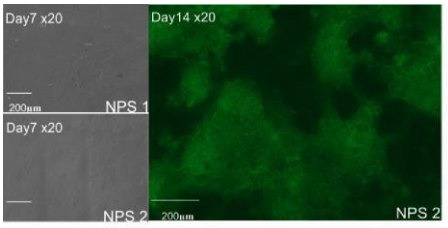

Fig. 1: Optimal nanopatterns (NPS-1/2): HepaRG101 (early) liver cell maturation (d7), which express cell polarity marker ZO-1 (d14) on NPS1/-2.

DISCUSSION & CONCLUSIONS: Discrete nanopatterned topography, which differed from NPS' shown to induce MSCs differentiation[2], significantly increased liver-specific differentiation in HepaRG progenitors. NPS surface engineering may provide inductive biophysical cues to switch on lineage-/ maturation-specific programs; essential to provide here, differentiated HepaRG co-cultures. A robust, scalable, cost-effective human hepatic *in vitro* organotypic platform would help generate more physiologically-relevant preclinical data for drug screening for DILI, and facilitate the study of biological mechanisms controlling hepatobiliary development.

Mechanical properties of the porcine oesophagus assessed using biaxial testing

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INTRODUCTION: The use of interventional endoscopic procedures has increased the interest in tissue regeneration in the oesophagus to develop techniques to mitigate the effect of post-operative leaks and perforations[1, 2]. This project characterizes the material properties of the oesophagus to allow the development of materials that better match the properties of the native tissue for use in regenerative applications. Biaxial mechanical testing was performed to assess the mechanical behaviour of porcine oesophagus.

METHODS: The oesophagus was retrieved from recently terminated porcine specimens and tested within four hours. Six 16 mm × 16 mm samples 3 mm in thickness were analysed using a biaxial testing system in a 37 ^{o}C water bath (Cellscale Biotester) (figure 1). Preconditioning, to ensure alignment of fibres, consisted of 5 repetitions of 10% load and equi-biaxial testing consisted of a single 40% load in the longitudinal and circumferential axes.

RESULTS: The results demonstrate minor anisotropy of the oesophageal tissue with stress of 4 MPa at 40% strain in the circumferential axis and 3 MPa at 40% strain in the longitudinal axis. The elastic modulus was 1.3 kPa and 1.6 kPa in the longitudinal and circumferential axes respectively at 40% strain.

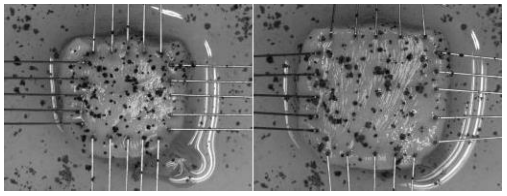

Fig. 1:Images of oesophageal sample loaded on CellScale biaxial testing system at 0% strain (Left) and maximum strain (Right).

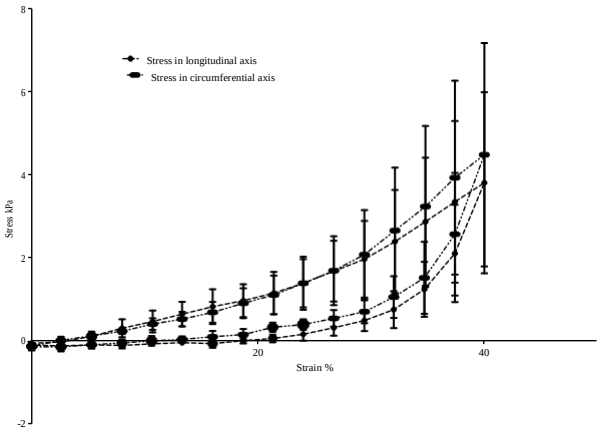

Fig 2:Stress-Strain graph for oesophageal specimens showing mean (kPa) and standard deviation

DISCUSSION: The oesophagus is a target for tissue regeneration following therapeutic endoscopic procedures, post-operative anastomotic leaks and iatrogenic injuries[3]. Self-expanding stents are the current mainstay of treatment, however these disrupt the normal anatomy and function of the oesophagus[4]. Understanding the biomechanical properties is vital for the development of improved materials that match the properties of the oesophagus and can allow the development of better stents or other novel devices including materials applied to the lining of the oesophagus to promote in situ tissue regeneration.

Methylation analysis according to the development stage of insulin-producing cells induced from bone marrow stromal cells derived from a GalT (GGTA1) knockout pig

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INTRODUCTION: Pigs with genetic modifications may prove useful in overcoming the metabolic and immunological barriers between species. DNA methylation, histone modifications, and non-coding RNAs have recently been identified as important epigenetic factors regulating pancreas development [1]. The present study aimed to identify the mechanism underlying methylation of Oct4 (stem cell factor) and PDX1 (pancreas transcription factor) by inducing insulin-producing cells (IPCs) from bone marrow stromal cells (BM-MSCs) of a double GalT (GGTA1) knockout (GalT KO) pig.

METHODS: GalT KO BM-MSCs were isolated from the femur of a double GalT KO pig and cultured in advanced DMEM with 10% FBS. IPCs were induced from GalT KO BM-MSCs by chemical induction for 2 months. Chemical induction was performed using a modification of the Pennarossa et al [2] method (i.e., DMEM/F12 medium was used instead of n2b27) after treatment with 1 μM 5-azacytidine (5-aza), a DNA methyltransferase inhibitor, for 1 day [2]. For analysis of Oct4 and PDX1 CpG islands, genomic DNA was extracted and treated with DNA bisulfite by using EpiTect Bisulfite Kits (Qiagen); methylation was analyzed by methylation sequencing. CpG islands were predicted using the EMBOSS CpGplot program, from 2,000 bps upstream to 500 bps downstream of the target sequence. The comparison groups were BM-MSCs, fibroblasts derived from a non-genetically modified pig, and pancreas tissue derived from a GalT KO pig.

RESULTS IPCs were successfully induced from GalT KO BM-MSCs after chemical induction for 2 months. Real time PCR and fluorescence analysis by immunofluorochemistry showed that IPCs had higher insulin, glucagon, PDX1, and Glut2 mRNA expression than GalT KO BM-MSCs. Three CpG islands each were detected in the predicted Oct4 and PDX1 promoter regions. In the full genome of PDX1, including the predicted promoter, a total of 10 CpG islands were detected. In the case of Oct4, 5-aza treatment did not affect the first CpG island but affected the second and third CpG islands, especially the second one (Fig.1). GalT KO cells were more sensitive to 5-aza than non-genetically modified cells. In the case of PDX1, CpG islands were not affected by 5-aza or IPC induction; a considerable part of the second and third CpG islands was unmethylated (Fig.2).

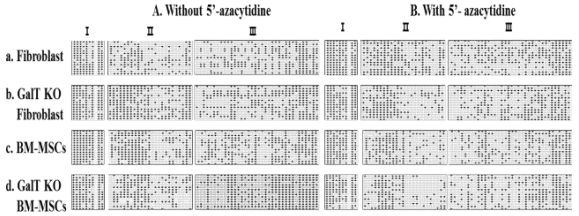

Fig. 1: CpG islands of Oct4. A and B were treated without and with 1 μM 5-azacytidine for 1 day, respectively.

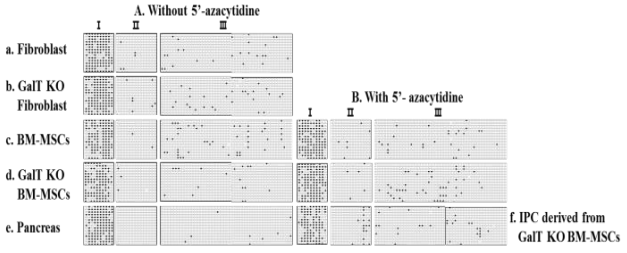

Fig. 2: CpG islands of PDX1. A and B were treated without and with 1 μM 5-azacytidine for 1 day, respectively.

DISCUSSION & CONCLUSIONS: Treatment of GalT KO BM-MSCs with 5-azacytidine improved stem cell capacity and accelerated IPC formation but did not affect the methylation pattern of PDX1, a key factor in pancreatic development.

ACKNOWLEDGEMENTS: This work was carried out with the support of the Next-Generation BioGreen 21 Program (no. PJ011311022016).

Decellularized liver protein matrix enhances the differentiation potential of pancreatic islet stem cells into beta-like cells

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INTRODUCTION: Type 1 diabetes mellitus is a metabolic disorder characterized by destruction of pancreatic β-cells, culminating in absolute insulin deficiency leading to the inability to control the glucose homeostasis. Cell transplantation promises a long term treatment. However, the potential source of these cells is a matter of debate. In the previous study, the pancreatic islet derived stem cells (PI-SC) were reported to be as insulin secreting cells, but the level of secretion was not sufficient to regulate the glucose homeostasis (1). In this study, the rat pancreatic islet derived stem cells, which were recovered from the tissue section after the recellularization process of the completely decellularized rat liver, were analyzed with respect to the reprogramming into insulin secreting cells. This study focused on the effect of the liver protein matrix after the decellularization on the differentiation into beta-like cells.

METHODS: Rat whole liver was decellularized by stepwise increasing the SDS-concentration using peristaltic pump under constant flow rate (2). The decellularized matrix collagen composition and integrity was analyzed by staining of matrix. Rat PI-SC were cultured in RPMI 1640 basal medium supplemented with 10% fetal bovine serum and 1% pen/strep. The recellularization of tissue took place for 4 days. The PI-SC were obtained by explant-culture of the recellularized tissue section, and subcultured for further analysis. The cell morphology and cell characteristics were investigated with respect to proliferation, gene expression profile and differentiation potential into endocrine cell lineage. The insulin secretion level was determined by ELISA.

RESULTS: After the decellularization process, the protein matrix was analyzed. It was observed that the collagen and fibronectin content was preserved, and the matrix structure was still intact. The DNA content analysis for the remaining cells in the matrix by PCR led to negative outcome, which mean complete removal of the hepatocytes from the matrix. The organ scaffold was recellularized by MafA+/Pax4+ PI-SC for 4 days. The stem cells after the interaction with the liver protein matrix were obtained from the migrated cells after the sectioning the recellularized tissue (Fig.1). The cells still preserved their MafA+/Pax4+ expression along with the co-expressed fluorescence protein. The insulin secretion level of the cells was increased after the contact with the matrix protein.

Fig. 1: PI-SC expanded from the recellularized liver tissue matrix with MafA+/Pax4+ cells after the sectioning on the culture plate.

DISCUSSION & CONCLUSIONS: The differentiation into beta cells was not fully achieved by this method. However, the interaction of the PI-SC with the protein matrix without any chemical induction increased the insulin secretion level close to the chemically induced PI-SC. The supportive effect of the matrix protein might be explained by the embryonic origin, which means both the liver and the endocrine pancreas originated from the endoderm germline.

Three dimensional differentiation model of adiposed derived mesenchymal stem cells in normoxia and hypoxia for cartilage repair

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INTRODUCTION: Three dimensional (3D) culture conditions of human adipose-derived mesenchymal stem cells (ADSCs) and low oxygen concentration are key factors for chondrogenic differentiation [1]. The hanging drop (HD) [2] culture system allows to form cell spheroids due to gravity. This model provides a 3D environment that allows cell-cell interactions observed in the cartilaginous condensation during the embryonic development and overcomes the limitations of two dimensional (2D) culture systems. Moreover, a low physiological oxygen tension has been reported in embryonic cartilage development, suggesting that a hypoxic environment enhances chrondrogenic differentiation of ADSCs [3]. The aim of this study was to evaluate the viability of ADSCs in HD culture system and further chondrogenic differentiation under normoxia (20% O_2) and hypoxia (5% O_2) conditions.

METHODS: ADSCs at a density of $15x10^3$ in droplets of 30 µl of complete culture media were seeded in petri dishes[2]. After spheroids were formed, chondrogenic differentiation media or fresh complete culture media (standard medium) were added, and cultures were placed either in 20% or 5% O_2 atmosphere. At 7, 14 and 21 days, the diameter of the spheroids was quantified. Cell viability and proliferation within the spheroids was measured by Live/Dead staining. To evaluate chondrogenic differentiation, in terms of extracellular matrix production, histological analysis (hematoxilin-eosin, safranin-O, alcian blue and Masson´s trichrome) were done at the same time points.

RESULTS: ADSCs cultured in HD system were viable and proliferate well in both conditions, under 20% and 5% O_2. The quantification of the diameter of the spheroids gives an approach of cell proliferation and matrix deposition. In normoxia conditions we saw that diameter of spheroids from the differentiated group (chondrogenic media) was significantly higher than spheroids from the control group (1.6, 2.2 and 2.3-fold increase at 7, 14 and 21 days, respectively). In addition, we also found that the chondrospheroids grown in 5% O_2 were larger at 7 and 14 days of culture compared to those cultured in 20% O_2 (1.2-fold increase; Fig1).

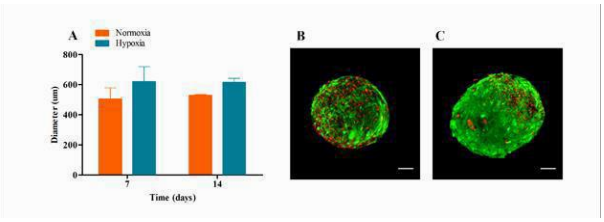

Fig. 1: Spheroids diameter quantification at 7 and 14 days in normoxia and hypoxia (A). Live/Dead staining in normoxia (B) and in hypoxia (C) at 14 days. Scale bar corresponds to 100 µm.

The histological evaluation performed in all measuring points showed positivity in alcian blue, safranine-O and Masson´s trichrome, due to a chondrogenic matrix synthesis in both culture conditions (20% or 5% O_2).

DISCUSSION & CONCLUSIONS: ADSCs in the 3D spheroids are viable, proliferate and exhibit chondrogenic phenotype under appropriate conditions (20% or 5% O_2, and chondrogenic media). Data obtained from the diameter quantification showed that spheroids differentiated in normoxia conditions are higher than the control group, which could be related with a chondrogenic matrix synthesis. Moreover, we note larger spheroids cultured in 5% O_2, compared to cultures in 20% O_2, suggesting an increase in matrix deposition activity of the cells under hypoxia. We can conclude that this 3D model (spheroids of ADSCs cultured in 5% hypoxia atmosphere) provides an excellent pro-chondrogenic microenvironment to create tissue constructs when combined with an adequate biomaterial.

ACKNOWLEDGEMENTS: V. Zubillaga is the recipient of an UPV/EHU predoctoral grant (PIF2014/46). Part of this work has been supported by Jesús Gangoiti Barrera Foundation.

Examination of the glial scar in a rat *ex vivo* model of spinal cord injury

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INTRODUCTION: Current interventions offer little hope for recovery of function for patients after spinal cord injury (SCI). In the search for better standard treatments for SCIs, several in vivo models have been developed and characterized over the past two decades[1]. However, we believe that an *ex vivo* model of spinal cord injury would be a beneficial research tool in the field by reducing the cost and time involved in carrying out animal studies, as well as reducing the numbers of animals used. It is believed that the use of organotypic slice culture models provide an simple way to study the cellular consequences following SCI and they can also be used to evaluate any potential therapeutics for the treatment of SCI[2]. Here we examined the cellular response of cells in at the lesion site following injury in organotypic slice cultures of rat spinal cord.

METHODS: Postnatal day 4 rat pup spinal cords were sliced at 350µm on a tissue chopper. A stab injury was performed in transverse sections and a transection injury was performed in longitudinal sections. Tissue was examined at day 3 and day 10 post injury (see Table 1). Tissue was processed for immunohistochemistry for markers of astrocytes (GFAP) and the chondroitin sulphate proteoglycan NG2. A live dead assay was performed to examine cell survival in slice cultures. Histological analysis for collagen scarring was examined. Ultrastructural analysis was performed at TEM level within the injury zone. Stereological analysis was performed to determine proportion of tissue composed of the various cellular components.

Table 1. Timepoints (days) for immunohistochemical study of glial scar

Spinal cord orientation	Experimental group	Days post injury	
Transverse slices	Uninjured	D3	D10
	Stab injury	D3	D10
Longitudinal slices	Uninjured	D3	D10
	Transection injury	D3	D10

RESULTS: A significant reduction in lesion size was observed between D3 and D10. Stereological analysis revealed that the expression of GFAP and NG2 increased in the tissue slices between D3 and D10 in both experimental groups. This is most obvious at the injury zone and scar zone (Fig. 1). Analysis of cell viability indicated a minimal amount of cell death.

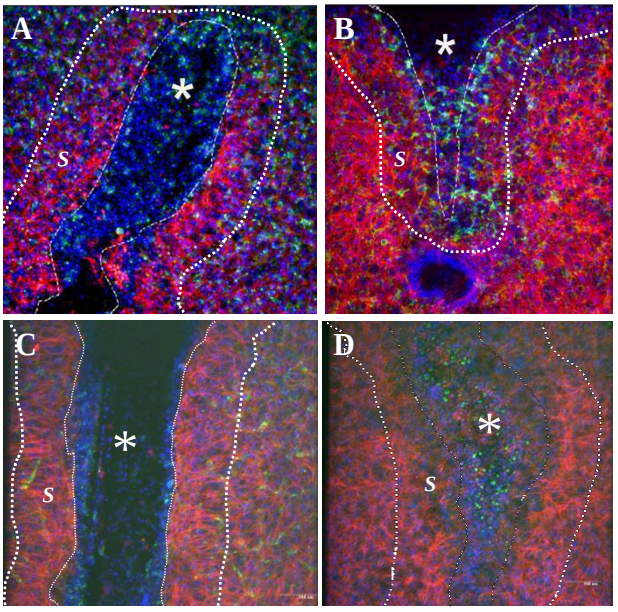

Fig. 1: Confocal photomicrographs of spinal cord transverse sections and longitudinal sections at D3(A and C respectively) and D10(B and D respectively); GFAP (red), NG2 (green)j, DAPI (blue). White dashed lines outline borders of the injury zone (*) and the scar zone (S).

DISCUSSION & CONCLUSIONS: A standardised spinal cord stab injury and transection injury model was established. The size of the lesion gradually decreased by D10. Presence of astrocytes and other scar components showed a proportional increase in size within injury and scar zones. Further analysis of cell response and ultrastructural detail will reveal the extent of different cell types within injured tissue.

ACKNOWLEDGEMENTS: Centre for Microscopy & Imaging funded by NUI Galway and PRTLI, Cycles 4 and 5, National Development Plan 2007-2013. This project was funded by the Ministry of Higher Education (MOHE) of Malaysia.

Effects of macromolecular crowding on human adipose stem cell culture in fetal bovine serum, human serum and defined xeno-free/serum-free conditions

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INTRODUCTION: Culture microenvironment plays an important role in expansion and differentiation of stem cells. Macromolecular crowding (MMC) has evolved as a method to assist cells in producing their own matrix microenvironment in vitro [1].

METHODS: The ability of MMC to support adipose stromal/stem cell (ASC) proliferation, metabolism and multilineage differentiation was studied under different culture conditions: fetal bovine serum (FBS), human serum (HS) and xeno- and serum-free (XF/SF) conditions.

RESULTS: Proliferation capacity of ASCs was reduced in each condition under MMC compared with standard conditions although higher metabolic activity in FBS media was observed under MMC compared with standard cultures. ASCs adopted rounded morphology and became larger in size under MMC in all studied culture conditions. Especially, the morphology of XF/SF expanded ASCs was changed dramatically under MMC. Alizarin Red staining showed stronger deposition of mineralized matrix under osteogenic induction under MMC in FBS and HS cultures compared with standard osteogenic induction. Likewise, Nile Red staining revealed significantly more lipid droplets under MMC induction in FBS and HS cultures compared with standard adipogenic induction.

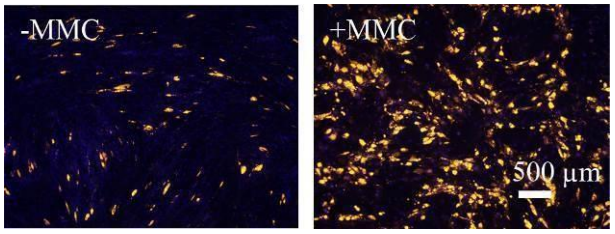

Fig. 1: Nile Red staining revealed significantly more lipid droplets under MMC induction (+MMC) compared with standard induction (-MMC) in HS cultures. Similar results were observed in FBS cultures.

Similar to Nile Red staining, collagen IV staining showed increased adipogenic matrix deposition under MMC induction in FBS and HS cultures compared with standard inductions. Due to poor viability of XF/SF expanded cells under MMC the adipogenic and osteogenic differentiation capacity was significantly reduced in XF/SF cultures. Alcian Blue staining showed stronger chondrogenic differentiation of ASCs after expansion in standard media compared with MMC culture in all conditions. The characteristic immunophenotype of ASCs was maintained under MMC conditions, except the significantly higher expression of CD54 (ICAM-1) under MMC.

DISCUSSION & CONCLUSIONS: This study demonstrated both supportive and inhibitory effects of MMC on ASC cultures. Although MMC did not support ASC proliferation in any of the studied conditions, a strong capacity for osteogenic and adipogenic differentiation was observed under MMC in serum containing media. A characteristic immunophenotype of ASCs was maintained under MMC, except for the increased expression of CD54 under MMC. For the studied XF/SF conditions MMC was a suboptimal method. Therefore, the success of MMC treatment proved to be culture condition-dependent. However, MMC is a potential technique to drive differentiation by modifying the ECM, which directly appears to modify cell morphology and to have an influence on the determination of a cell fate.

ACKNOWLEDGEMENTS: This work was supported by TEKES, the Finnish Funding Agency for Innovation, the Finnish Cultural Foundation, and NUS Tissue Engineering Programme.

Development of a new silk fibroin-autologous blood serum scaffold for healing of long-bone critical-sized defects: preliminary results

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INTRODUCTION: Bone critical-sized defects constitute one of the main challenges to be faced by specialists in traumatology and orthopaedics [1]. Although the "gold standard" treatment is autologous bone grafting, it presents some restrictions such as the limited amount of bone that can be taken from the patient. Silk fibroin (SF) [2] and blood serum (BS) [3] constitutes two scaffolds that have been tested in bone tissue engineering. While BS shows a higher osteoconduction, SF shows better biomechanics properties. Combination of both biomaterials could provide a new scaffold with promising properties for bone tissue engineering.

METHODS: Using a 10mm radial model of adult New Zealand rabbit, a SF (*Bombix mori*) scaffold was obtained by lyophilization of aqueous SF solution (7%) and posterior methanol crystallization. Autologous BS was obtained from whole blood allowed to clot and centrifuged. BS was cross-linked with glutaraldehyde, adapted to SF scaffold contour and finally lyophilized (Fig.1A). Autologous bone marrow was harvested from iliac crest and BM-MNCs were isolated with Biocoll separating solution. BM-MSCs were *in vitro* expanded and used after 3-4 passages. BM-MNCs or BM-MSCs were cultured with SF-BS scaffold for 1-2 h before transplantation. A 10mm radial defect was performed and substituted by a SF-BS scaffold alone or combined with BM-MNCs or BM-MSCs. Transplanted animals were followed-up along 6 months.

RESULTS: All animals transplanted with SF-BS scaffold achieved bone consolidation. However, this consolidation was significantly better when BM-MNCs or BM-MSCs were previously combined with the scaffold (Fig. 1B-C). No significantly differences were observed among BM-MNCs and BM-MSCs groups. In spite of bone consolidation, the scaffold wasn't completely integrated at the end of the study.

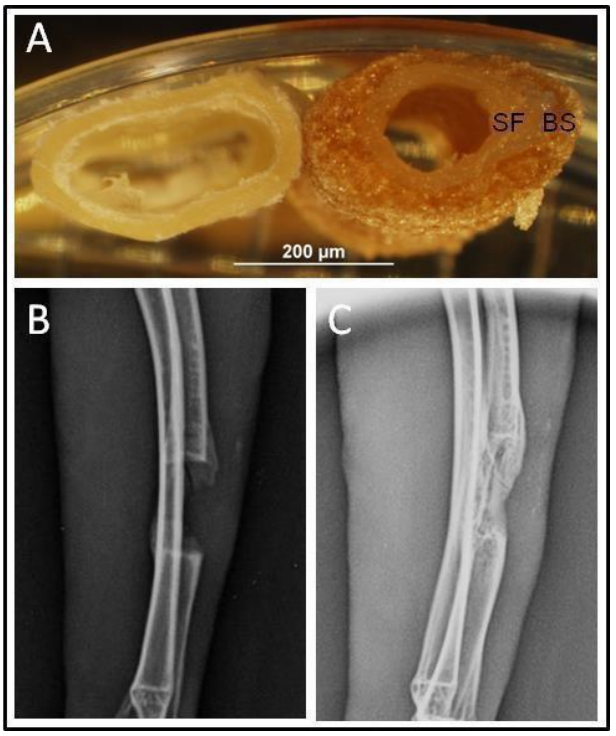

Fig. 1: A) Front view of native radius (left) and SF-BS scaffold (right). B) Radial defect transplanted with SF-BS scaffold combined with BM-MSCs, 1 month and C) 6 months after transplantation.

DISCUSSION & CONCLUSIONS: This new SF-BS scaffold, both alone and combined with cells, allowed healing of long-bone critical-sized defects. However, its resorption should be improved. Although micro-CT and histological studies will provide shortly interesting data to improve the scaffold, this strategy could constitute a promising alternative to autologous bone graft.

ACKNOWLEDGEMENTS: This study was supported by a grant from ISC III (PI13/02836).

3D-printing for tissue engineering of nasal cartilage

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INTRODUCTION: Nasal reconstruction remains a challenge for every oral and maxillofacial surgeon. Demands for replacements to fill nasal cartilage defects, after cancer resection and trauma or in cosmetic rhinoplasty, are increasing. The current attitude for reconstruction is to use autologous cartilage grafts of various origins (septal, auricular or costal) which aim to restore physiological anatomy. The Neck and Head Surgery Service of Pr. Disant, recognized in the field of nasal reconstruction is, at present, the pioneer of extracorporeal rhinoplasty. This technique consists in reconstructing in the operating room a nasal septum architecture from cartilaginous elements taken from the patient. A clinical study showed that the dimensions required for the reconstructed frameworks are very similar from patient to patient, opening the way for the standardization of the reconstruction process. However, the drawback of these grafts is the quantity of cartilage which may be limited depending on the patient's condition, sometimes resulting in incomplete and non-anatomical reconstruction. In this context, synthetic implants could be used: they offer the advantage of providing the necessary amount of material and respond to the immediate need of an aesthetic structure. A candidate material is high-density porous polyethylene (HDPP) already used in facial reconstruction.

METHODS: Our project aims to create by 3D printing an original HDPP framework. However, HDPP implants are still associated with high rates of extrusion and infection and poor biocompatibility. Thus, the idea is to fill the pores and cover the surface of the scaffold with a cartilage gel produced by tissue engineering. This cartilage gel is formed by embedding autologous chondrocytes in fibrin hydrogel. We have developed a new protocol to amplify chondrocytes from a small biopsy of cartilage and to stimulate their cartilage matrix production in hydrogel, under the control of selected soluble factors (used in their drug forms) [1].

RESULTS: Our immunohistological and biochemical *in vitro* analyzes show that human nasal chondrocytes embedded in fibrin hydrogel synthesize proteins characteristic of cartilage such as type II collagen and aggrecan. When the hydrogel is cast into a HDPP support, we observe by confocal microscopy the presence of cartilage that covers and fills the pores of the framework. After implantation of these constructs in nude mice, our observations highlight the stability of the newly synthesized matrix.

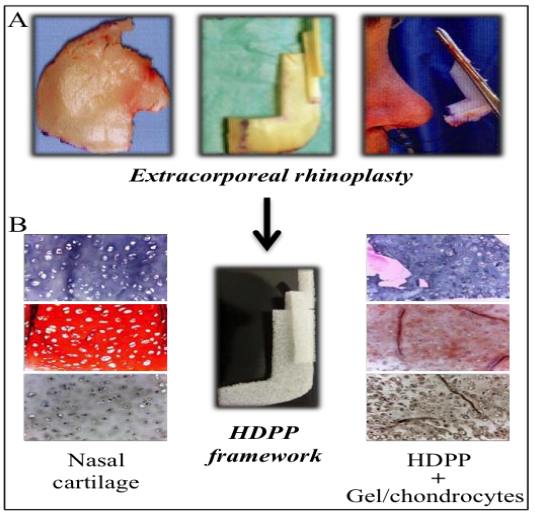

Fig. 1: (A) Cartilage nasal framework constructed with cartilaginous elements during extracorporeal rhinoplasty. This framework is re-implanted into the nose of the patient (example of nasal framework made with HDPP). (B) Histological sections: Hematoxylin-Eosin staining of the chondrocytes (on the top), Safranin-O staining of aggrecan (in the middle) and immunostaining for type II collagen (at the bottom). Note the quality of the cartilage matrix produced by nasal chondrocytes in the gel (on the right), in comparison with true cartilage (on the left). In this example, chondrocytes were embedded in hydrogel and poured into a HDPP disk, then were cultivated with specific factors during 4 weeks.

DISCUSSION & CONCLUSIONS: We have developed an original protocol allowing expansion of chondrocytes and their production of cartilage in 3D-printed framework mimicking the shape of nasal cartilage. This new technique should improve the biocompatibility of the implant. The next step is the establishment of a Medical Device and the development of an implant for validation as an Advanced Therapy Medicinal Product.

Fibrin glue as a carrier for cultured human epidermal progenitor cells for cellular therapy of non-healing wounds

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INTRODUCTION: Chronic wounds constitute a serious medical, economic and social problem. However, there has been a significant progress in the treatment of these conditions. It is connected mainly to the implementation of tissue engineering products based on in vitro expanded, autologous epidermal progenitor cells (EPCs) to the treatment [1,2]. This method has many advantages such as relatively high efficacy, lack of graft rejection and lack of diseases transmission. However, there are still many limitations including a long period of culture (3-4 weeks) and difficulties in effective and safe delivery of cells onto the wound [3,4]. In this study we propose a comprehensive method of chronic wound treatment based on autologous in vitro expanded patient's cells applied in fibrin gel.

METHODS: Skin biopsies were aseptically taken during a variety of surgical procedures after obtaining a written informed consent of patients (a total number of patients n=15). After removal of the subcutaneous tissue, the cells were isolated using dispase and trypsin. Cells were cultured in basal serum-free medium (KBM2, Lonza) supplemented with hydrocortisone, insulin, transferrin, epidermal growth factor (KGM2) [2]. Cells were analysed by means of flow cytometry (LSRII, BD, USA) and transmission electron microscopy (TEM, JEOL, Japan). The second passage cultured human autologous keratinocytes (4×10^6 in 4.0 mL of Tisseel Lyo fibrin glue) were applied to the wound surface of five patients (HBV-, HCV-, HIV-negative) suffering from chronic wounds.

RESULTS: Our research has demonstrated that a proposed method of isolation and culture enables to obtain a high number of autologous EPCs on a low level of differentiation (high expression of alpha-6 and beta-1 integrin, low expression of receptor for transferrin - CD71) and a high proliferative activity (high percentage of Ki67 positive cells). The method also includes microbiological assessment of cell culture (LAL test) what increases the patient's safety. Mixing cells with the fibrin gel (commercially available) did not cause any significant changes in the ultrastructure of the cells. TEM images visualized morphologically normal keratinocytes with dispersed intracytoplasmic membranous organelles. Lack of intermediate filaments in keratinocytes in TEM was consistent with the undifferentiated state and potential of proliferation. Moreover, the application of the cells together with the gel has increased the adherence of cells to the wound bed; acted hemostatically and stimulated chronic wound healing.

DISCUSSION & CONCLUSIONS: The presented method of isolation and EPCs culture allows to obtain appropriate number of autologous cells to the transplant from a small skin fragment. Then, these cells may be applied directly on the wound by means of the gel which constitute a widely used and safe tool in medicine. During the cell culture and clinical application no elements of animal origin were used that affected the safety of the therapy. In conclusion, the above described comprehensive method of the therapy with the use of EPCs and fibrin gel may become a promising alternative in the treatment of chronic wounds.

ACKNOWLEDGEMENTS: This work was supported by the[2] funds of Polish National Science Centre granted to Michał Pikuła based on the decision no. Dec-2011/03/D/N25/00555.

Double-compartment culture system for conditioning vascular grafts with different trans-wall oxygen levels

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INTRODUCTION: The aim of this work is to develop a new *ex-vivo* model for conditioning small vessel structures with inner-outer oxygen gradients, featuring separated intra-luminal and extra-adventitial compartments, where oxygen levels are differentially controlled.

METHODS: The culture system consists of a double-compartment chamber, a vessel housing and a purpose-developed de-oxygenator module [1], the latter consisting of oxygen-permeable silicone tubing hosted in a reservoir (Fig. 1A-B). Silicone tubing length, and recirculating flow rates were pre-dimensioned through mathematical modelling. The performances of the de-oxygenator was tested single-pass and in a recirculated condition imposing different levels of oxygen. Oxygen concentrations were measured via PreSens Microx 4 meter with PSt7-10 spots (Fig 1A). Experiments with human SV (hSV) segments were performed for a conditioning period of 7 days (Fig.1C). Briefly, hSVs were mounted within the culture system, and arterial oxygen level conditions (21% intraluminal, 5% extra-adventitial) were compared with standard oxygen conditions (21% in both compartments). In all experiments, culture medium was recirculated at 5 ml/min with a luminal pressure of 5 mmHg. At the end of the conditioning period, hSV central portion was processed for the histological analyses.

RESULTS: The results of bench tests demonstrated that controlled trans-wall oxygen gradients were generated, exploiting the oxygen saturation of the gaseous mixture as a control parameter (Fig.1D). Regarding the conditioning experiments with hSVs, the main result is that the arterial oxygen condition displayed a significant increase in the density of adventitial small-and-large caliber *vasa vasorum* (Fig.1E, upper panel), as well as a higher proliferation of cells within and around these vessels (Fig.1E, lower panel).

DISCUSSION & CONCLUSIONS: From the technical point of view, the results proved the device to be a versatile, easy-to-use and functional system. Moreover, it is compatible with the best standards for good laboratory practice and a valuable alternative to bubbling a gas mixture in the culture medium. Regarding the hSV experiments, these results suggest that vein adventitial hypoxia promotes neovascularization and growth of *vasa vasorum*, which is known to predispose the arterialized vein to restenosis, increasing the risk of graft failure.

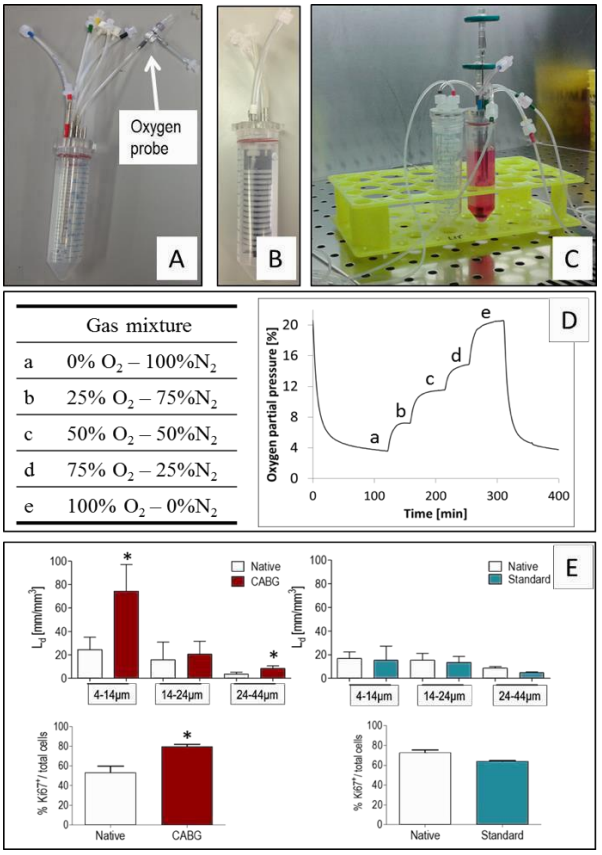

Fig. 1: Pictures of the culture chamber (A), the de-oxygenator module (B) and the assembled system during experiments with hSVs(C). Results of the experiments carried out to verify the system's performances (D), and results of the hSV oxygen conditioning experiments (E).

ACKNOWLEDGEMENTS: This work is supported by the Italian Ministry of Health (RF- 2011-02346867).

An advanced platform for investigating fibrin based engineered cardiac tissues

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INTRODUCTION: In the past years, significant advancements were made in the generation of 3D Engineered Cardiac Tissues (ECTs) used as functional *in vitro* models for drug screening or investigating fundamental mechanisms [1,2]. In particular, the application of physiological physical signals (e.g. electrical and mechanical stimuli) was confirmed to be crucial to further promote cardiac differentiation, maturation and organization [3]. However, until now the bioreactor-culture was meant to passively apply a time- constant or variable stimulus predefined by the user regardless of the continuous changes of the maturing ECTs during the time of *in vitro* culture. Here, we present a novel bioreactor design, capable in its most advanced operational mode to (i) apply native-like or pathologic stimuli (namely cyclic strain), (ii) monitor in real-time both the milieu parameters (e.g. oxygen tension, pH, temperature) and the progress in mechanical stiffness of the mm-scale ECTs, and eventually adapting the stimulation to their actual maturation stage. Towards the final validation of the bioreactor potential, we first assessed the effects of cyclic strain on the maturation, alignment and contractility of fibrin-based ECTs.

METHODS: The bioreactor consists of a culture unit and of a monitoring and control unit. A linear actuator applies controlled cyclic stretch to ECTs (up to 200% deformation) in a frequency range of 1-6Hz. The presence of an ad hoc control unit allows (1) the acquisition of the signals from sensors, (2) the communication between the final user and the system (by a user friendly interface), and (3) the feedback loop motion control strategy (Fig 1a). ECTs were generated by neonatal rat cardiomyocytes embedded in fibrin gel and, after a pre-culture of 5 days in static condition, were moved into the bioreactor and exposed to cyclic stretch (10% strain at 1 Hz, Fig 1b). Static culture was used as control.

RESULTS: Initial tests aimed at assessing the proper function of the hardware and software components. Compared to static controls (Fig 1, c), the cyclic mechanical stimulation promoted the cardiomyocyte alignment and maturation as confirmed by the enhanced expression of typical cardiac markers (i.e. sarcomeric α-actinin) (Fig. 1 d). Remarkably, only in presence of cyclic strain the ECTs were functionally mature, capable to respond to external electrical pacing. The ECTs showed a significant matrix remodeling and increase of collagen deposition after mechanical stimulation. The ECTs stiffness (Young modulus) resulted also to be superior after cyclic strain.

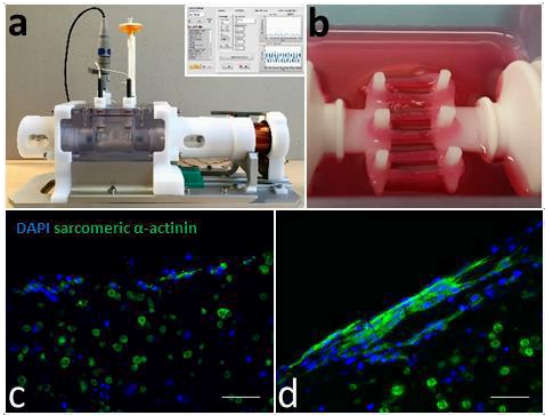

Fig 1. Bioreactor set-up and user interface (a). ECTs housed within the bioreactor culture chamber (b). Immunofluorescence staining for DAPI and α- sarcomeric actinin for static (c) and dynamic (d) conditions (scale bar 50 µm).

DISCUSSION & CONCLUSIONS The proposed platform represents a very promising in vitro functional cardiac model to monitor *in real time* ECT maturation and their dose-response to different drug supplementation during physio/pathological stimuli. Ongoing studies aim to implement an automated adaptation of the stimulus with respect to the ECT maturation.

ACKNOWLEDGEMENTS: Mechanical components were realized by BioExpansys srl®.

Establishment of an *in vitro* model of teratoma-like tissue formation to assess stem cell pluripotency

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INTRODUCTION: The gold standard to demonstrate pluripotency of newly derived stem cell lines is the formation of teratoma within five to ten weeks in immunodeficient mice [1]. In addition, the teratoma assay is considered as a criterion that should be fulfilled in order to ascertain that a genuine induced pluripotent stem cell line (iPS) has been obtained [2]. Nevertheless, the place of implantation, the different mouse strains as well as the duration of the assay introduce high level of variability [3]. We report the development of an *in vitro* model, capable of supporting formation of teratoma-like structures, towards integration or replacement of conventional *in vivo* assays.

METHODS: Spheres derived from a human ESC line (CHES-6) and one iPS line were embedded into a collagen scaffold (Ultrafoam®) and cultured dynamically in a 3D perfusion-based bioreactor system (Cellec Biotek, U-CUP) for up to 8 weeks. Embryoid bodies (EBs, the c onventional *in vitro* culture) were also cultured during the same time interval and used as controls. Harvested tissues from the two groups were assessed by using immunohistochemistry (IHC) and qPCR of selected markers, such as α-fetoprotein (*AFP*), *Brachyury* and *Pax6,* to demonstrate commitment to the three germ layers. In addition, the capability of prolonged tissue differentiation and structure development were investigated by IHC. Glucose consumption and the release of *AFP* were measured in the culture medium by ELISA.

RESULTS: Positive expression of the endodermal marker *AFP*, the mesodermal marker *brachyury* and the neuroectodermal marker *PAX6* were detected at mRNA and protein level between 1 and 3 weeks in both *in vitro* culture conditions. However, in contrast to the EB formation, a broad spectrum of different structures was detected in the bioreactor system (Fig.1) such as epithelium of early intestine, glandular tissue and neural rosettes. The measurements of glucose and *AFP* in culture media of the bioreactor system showed a characteristic curve over time reaching peak values at 3 weeks for the AFP release.

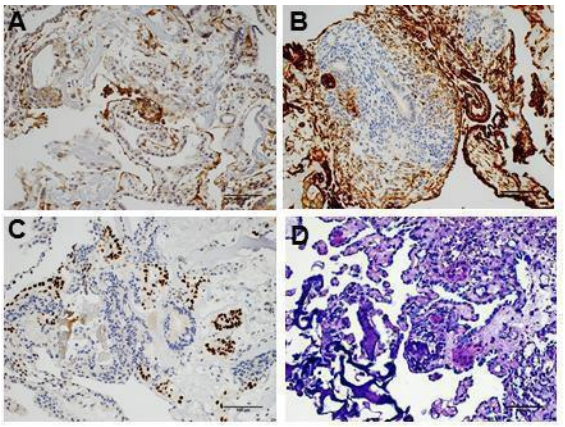

Fig. 1: (A-D) IHC of teratoma-like tissue generated in the bioreactor system after 3 weeks of culture. (A) α-SMA expression displays smooth-muscle like structures (B) positive expression of Lu-5 indicates epithelial structures (C) CDX2 expressed by cells of early intestinal tissue (D) PAS staining showing early glandular structures.

DISCUSSION & CONCLUSIONS: Bioreactor-based cultures from the two pluripotent stem cell lines support the development of multiple tissue structures and positive expression of the markers from the three germ layers already after 3 weeks of culture. The peak of the *AFP* release at 3 weeks indicates that the differentiation process might be monitored in a non-invasive way. The system represents a controlled and reproducible method to obtain a teratoma-like tissue within 3 weeks of culture. Studies are ongoing to further validate the system as a surrogate or complimentary assay of conventional *in vivo* teratoma formation. The system would bear relevance in basic science and towards definition of more practical and better standardized regulatory guidelines for assessment of pluripotent cell lines.

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ACKNOWLEDGEMENTS: This work is supported by the 3R Research Foundation Switzerland.

Self-assembling peptide hydrogels coupled with stem cell inducing/recruiting factor and cell-adhesion peptide for accelerated in situ tissue regeneration

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INTRODUCTION: Stem cells hold great promise for repairing injured/damaged tissues. However, limited number of stem cells and poor survival and engraftment rate after *in vivo* transplantation hamper their full utilization for an effective tissue repair. These bottlenecks can be overcome by enhancing host's own repair capabilities by stem cell mobilization, by localizing cells at the injury site, and by instructing these populations to differentiate into tissue/organ specific lineages. The objective of this study was to develop instructive nanofibers presenting stem cell inducing/recruiting factors, cell adhesion peptides, and growth factors to enhance in situ tissue repair.

METHODS: Self-assembling peptides (RADA-16) with or without neuropeptide substance P (SP) (R-SP) and mesenchymal stem cell-affinity peptide (R-M7) were purchased from Peptron (Daejeon, Korea).[1] Instructive nanofibers were developed by mixing 1% (w/v) solutions of RADA-16, RADA-SP, and RADA-M7 (200:1:1) in sucrose. Cell proliferation and migration was assessed *in vitro*. In order to select the optimum amount of M7, cell proliferation was analysed as a function of the concentration of M7. For *in vivo* analysis, these nanofibers were applied in an excisional wound model in SCID rats (age = 7 weeks, n = 30) for up to 3 weeks. Following groups were studied: a) saline, b) RADA-16, c) RADA-16 + R-SP, d) RADA-16 + R-SP + R-M7, and e) RADA-16 + R-SP + R-M7 + MSCs + EGF.

RESULTS: The obtained results revealed higher cell proliferation and migration in nanofibers containing SP compared with RADA-16 nanofibers only. Furthermore, cell adhesion was increased with increasing content of M7. SP and M7 showed higher wound closure rate and tissue healing compared with other groups. The introduction of exogenous MSCs further enhanced the wound closure rate and paralleled with groups receiving both SP and M7. While both SP and SP/M7 groups showed recruitment of endogenous MSCs, later group showed higher extent of endogenous cell recruitment. Noticeably, the combination group also exhibited higher number of newly formed blood vessels, which may arise from either pre-existing vasculature or from recruited stem/progenitor cells. TUNEL assay was performed to evaluate the content of apoptotic cells, which demonstrated less apoptosis in groups containing M7, which signal that the incorporation of cell adhesion peptide may localize the recruited stem/progenitor cells at the injury site.

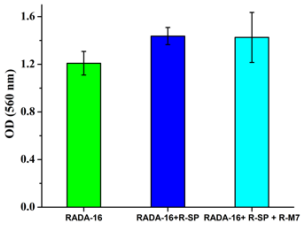

Fig. 1: In vitro cell migration of RADA-16, RADA16 + R-SP, and RADA-16 + R-SP + R-M7 hydrogels

DISCUSSION & CONCLUSIONS: These findings reveal that incorporation of cell adhesion motif positively affects the tissue repair capability of instructive nanofibers containing stem cell inducing/recruiting factor possibly by localizing both endogenous and exogenous stem/progenitor cells at the injury site. Given that the stem cell-mediated tissue repair decreases because of the poor survival/retention of stem cells and limited engraftment into tissue-specific lineage, the combinatorial methodology presented here may obviate such limitations. Our ongoing projects include the application of developed material for limb ischemia and myocardial infarction models.

ACKNOWLEDGEMENTS: This work was supported by the Ministry of Trade, Industry and Energy, Republic of Korea (Project No. 10052732).

White blood cell concentration correlates with CFU-F concentration in the output of a point-of-care bone marrow concentrating device

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INTRODUCTION: Concentrated bone marrow aspirate (cBMA) is commonly used in regenerative medicine [1]. cBMA contains mesenchymal stem cells (MSCs) which are measured using colony forming unit (CFU-F) assays. Previous research has shown correlation between CFU-F number and treatment success [2]. However, CFU-F assays typically take 10 – 15 days limiting their clinical utility. It would be advantageous if there were a rapid method to approximate the CFU-F concentration in a clinically relevant time frame.

METHODS: From one hip (Hemacare, 5 Donors) 54 ml of bone marrow was combined with 6 ml heparin (1000 U/ml). BMA was then processed using concentrating devices (n = 10 devices) (BioCUE™ Mini Concentration Kit (800-0612A), Zimmer Biomet) using manufacturer's instructions. A portion of the BMA was set aside for baseline analysis. Complete blood count analysis was performed using a Cell Dyn Sapphire (Abbott). CFU-F assays were performed using Mesencult Media (StemCell Inc.) following manufacturer's instructions.

RESULTS: The concentrating device significantly increased the concentration of all cell types in BMA (t-test, $p < 0.05$).

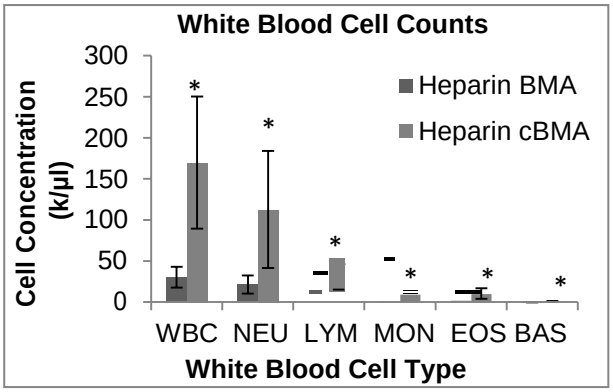

Figure 1. White blood cell concentration in BMA and cBMA anti-coagulated with heparin.

The average CFU-F concentration in the output of the concentrator was 3,905 CFU/ml. The CFU-F concentration was significantly increased in cBMA compared to BMA (t-test, $p < 0.05$). The average CFU-F concentration fold increase was 8.9X when using heparin as an anticoagulant (Figure 2).

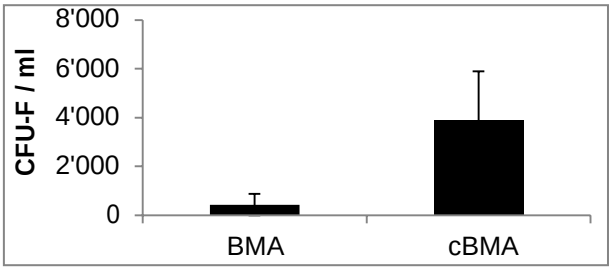

Figure 2: CFU-F concentrations for BMA and cBMA using heparin as an anticoagulant. (n = 10 devices / group).

There was significant ($p = 0.001$) correlation between the WBC concentration and CFU-F concentration in cBMA (Figure 3).

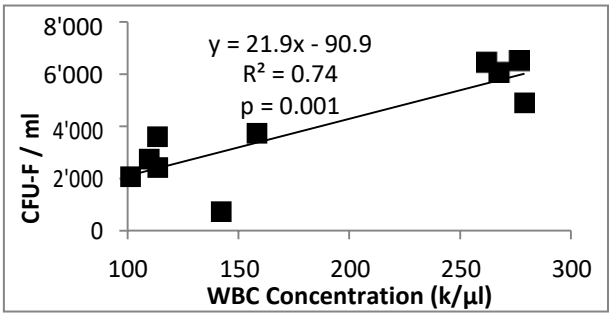

Figure 3. Correlation analysis between WBC (k/µl) and CFU-F (CFU-F / ml) concentration.

DISCUSSION & CONCLUSIONS: This study demonstrated that the point-of-care devices enriched all of the bone marrow-derived cell types measured in this study including MSCs. The strong correlation between WBC and CFU-F concentration could enable the estimation of CFU-F in a clinically relevant time frame instead of the 10 – 15 days typically required. Further research will be necessary to characterize the correlation between WBC and CFU-F concentration in the output of point-of-care concentration devices.

DISCLOSURES: The study was sponsored by Zimmer Biomet, the manufacturer of MarrowStim. All authors received meals from Zimmer Biomet during the planning phase of the study

Comparison of human bone marrow mesenchymal stromal cells, adipose tissue-derived stromal cells and bone marrow mononuclear fraction in a diabetic wound healing model

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INTRODUCTION: Wound healing impairment is one of the prominent diabetic complications associated with neuropathy, immune system dysfunction and ischaemia [1]. There is growing evidence that mesenchymal stromal cells (MSCs) might provide a supportive environment for angiogenesis and will enhance wound healing in diabetic patients [2]. We compared the process of full-thickness wound healing in diabetic rats treated with the various types of human MSCs isolated from bone marrow (BM-MSCs), adipose tissue (ASCs), or bone marrow mononuclear cells (BMC) obtained from diabetic (DM) and healthy donors (nonDM).

METHODS: BMC were isolated using Gelofusine separation, BM-MSCs and ASCs were isolated and cultured according to standard protocols. Diabetes was induced in 56 Wistar rats by a single intraperitoneal injection of streptozocin 64 mg/kg and confirmed 48 h later by a blood glucose level higher than 15 mmol/l. Two full-thickness skin wounds, 0.8 cm in diameter, were created by a punch biopsy at the back of each animal. The cell suspension ($2x10^5$ for hBMC, $2x10^6$ for hBM-MSCs and hASCS) was injected into the base of the wound, and its close surroundings (*Fig. 1*). The speed of wound closure and neovascularization performed by Vessel Index (VI) counted as the ratio of CD31+ cell number to wound area was assessed after 3, 7 and 14 days.

RESULTS: A significant acceleration of wound healing was seen in all cell treated groups in comparison with controls after 7 and 14 days (BMC $p<0.001$; BM-MSCs $p<0.01$; ASCs $p<0.05$). No difference between diabetic cell- and nondiabetic cell-treated groups was observed. VI was significantly higher in all cell-treated groups in comparison to controls ($p<0.05$). However, lower VI was found after treatment with diabetic BMC in comparison to the nondiabetic BMC and nondiabetic BM-MSCs, ($p<0.05$).

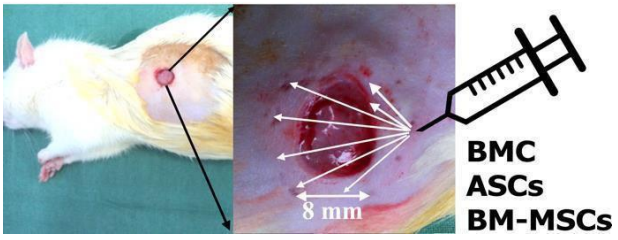

Fig. 1: Injection of cell suspension into the full-thickness wound in a streptozocin induced rat.

Table 1. Study design of animal groups.

Animal group	Cell type	Source DM/nonDM	Wound area reduction at 14 days
1	hBMC	DM	99.2%
2	hBMC	nonDM	94.4%
3	hBM-MSCs	DM	97.8%
4	hBM-MSCs	nonDM	98.6%
5	hASCs	DM	96.6%
6	hASCs	nonDM	95%
7	control	-	83.8%

DISCUSSION & CONCLUSIONS: Both diabetic and nondiabetic cells improved healing of full-thickness wounds in diabetic rats. However, better neovascularization was found after treatment with nondiabetic cell source. BMC, BM-MSCs and ASCs represent a safe and efficient method for allogeneic as well as autologous wound treatment in diabetic patients.

ACKNOWLEDGEMENTS: This project was supported by GAČR 1403540S.

Transplantation of allogeneic mesenchymal stem cells for the treatment of critical lower limb ischemia

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INTRODUCTION: Insufficient efficiency of the existing approaches in the treatment of inoperable patients with critical lower limb ischemia (CLLI) necessitates a search for new techniques to increase the treatment efficiency. In this study, we have assessed the safety and efficiency of the CLLI treatment with the use of donor mesenchymal stem cells (MSC).

METHODS: MSC were isolated from healthy donors' bone marrow using a standard procedure [1]. The MSC population expressed CD44, CD105, CD73 and CD90, and lacked expression of CD45 and CD34, as measured by flow cytometry. MSC also demonstrated ability to differentiate to osteoblasts, adipocytes and chondroblasts in vitro. The MSC transplantation (1 million cells per 1 kg of the body weight) was performed in 10 CLLI patients, of which 7 patients had pronounced rest pains and limited trophic disorders (class 5 by Rutherford [2]), 1 patient had an extended necrosis of the anterior tibial surface (class 6 by Rutherford). The mean age of patients was 61 years. 6 out of 10 patients had been repeatedly operated at the side of the affected limb prior to transplantation. A multi-level damage of the arterial flow was found in all the patients.

Fig. 1: Intramuscular MSC administration.

In 7 patients, the MSC transplantation into the tibial muscles was performed as a sole treatment (Fig.1), in 3 patients it was performed intra-arterially together with the arterial reconstruction to improve the outflow.

RESULTS: All the patients noticed a satisfactory tolerance of the cell transplantation procedure, no serious direct adverse effects were observed. No fatalities were seen during the observation period (up to 18 months). The limb was preserved in 7 patients with either complete or almost complete relief of the pain syndrome at the 13 months mean duration of observation. 3 patients experienced a complete healing, 4 patients – a significant healing of the trophic ulcers. No complications were observed in the distant period. The very important index of the pain syndrome was estimated by means of a visual analogue scale.

It reduced significantly even in patients with the remaining trophic disorders. The mean pain index decreased from the initial value of 51.4 to 21.4 post-treatment. It should be noted that the unfavorable outcomes were observed in patients with the initial severe pain syndrome (more than 80 points), in all such cases the amputation was required.

DISCUSSION & CONCLUSIONS: Application of donor MSC from the human bone marrow is safe and efficient in CLLI, allowing preservation of the limb in 7 of 10 patients at the 13 months mean duration of observation. A further study of the problems is needed both for the technique perfection and for a more detailed assessment of this therapy safety and efficiency.

ACKNOWLEDGEMENT: This study is financially supported by the Russian Science Foundation (Grant No. 16-15-10432).

Fluid-based culture medium (FCM) for clinical application

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INTRODUCTION: Cell transplantation requires *in vitro* culture and preservation of the cells or tissues within a certain period using appropriate culture medium and methods. However, most cell culture media contain substances (i.e. pH indicators, buffers and other components) that are yet to be to be verified for their safety when used for clinical purposes. We modified a culture medium using the injectable and fluid drugs, under the concept of drug repositioning, which might be safe alternative for culturing human cells and tissues prior to transplantation.

METHODS: The medical fluid-based culture medium (FCM), is a modified culture medium, consisting of commercially available fluids and injectable drugs and adjusted to concentrations equivalent to the commercial Dulbecco's modified Eagle's medium (DMEM). Rat H9c2 cells and human fat synovium - derived mesenchymal stem cells were cultured in 1) DMEM-high glucose (HG), an original commercial medium, and 2) optimized FCM-HG and the differences in cell proliferation rates, conditioned medium pH values, and conditioned medium osmolalities were analyzed using the Student t-test, with significance defined at $P < .05$.

RESULTS: We observed that the morphologies and proliferation rates of H9c2 cells and FS-MSCs cultured under FCM-HG showed no significant differences compared to those grown in DMEM-HG.

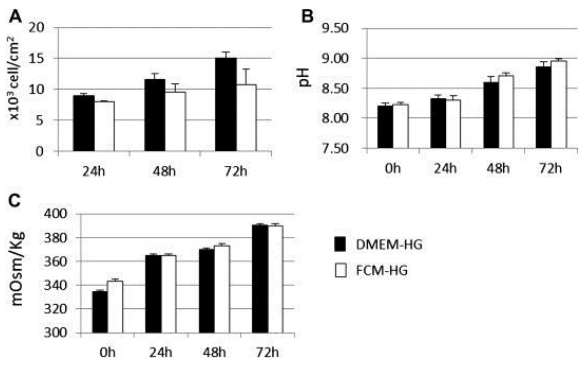

Fig. 1: Human fat synovium-derived mesenchymal stem cells grown in high-glucose fluid-based culture medium (FCM-HG) and Dulbecco's modified Eagle medium (DMEM-HG) showed no differences in terms of proliferation rates (A), pH values of the conditioned media (B), osmolalities of the conditioned media (C).

DISCUSSION & CONCLUSIONS: The full potential of the FCM can explored further by investigating its efficiency in culturing other cell types. It is also crucial to investigate the effects of serum-free FCM on cell proliferation, cell morphology, cell passage conditions, and cell physiologic characteristics to determine the possibility of the FCM as a xeno-free alternative to the fetal bovine serum (FBS), or combined with human cord blood serum or albumin instead of FBS. There is also potential of harvesting conditioned FCM which may contain various metabolites, cytokines, growth factors and effective components secreted by cultured cells. We have established that the FCM for the first time, using drug repositioning strategy, is a more economically beneficial and safer than commercial medium and can be used to culture cells intended for human cell and tissue transplantation.

ACKNOWLEDGEMENTS: This research was supported by the Basic Science Research Program through the National Research Foundation of

Korea (NRF), the Ministry of Education, Science and Technology (2013R1A2A2A01015327, 2015R1D1A1A09057348), the Center for Women in Science, Engineering and Technology (WISET), the technology development program (S2342882)
funded by the Small and Medium Business Administration (SMBA, Korea), and Veterinary
Science Research Institute of Konkuk University.

An easily accessible adult stem cell pool may be suitable for tissue engineering-based regeneration of articular cartilage defects

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INTRODUCTION: Cartilage defects due to traumatic injuries or also in degenerative diseases like osteoarthritis lack appropriate self-repair and require therefore cell based therapies for tissue regeneration. Cartilage tissue engineering based on isolated human chondrocytes has been widely studied. However, detailed investigations have been hampered by a number of factors such as the scarce availability of human cartilage and its weak cellularity. Moreover, primary chondrocytes have a short *in vitro* life span and undergo a rapid shift of their collagen phenotype. Therefore, adult stem cells are an alternative cell source and in particular stem cells of the dental pulp (DPSCs) of adult individuals provide an easily accessible cell source to be used for new cartilage tissue engineering approaches.

METHODS: Third molar derived DPSCs from three different donors were characterised via flow cytometry and expanded in monolayer cell culture. For chondrogenic differentiation the cells were transferred to a scaffold-free 3D-culture in the presence, or absence, of dexamethasone, TGF-β and BMP-2. After three weeks in 3D-culture the cartilage-like microtissues were harvested for isolation of RNA or for cryosectioning. The tissue maturation process was analysed via TaqMan-based qPCR (for gene expression of collagen type II, aggrecan, Sox9, and Twist1) and on tissue cryosections using histology (Alcian Blue to detect proteoglycans) as well as fluorescence-based immunohistochemistry (for protein expression of Sox9 and COMP).

RESULTS: Flow cytometry analyses of DPSCs revealed a high expression of common mesenchymal stem cell markers in all of the three donors. In detail, CD29, CD73, CD90, CD146, and CD166 were expressed by 85 – 99% of analysed DPSCs. Stable dental pulp stem cell aggregates were formed already after three days in 3D- culture independent from medium composition. Chondrogenic microtissues cultured in medium

supplemented with TGF-β and BMP-2 showed a proteoglycan rich extracellular matrix indicated by Alcian Blue staining. Furthermore, cells in these microtissues expressed the cartilage transcription factor Sox9 and synthesized cartilage oligomeric matrix protein COMP. However, no collagen type II expression was detectable via immuno-fluorescence. Corresponding results were obtained at the transcriptional level using qPCR. These analyses showed gene expression of Sox9, comparable to that of primary chondrocytes as positive control, and in one TGF-β1 + BMP-2 condition a measurable induction of aggrecan expression. However, collagen type II gene expression was not detectable in any differentiation approach. In addition, we detected Twist1 gene expression.

DISCUSSION & CONCLUSIONS: The use of new stem cell sources for cartilage tissue engineering in order to repair articular cartilage is of growing interest. One promising cell source are DPSCs as they can be easily obtained from human third molars that are routinely extracted because of orthodontic reasons, show a high proliferation capacity *in vitro* and multilineage differentiation potential. Flow cytometry analyses indicated a considerably high percentage of mesenchymal stem cells within all cell populations used for this study. The present study has shown that DPSC are able to undergo chondrogenic differentiation and growth factors enhance this process. Although the chondrogenesis of cells in the generated micro-tissues seemed to be just at the beginning and needs to be optimized, DPSCs represent a suitable potential cell source for future cartilage tissue engineering.

Hypoxia-conditioned Adipose Mesenchymal Stem Cell Medium Impacts on Salivary Glandular Cell Survival and Function after Irradiation in Organotypic Three-dimensional Culture

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INTRODUCTION: Mesenchymal stem cells (MSC) have been reported to ameliorate irradiation-induced salivary gland hypofunction through trophic effects and immunomodulation. We investigated the radioprotective effects of MSC secretome could be enhanced by hypoxia preconditioning in an organotypic three-dimensional (3D) co-culture model.

METHODS: Human parotid gland epithelial cells (hPEC) were cultured on Matrigel to form 3D acinar-like spheroids, which were followed by irradiation. Human adipose mesenchymal stem cells (hAdMSC) were cultured under normoxia (NMX, 20% O2) or hypoxia (HPX, 1% O2) condition for 24 hours and then co-cultured with the irradiated 3D spheroids.

RESULTS: IR induced the destruction of structural integrity and decreased salivary secretory function of the 3D spheroids. The irradiation-induced structural damages and phenotypic changes were ameliorated by coculture with HPX-conditioned hAdMSC. The coculture with HPX-conditioned hAdMSC significantly attenuated the loss of epithelial polarity and acinar-specific cellular functions of the 3D spheroids to secrete α-amylase in response to Ca^{2+} agonists. IR-induced apoptosis of the 3D spheroids was also reduced by HPX-conditioned hAdMSC, which was associated with an decreased expression of PTEN and p53, along with an increase in AKT, MDM2 expression. Protein assay revealed that HPX-conditioned hAdMSC secreted more FGF7 and FGF10 than NMX-cultured hAdMSC. IR-induced apoptosis was significantly attenuated by FGF7 and FGF10.

DISCUSSION & CONCLUSIONS: Our results suggest that HPX-preconditioning promotes hAdMSC to produce more FGF7 and FGF10, which protect salivary hypofunciton by attenuation of IR-induced apoptosis via the mechanism of modulation of PTEN/AKT/p53.

REFERENCES: [1] An HY, Shin HS, Choi JS, et al. (2015) *PLoS One* **10**: e0141862.

ACKNOWLEDGEMENTS: This research was supported by grants from the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and future Planning (2015R1A2A2A01006528), the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI) funded by the Ministry of Health & Welfare, Republic of Korea (HI15C2807), and an Inha Research Grant

Mechanical stretching on cardiac adipose progenitors upregulates sarcomere-related genes

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INTRODUCTION: The cardiac tissue is an unfriendly environment for the implantation of therapeutic cells due to its unavoidable contractility. Cardiac cells respond to mechanical stimuli and adjust their performance accordingly. It is also known that mechanical stimulation of tissue-engineered constructs improves their organization and contraction force [1]. We hypothesize that mechanical conditioning of therapeutic cells could improve their retention and cardiovascular potential, to help in cardiac tissue restoration.

METHODS: Cardiac adipose tissue-derived progenitor cells (ATDPCs) were mechanically stretched for 7 days at 1 Hz in 3 different surfaces (vertical, horizontal and smooth) (Figure 1). Gene and protein analysis were carried out for each cell type and condition. Secretome analysis was also performed.

RESULTS: A device was designed and validated to effectively apply a ~10% stretch. Mechanically stimulated cardiac ATDPCs increased the expression of cardiac transcription factors (GATA-4 and Tbx5) and structural genes (cTnI and α-actinin) after 7 days of mechanical stimulation. This gene modulation was different depending on the patterned surface, however the secretome analysis revealed that the vertical pattern was the most convenient for cardiac ATDPCs conditioning. Indeed, the secretome of cardiac ATDPCs stretched on vertical patterned surfaces was significantly associated to myocardial infarction, left ventricular extracellular matrix remodelling and the regulation of myoblastdifferentiation.

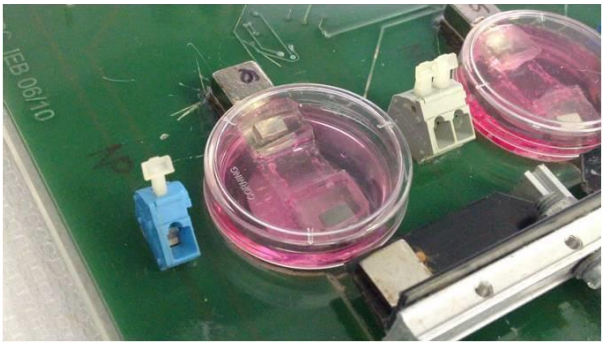

Fig. 1: Mechanical stimulation device.

DISCUSSION & CONCLUSIONS: It seems that mechanical conditioning acts on transcription factors to further activate the expression of downstream contractile proteins in order to adapt their phenotype to the stretching environment. Mechanical conditioning of cardiac ATDPCs enhances the expression of early and late cardiac genes related with the cardiac sarcomere, and it is strongly dependent on the culture surface pattern. The vertical pattern seems to be the best option to mimic the cardiac environment, as revealed by the secretome analysis.

ACKNOWLEDGEMENTS: The authors thank the patients and the members of the Department of Cardiac Surgery who made this study possible. This work was supported by grants from the Ministerio de Educación y Ciencia (SAF2014- 59892), Fundació La MARATÓ de TV3(201502- 30, 201516-10), Red de Terapia Celular - TerCel (RD12/0019/0029), Red de Investigación Cardiovascular - RIC (RD12/0042/0047) projects as part of the Plan Nacional de I+D+I and cofunded by ISCIII-Subdirección General de Evaluación y el Fondo Europeo de Desarrollo Regional (FEDER). We also appreciate support from the Fundació Privada Daniel Bravo Andreu.

Secretory activity of the stromal vascular fraction

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INTRODUCTION: Human adipose tissue is an attractive and abundantly available source of adult stem cells used in regenerative medicine and tissue engineering. The stromal vascular fraction (SVF) and adipose derived stromal/stem cells isolated from adipose tissue show a high regenerative potential due to their capacity to secrete molecular factors. These secreted factors have the potential to influence and support surrounding cells and tissue regarding immunomodulation, vascularization and wound healing. On the other hand, extracorporeal shockwave treatment (ESWT) demonstrated beneficial effects on wound healing especially due to neovascularization and an early upregulation of angiogenesis-related growth factors [1]. Therefore we asked the question whether ESWT could influence the secretion of factors from SVF.

METHODS: Human liposuction material was obtained and a low energy ESWT was applied. The freshly isolated (with collagenase) cells thereof were analyzed regarding yield, viability, and the trilineage differentiation potential. For the analysis of the protein secretome, a membrane-based antibody array was used. For this, SVF cells were cultured in serum-free media subsequently after cell isolation and 41 different human growth factors in the conditioned medium were simultaneously measured by semi-quantitative analysis.

RESULTS: Analysis of the SVF supernatant showed a positive secretion of epidermal growth factors (EGF), nerve growth factors (NGF), fibroblast growth factors (FGF), vascular endothelial growth factors (VEGF) and transforming growth factors (TGF) beta, which supports the regenerative potential of SVF. Significantly enhanced protein secretion after ESWT was detected for insulin-like growth factor 1 (IGF-1) and placental growth factor (PLGF) compared to untreated cells. SVF derived cells were able to differentiate towards the adipogenic, osteogenic and chondrogenic lineage.

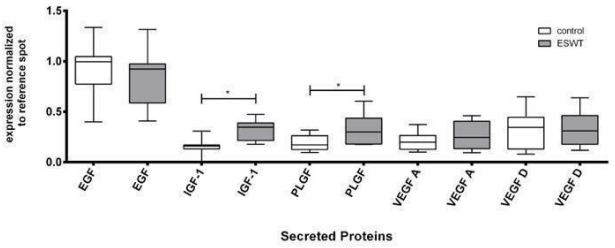

Fig. 1: SVF secretion of selected proteins. A significantly increased secretion after ESWT was detected for IGF-1 and PLGF.

DISCUSSION & CONCLUSIONS: SVF is capable of secreting factors with a potential therapeutic effect. PLGF is, like the other members of VEGF, involved in angiogenesis. Therefore, SVF activated with ESWT could be a promising strategy to treat disorders, which require neovascularization, such as wound healing and soft tissue repair.

ACKNOWLEDGEMENTS: This work was funded by grant from the Austrian Research Promotion Agency (FFG) (Bridge1 programme, grant No.846062).

Adipose stromal cells effects on osteoarthritic synovial cells are dependent by macrophages

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INTRODUCTION: Adipose stromal cells (ASC) are promising candidate for cell therapy in osteoarthritis (OA), because they have immunomodulatory, trophic and differentiation capacities [1,2]. Synovial inflammation is now accepted as important OA feature for the symptoms and disease progression [3]. Synovium is mainly composed of synovial fibroblasts (SF) and macrophages (SM) and a low percentage of other cell types [4]. Aim of the study was to analyze the role of SF and SM in counteracting the effects due to adipose stem cell co-culture.

METHODS: Good manufacturing practice (GMP)-clinical grade ASC were isolated from subcutaneous adipose tissue. Synoviocytes were isolated from synovia of OA patients undergoing total joint replacement. Synoviocytes both at passage 1 (mainly composed of SM and SF) and 5 (only SF) were characterized for the following markers: CD3, CD11b, CD14, CD16, CD68, CD80, CD86, CD90, CD163, CD206, by flow cytometry, immunocytochemistry and qRT-PCR. Co-culture of synoviocytes p1 and p.5 with ASC in transwell were performed. Macrophages type 1 (M1) were also isolated and co-cultured with ASC. Secreted inflammatory and degradative factors (IL6, CXCL8/IL8, CXCL1/GROα, CCL2/MCP-1, CCL3/MIP1α, CCL5/RANTES, ADAMTS5, MMP13 S100a8, S100a9) were analyzed by ELISA or multiplex bead-based sandwich immunoassay.

RESULTS: Synoviocytes at passage 1 were positive to typical markers of SM (CD14, CD16, CD68, CD80 and CD163) and SF (CD55, CD73, CD90, CD105, CD106), whereas at passage 5 were positive only to SF markers and showed a higher percentage of CD55 and CD106. At p.1 synovial cells released a significantly higher amount of all inflammatory (IL6, CXCL8, CCL2, CCL3, CCL5) and some anabolic (IL10) factors than those at p.5. Moreover, p.1 synovial cells expressed also higher amount of some degradative factors (MMP13, S100A8, S100A9) than p.5 synovial cells. Co-culture experiments showed that the amount of SM in p.1 synovial cells specifically orchestrate the up or down-modulation of some inflammatory (IL6, CXCL8, CCL2, CCL3, CCL5) and degradative factors (ADAMTS5, MMP13, S100A8, S100A9) analyzed. Interestingly, p.5 synovial cells induced all factors analyzed, except CCL5. Finally, we demonstrated that ASC effects were strictly dependent by M1 that decreased the release of IL1β, TNFα and CCL3/MIP1α, typical macrophages cytokines.

DISCUSSION & CONCLUSIONS: These data demonstrate that the GMP-ASC effects on OA synovial inflammation is strictly dependent by macrophages. ASC counteracting effects mainly occur through released soluble factors by macrophages as well as by cells cross talk.

ACKNOWLEDGEMENTS: This research was partially supported by the European Seventh Framework Programme (project ADIPOA; Health- 2009-1.4-3, 241719) and partially by Horizon 2020 (project ADIPOA2; PHC-15-2014, 643809).

DISCLOSURES:
The authors declare that they have no competing interests.

Mechanosensitivity is induced by differentiation in cardiomyocyte progenitor cells: mechanosome development and strain response

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INTRODUCTION: For emerging strategies of endogenous cardiac regeneration, it is essential to know if and how resident cardiac stem cells, such as cardiomyocyte progenitor cells (CMPCs), sense and respond to the mechanical stimuli provided by the beating heart. Identifying the key players and the underlying mechanisms of cardiac stem cell mechanotransduction is essential to achieve optimal migration and integration of endogenous cardiac cells into the injured site. Therefore, the cellular mechanosensing apparatus, which we termed the mechanosome, and is composed of actin stress fibres and focal adhesions (FAs) in direct contact with the ECM needs to be explored. Here, we study the response to cyclic strain of undifferentiated and predifferentiated CMPCs, as well as the formation of the CMPC mechanosome.

METHODS: L9TB CMPCs were cultured and differentiated for 14 days (predifferentiated CMPCs) according to established protocols [1]. Uniaxial cyclic strain (10% strain at 0.5Hz) was applied to undifferentiated and predifferentiated CMPCs for 48h with a FX-5000 Flexcell device (Flexcell International). Strain was applied starting 24h after cell seeding on Bioflex membranes coated with collagen IV (Flexcell International). Static samples were used as control. Cell reorientation was determined every 24h and quantified using the Directionality plug-in of Fiji. Protein and gene expression were determined with immunofluorescence and qPCR. Statistical analysis was performed with ANOVA, using Kruskal-Wallis test with Dunn's post-hoc test for multiple comparisons.

RESULTS: In response to the applied strain, undifferentiated CMPCs maintained their original orientation, whereas predifferentiated CMPCs at an early stage of differentiation exhibited a distinct strain avoidance response during 48 hrs of cyclic straining. Moreover, the development of the mechanosome coincided with early cardiac differentiation of the CMPCs, but was not induced by the cyclic strain per se.

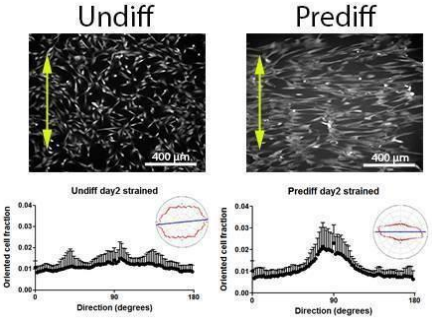

Fig. 1, Top panes: representative fluorescence images of undifferentiated (undiff) and predifferentiated (prediff) CMPCs after 48h of cyclic strain. Lower panes: frequency distribution (mean ± SD, N=16-23 from 3 independent experiments) and polar plots of cell orientation indicating the direction of maximum orientation (blue line).

DISCUSSION & CONCLUSIONS: In summary, our results suggest that CMPC mechanosensitivity is dependent on the presence of a developed mechanosome. The mechanosome is formed during early cardiomyogenic differentiation. Our findings provide the first understanding of the mechanotransduction mechanisms in human CMPCs. Mechanotransduction is essential for optimal recruitment, migration, and mechanical integration of endogenous stem cells into the injured myocardium. Therefore, these results can contribute to improve current treatments of cardiac disease, as well as to develop novel *in situ* regeneration strategies.

ACKNOWLEDGEMENTS: This research was funded by the People Program (Marie Curie Actions) of the EU's Seventh Framework Program FP7-People-2012-ITN "TECAS", under grant agreement n. 317512.

Quiescencent preconditioned human multipotent stromal cells adopt a metabolic profile favorable for enhanced survival under ischemia

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INTRODUCTION: A major impediment to the development of therapies using mesenchymal stem cells/multipotent stromal cells (MSC) is the poor survival and engraftment of MSCs at the site of injury. The ischemic environment (i.e. lack of both oxygen and nutrients) encountered by cells upon implantation is the prime cause of this cell death. We hypothesized that lowering the energetic demand of MSCs by driving them into a quiescent state would enhance their survival under ischemic conditions.

METHODS: Quiescence was induced in Human MSCs by a 48h culture in serum-deprived α-MEM medium (SD-hMSCs). Cells were then characterized by cell cycle analysis, ATP/ADP and protein content measurement, seahorse analyses, mTOR western blotting and autophagy asssay in comparison with unpreconditioned cells (UP-hMSCs). RT-qPCR targeting expression of 84 genes implicated in the human glucose metabolism was performed. Viability of both SD-hMSCs and UP-hMSCs cultured under *in vitro* ischemic environment (in serum- and glucose-free medium at 0.1% O_2) for 14 days were quantified by flow cytometry. UP-hMSCs maintained in same conditions, except in the presence of glucose (5g/L), were used as (ctrl+). Cell-containing constructs were prepared in diffusion chambers and implanted in nude mice over the course of 14 days for *in vivo* cell viability analyses. Post-ischemic hMSC functionalities (proliferation, osteogenic and adipogenic differentiation potentials) were then assessed after cell reperfusion in standard culture conditions (including 21% O_2 and 1g/L glucose).

RESULTS: SD-hMSCs sustained their viability (Fig. 1) and their ATP levels upon exposure to ischemic conditions for up to 14 consecutive days *in vitro*, while maintaining their hMSC multipotential capabilities upon reperfusion. Most importantly, SD-hMSC showed enhanced survival *in vivo* after implantation in an ischemic environment in mice. Quiescence preconditioning modified the energy-metabolic profile of hMSCs: it suppressed energy-sensing mTOR signaling, stimulated autophagy, promoted a shift in bioenergetic metabolism from oxidative phosphorylation to glycolysis and up-regulated the expression of gluconeogenic enzymes, such as PEPCK.

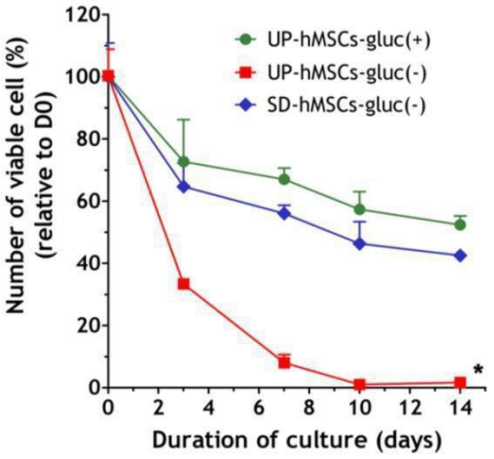

Fig. 1: Time-course of cell viability assessed during the ischemic period in vitro. Results are expressed as means ± S.D. (n=3)

DISCUSSION & CONCLUSIONS: Rather than using a single pathway to ensure their survival under ischemia, SD-hMSCs likely use a multifaceted strategy to overcome this metabolic insult. The present investigation focused on the role of autophagy, the potential metabolic fuels, and on their related energetic pathways. Since the presence of pyruvate in cell culture media was critical for SD-hMSC survival, we speculate that these cells may utilize some steps of gluconeogenesis to overcome metabolic stress. These findings support that quiescence preconditioning causes a protective metabolic adaptation that might be taken advantage of to improve hMSC survival in ischemic environments.

ACKNOWLEDGEMENTS: The authors acknowledge the Centre National de la Recherche Scientifique (CNRS) and the Direction Générale de l'Armement (DGA) for the financial support of the study.

Cryogel microcarriers as an injectable scaffold for neural cell growth and transplantation

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INTRODUCTION: Cell transplantation to replace the dopaminergic neurons lost in Parkinson's disease (either fetal cells, or stem cells) could improve function in individuals. However, graft survival is generally poor, requiring acquisition from multiple fetuses or the preparation of large numbers of stem cell-derived neurons. Recent evidence suggests that pre-adhering cells to a material *in vitro*, then transplanting the cells and the material together, dramatically improves the survival of the graft [l].

Our aim is to develop a transplantation platform material, which allows stem cells or neurons to attach, which can be injected through a 28 gauge cannula, with the aim of using this platform to improve the survival of grafting into the brain.

METHODS: Microcarriers consisting of heparin crosslinked with starPEG (polyethylene glycol)[2], were made using a water-in-oil emulsion. Furthermore, during the crosslinking process, the solution was frozen to -20^{o}C, to induce ice crystal formation within the structure. Upon freeze drying, the area that was once occupied by the ice becomes a pore, allowing a highly porous material of microscale dimensions to be made (see Figure 1) [3]. Microcarriers were functionalized with the adhesion peptide RGD, via an EDC/sNHS mediated reaction to carboxylic acid groups of the heparin.

RESULTS: Rat mesenchymal stem cells (see Figure 2), PC12 cells and primary fetal neurons adhere to the microcarriers, particularly at the periphery. The growth factors GDNF and NGF can be loaded to the microcarriers, to be released over the period of a week (longest time point analyzed). PC12 cells, loaded to NGF loaded microcarriers produce neurites, showing the potential to provide a specific environment prior to transplantation. Finally, the microcarriers were shown to be injectable through a 28 gauge needle, exhibiting shape memory properties (collapse then reform post injection), without loss of cellviability.

Current studies are analyzing whether pre-culturing primary ventral mesencephalic tissue to microcarriers improves survival in the 6-hydroxydopamine lesioned Parkinsonian rat brain.

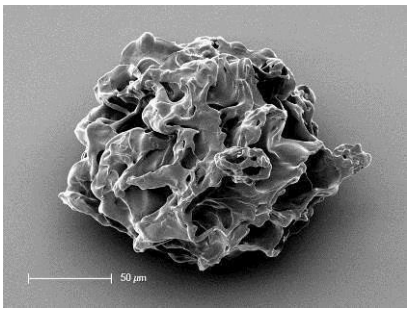

Fig. 1: Scanning electron microscopy image of a dehydrated microcarrier scaffold

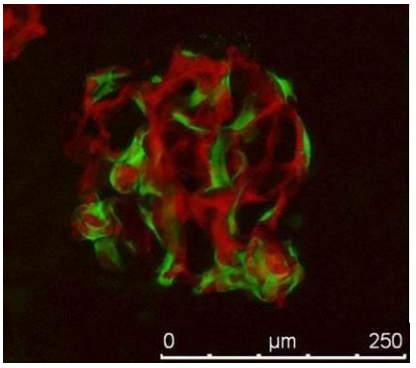

Fig.2 Fluorescent microscopy image of mesenchymal stem cells (green) growing throughout the cryogel structure (red)

DISCUSSION & CONCLUSIONS: PEG-heparin microcarriers allow cell attachment and growth and also allow growth factor release to differentiate the adhered cells. The highly porous nature serves two purposes: minimize dead space in the brain and allow shape memory properties for ease of injection. We are currently ascertaining the effect on survival in adult rat models of Parkinson's disease.

ACKNOWLEDGEMENTS: This work was funded by the Wellcome Trust, UK.

Effect of adipose stromal vascular fraction on random pattern flap viability in rats with diabetes and chronic renal disease: an experimental study

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INTRODUCTION: Diabetes (DM) and chronic renal disease (CRD) are epidemic diseases with increasing prevalence. Wounds due to microangiopathy and macroangiopathy tend to heal slowly which can lead to severe morbidites such as amputations. High flap failure rates reported in the reconstruction of these wounds[1]. Studies have shown increased flap viability by adipose derived stromal vascular fraction (SVF)[2]. However; there is no study in the literature about the effect of adipose stromal vascular fraction on skin flap viability in chronic renal disease and diabetes with chronic renal disease.

METHODS: 48 male Sprague Dawley rats were used. Diabetes was induced by 65mg/kg intraperitoneal streptozocin administiration. Chronic renal disease was induced by 5/6 nephrectomy. Four groups consisting of 12 rats were formed. 2 rats were used for obtaining adipose tissue from the inguinal regions for stromal vascular fraction preparation in each group. Group I (Control group): Two dorsal flaps were elevated, phosphate buffered saline (PBS) were injected to the flaps. Group II (DM), Group III(CRD), Group IV(DM+CRD): After disease induction and period;, two dorsal flaps were elevated, SVF were injected to the left flap, PBS were injected to the right flap. Flaps were harvested for macroscopic and histopathological assesments at postoperative 7th day. Percentage of flap viability measurement and microangiography were performed for macroscopic assesment. Capillary density assesment were evaluated in both hemotoxylin-eosin and CD31 stained specimens for microscopic assesment. Plasma levels of VEGF were studied in all rats at day 1 and day 7.

RESULTS: SVF was improved flap viability significantly ($p<0,05$). New capillary formation found signficantly more in SVF groups in capillary dansity assesment ($p<0,05$) *(Figure 1)* When groups were compared each other there were not significant difference except Group II(diabetes).

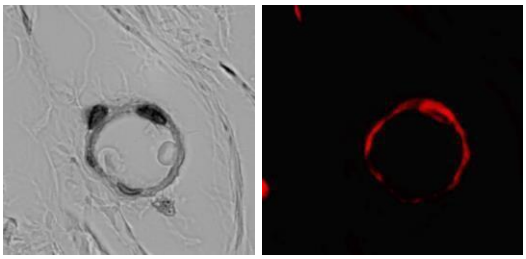

Figure 1: Images of DiI labelled endothelial cell in light microscopy and IF microscope in Group II. (100x)

This result was compatible with the scarcity of the vasculature in microangiography. When blood VEGF levels were compared, increase in day 1 and day 7 were significant according to control.

DISCUSSION & CONCLUSIONS: The result of the study has shown that DM and CRD impaired flap viability. Diabetes with chronic renal disease detoriated the flap viability much more. It has shown that SVF were increased flap viability via neovascularization by endothelial diffentiation. Flap viability percentage was found lower in diabetic and uremic groups when compared with healthy control group. Blood VEGF levels were not elevated in uremic groups. This results were indicated that in vivo function of stem cells were possibly impared by uremia dominantly and diabetes due to microenviromental changings.

Hydrogel-based cell traps for capturing MPCs participating in bone healing and regeneration model

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INTRODUCTION: Non-healing bone defects still remain significant clinical problem which is currently addressed by filling the gap with a bone graft, natural or synthetic materials that can contain stem cells and clinically approved growth factor bone morphogenetic protein-2 (BMP-2). Although BMP-2 has shown promising results in preclinical animal trials, high doses are required for human patients that can lead to serious side effects such as ectopic bone formation or cancer stimulation. Therefore novel approaches that enhance and accelerate bone repair through the sustained and controlled release of osteogenic factors are currently of high interest.

Synthetic polymers such as poly (ethylene glycol) PEG provide a blank slate which can be engineered with reproducible properties. Our laboratory has previously described modular designed by transglutaminase factor XIIIa (FXIII)-cross-linked poly (ethylene glycol) PEG-based biomimetic hydrogel which are tailorable in terms of stiffness, proteolytic stability, and presentation of cell adhesion ligands and growth factors [1,2]. Such hydrogels are potent 3D platforms to investigate multiple cellular processes such as proliferation, migration and differentiation under fully controlled extracellular matrix (ECM)-free conditions. Additionally, they have recently been engineered to bind and release growth factors in a cell, light or small molecule mediated manner.

METHODS: PEG-hydrogel cell-traps were employed in murine critical-sized (4mm) calvarial defects. Cells were allowed to infiltrate for 8 days and then cell-traps were enzymatically digested. Nucleated cells of non-hematopoietic and non-endothelial lineages were sorted based on the expression of Sca1 and Alcam as suggested by Nakamura Y et al. [3] giving rise to osteoblasts ($Alcam^+$, $Sca1^-$) and premature mesenchymal progenitor cells (MPCs) ($Alcam^-$, $Sca1^+$).

RESULTS: We report on a PEG-hydrogel based approach to entrap and prospectively isolate $Sca1^+$ MPCs from murine healing bone defects, in order to define their *in vitro* and *in vivo* differentiation potential. We show that $Sca1^+$MPCs are enriched in the defect area, suggesting their recruitment and/or expansion in a healing environment. Colony forming unit fibroblast (CFU-F) assays confirm that cells with clonogenic potential are restricted to the $Sca1^+$MPC subpopulation. Prospectively isolated $Sca1^+$MPCs demonstrated the ability to differentiate into osteoblasts, adipocytes and chondrocytes when cultured under differentiation conditions, fulfilling the *in vitro* criteria for MPCs. To determine their *in vivo* differentiation potential, MPCs were prospectively isolated from healing calvarial defects of transgenic GFP-mice based on the expression of Sca1 and encapsulated in PEG-hydrogels. After 12 weeks of implantation in calvarial defects of wild-type mice we show that the distribution of the transplanted GFP^+-$Sca1^+$MPCs leads to bone augmentation, with bone nodules formation in absence of BMP-2, whereas the transplanted GFP^+-$Alcam^-$/$Sca1^-$ failed to differentiate, though survived within the defected area.

DISCUSSION & CONCLUSIONS: Together these data indicate that PEG-hydrogel based traps can be employed to prospectively isolate MPCs that promote bone healing and can be used to further study bone regeneration and formation *in vitro* and *in vivo*.

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ACKNOWLEDGEMENTS: The research has received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/
under REA grant agreement No. 607868 (iTERM).

Rapid use of intracellular energy reserve through glycolysis explains poor human Mesenchymal stem cells survival after implantation

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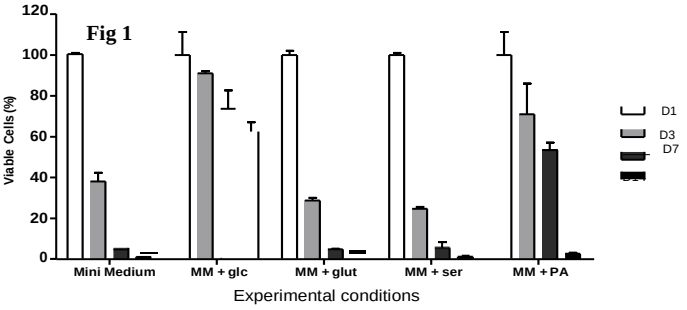
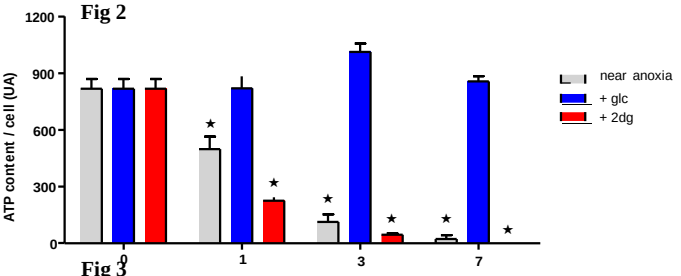
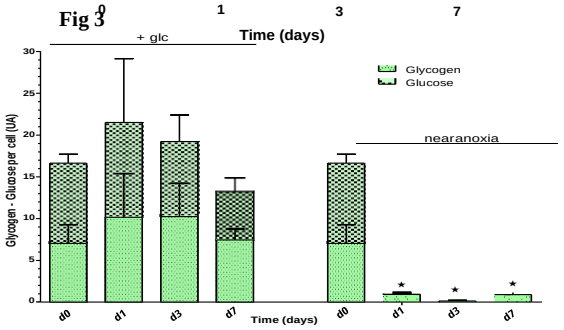
INTRODUCTION: Mesenchymal stem cells (MSC) hold considerable promise in regenerative medicine. However, exogenously administered MSCs, when loaded into scaffolds, exhibited poor survival. A possible explanation for this limited cell survival is that, upon implantation, MSCs encounter a considerable bioenergetic challenge to ensure their homeostasis and fuel the regenerative response. To explore how Human MSCs (hMSCs) align their bioenergetic needs with available energy resources post-implantation, an *in vitro* model that reflects the *in vivo* situation was developed and its physiological relevance established (data not shown).The complete metabolic requirements of hMSCs and their putative energy reserves when loaded in tissue constructs were then explored in this model.

METHODS: hMSCs were cultured at 0.1% oxygen without serum and in the presence or no of glucose, glutamine, serine and/or pyruvic acid to determine which nutrients are necessary to ensure hMSC survival in a post implantation environment. Specific inhibitors of glycolysis (2-DG, NaOx) or TCA cycle (AnA) were used to assess metabolic pathways used by hMSCs at 0.1% oxygen.. hMSCs survival rate was assessed by flow cytometry and energy reserves of hMSCs (glucose/glycogen, ATP) were assessed both *in vitro* and *in vivo* in post implantation conditions.

RESULTS: Upon implantation, hMSCs present in the core of cell-containing constructs up-regulated HIF-1α and LDH-A and down-regulated the mitochondrial activity. We observed that exposing hMSCs to 0.1% oxygen tension best reflected the *in vivo* milieu. Moreover, gene expression profiling revealed upregulation of glycolysis when hMSCs are exposed to near anoxia. We observed that glycolysis (and glucose) is necessary to ensure survival of hMSCs exposed to near anoxia (Figure 1). The assessment of hMSCs ATP and glucose/glycogen stocks revealed that hMSCs bioenergetic solely depend on glycolysis and that hMSCs have very low energy reserves (Figure 2).







DISCUSSION & CONCLUSIONS: In the present study, we determined, for the first time, that the 0.1% oxygen level best reflected the *in vivo* situation encountered by hMSCs located in the core of cell-engineered constructs. This new knowledge has practical implications because energy metabolism is modulated by oxygen levels (more specifically, TCA is drastically down-regulated at 0.1% but not at 1%) and has been implicated in cell fate decision . Most importantly, we established that energy was produced *via* glycolysis and that energy reserves were exhausted within 24 hours under near anoxia conditions resulting in hMSCs death within a week post-implantation. In this respect, development of strategies aiming at providing extracellular glucose supplies is an attractive, but yet untapped, alternative to enhance both the hMSCs viability and functions pertinent to neo-tissue formation.

Therapeutic potential of tonsil-derived mesenchymal stem cells loaded hydrogel-G in osteoporosis animal model

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INTRODUCTION: Stem cells promote the innate repair system as well as replace non-functional tissues, giving hope to patients suffering from organ dysfunction. We reported that tonsil-derived mesenchymal stem cells (TMSC) are successfully differentiated into parathyroid hormone (PTH)-releasing cells, demonstrating the therapeutic role in hypoparathyroidism. Because PTH is the only therapeutic bone-forming agent for osteoporosis, here we tested whether TMSC are also effective in treating osteoporosis.

METHODS: TMSC were isolated, and control TMSC (cTMSC) and differentiated TMSC (dTMSC) were prepared without or with differentiating chemicals (activin A and sonic hedgehog), respectively. In separate experiments, TMSC were embedded in hydrogel-G. Osteoporotic animals were prepared using female mice with ovariectomy (OVX), and osteoporosis was confirmed by increased serum osteocalcin levels. OVX mice were transplanted either with TMSC alone intraperitoneally or with TMSC embedded in hydrogel-G subcutaneously. During experimental periods (3 months), osteoporotic indicators such as serum levels of osteocalcin, alkaline phosphatase, total calcium, and phosphorus were investigated. The femoral heads were imaged with micro computed tomography, and bone parameters were also analyzed.

RESULTS: Although osteogenic capacity was not detected in OVX mice if those mice were implanted with either cTMSC or dTMSC alone (without hydrogel-G), dTMSC embedded in hydrogel-G showed an improved therapeutic efficacy in OVX mice.

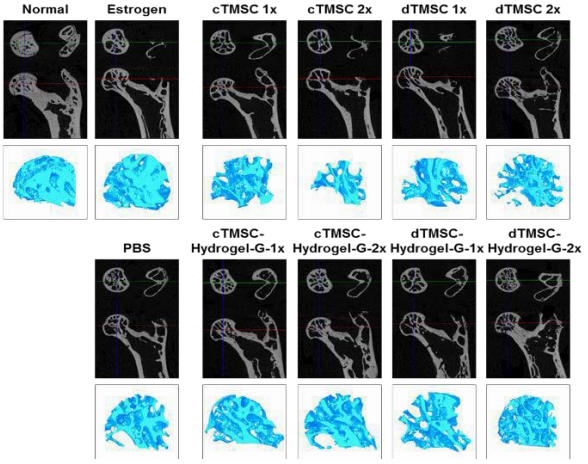

Fig. 1: Representative cross-sectional images of right femoral heads using micro computed tomography. Treatment with dTMSC embedded in hydrogel-G apparently recovers the trabecular architecture to become less porous.

DISCUSSION & CONCLUSIONS: TMSC demonstrate a high potential for a stem cell therapy targeting osteoporosis, only if those are differentiated and embedded in hydrogel-G.

ACKNOWLEDGEMENTS: This study was supported by research grants of the National Research Foundation of Korea (NRF-2012M3A9C 6049728, NRF-2013R1A1A3007591, NRF-2014 R1A2A2A005564), the Korea Health Industry Development Institute (KHIDI), (HI14C-1557- 020014), and Dankook University (for BK21 PLUS Global Research Center).

Adipose stem cells (ASC) from young and ageing rats show no difference in phenotype, proliferation potential or differentiation capability

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INTRODUCTION: Mesenchymal stem cells (MSCs) play crucial role in tissue regeneration processes and are widely used for the regeneration medicine purposes. There exist scarce data concerning the effect of donor age on the quantity and quality of mesenchymal stem cells collected from bone marrow or adipose tissue. Published data [1-3] concerning such phenomena like telomere lengths, switch from osteogenic to adipogenic differentiation or reduced proliferative potential related to cell donor age are challenging the rationale of the use of MSCs from older donors as transplants. The aim of our study was to compare the results of commonly used laboratory tests for transplantable adipose stem cells (ASCs) applied to cells collected from young versus aged rats. The data from the study will be the background for future in vivo experiments of rat tissue regeneration regulated by transplantation of ASCs from the young versus old donors.

METHODS: WAG inbred rats aged 6 months and 2 years were used for experiment. Adipose tissue was collected from 5 different regions: dorsal (brown or white fat), renal, inguinal and gonadal, and ASCs were isolated by enzymatic digestion. Cell numbers (per region and per fat volume), viability and phenotype (CD11b, CD29, CD90, CD34, CD45) were analyzed, and cells were in vitro cultured in standard conditions (DMEM + 15% FCS, 37^0C, 5% CO_2. Doubling time analysis, CFU-S score, MTS and Picogreen tests were applied for proliferation potential analysis. Osteogenesis, chondrogenesis and adipogenesis were stimulated by culturing cells in the differentiating media (LONZA) and analyzed by real time PCR and histochemical staining (Oil Red, Alizarin Red). Finally, the time in culture (numbers of cell passages) of ASCs from young and old animals were compared.

Experiment was approved by the Local Ethical Committee, license No: 71/215/30/06/2015

RESULTS: No statistical differences between data collected from analyses of ASCs from young and old rats have been observed. Cell numbers per collection were somewhat decreased in old animals, but the difference was statistically not significant. There were no significant differences between CFU-F frequency, DNA content (Picogreen test) and enzymatic activity (MTS test of formazan reduction) or cell doubling time, which could suggest the proliferative potential difference between "young" and "old" ASCs. Both osteogenic and chondrogenic differentiation capability was well preserved in old age, and there was no age-related increase in adipogenic potential of these cells.

DISCUSSION & CONCLUSIONS: The lack of differences between ASCs from young and old donors is in no conflict with the findings reported by authors observing age-related differences in MSCs quality or quantity. In our opinion minor changes in quantities or qualities of cells in old age and in the proliferative or differentiation potentials (1-3) may be not important enough to make such cells useless for autologous treatment by regenerative therapies. The clinician's choice between autologous MSCs of senescent patient or "better" but allogeneic cells from different donors will be considered according to the individual case. Our animal model was equivalent of differences between human patients 25 years old and 70 years old, and the analysis of data based on tests being obligatory for qualifying cells for autologous transplantations do not signal any decrease in value of cells from old donors. The final conclusions may be drawn after continuation of in vivo animal studies on tissue regeneration capabilities of cells from older donors, our recent observations suggest, however, that it is too early to exclude the possibility of the use of ASCs from humans of the age up to 70 years in clinical practice.

ACKNOWLEDGMENTS: This study was supported by the National Science Centre (UMO-2013/11/B/ST8/03401, project OPUS-6).

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Multi-cell approach for successful bioengineering of functional smooth muscle layers

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INTRODUCTION: Engineered tissues for bladder augmentation or substitution would allow circumventing the side effects of using bowel tissue for reconstruction. Tissue engineering using a combination of cells may provide novel approach for functional reconstruction. Adipose derived stem cells (ADSC) might be a key instrument to bioengineer contractile bladder tissue when differentiated to smooth muscle cells (SMC) [1]. However, it is uncertain whether these cells maintain their cell fate long term in vivo. It is our aim to evaluate different combinations of cells to improve the bladder tissue formation, by improving the microenvironment and cell-to-cell interactions.

METHODS: We have characterised rat ADSCs and optimally differentiated them to SMC (3 weeks) prior to subcutaneous injection into nude mice. Cells were injected in different combinations (ADSC, ADSC + differentiated ADSC, SMC, differentiated ADSC + SMC). The tissue formation was followed by MRI and PKH labelling. The formed tissue was analysed for contractile proteins measuring gene and protein expression by using RT-qPCR, western blot and immunohistochemistry.

RESULTS: In all experimental conditions, the PKH positive cells could be detected after 4 weeks, indicating the presence and survival of engineered tissues in vivo. MRI was able to visualize the engineered SM tissue over the study period. Collagen without cells showed no signal and was absorbed quickly. The tissue size differed between the experimental conditions with tissues grown from cells with 3 week ex vivo differentiation showing the largest constructs with good correlation in histology. Differentiated ADSC showed positive upregulation of smooth muscle makers (Calponin, Smoothelin, MYH11 and αSMA) similar to bladder derived SMC.

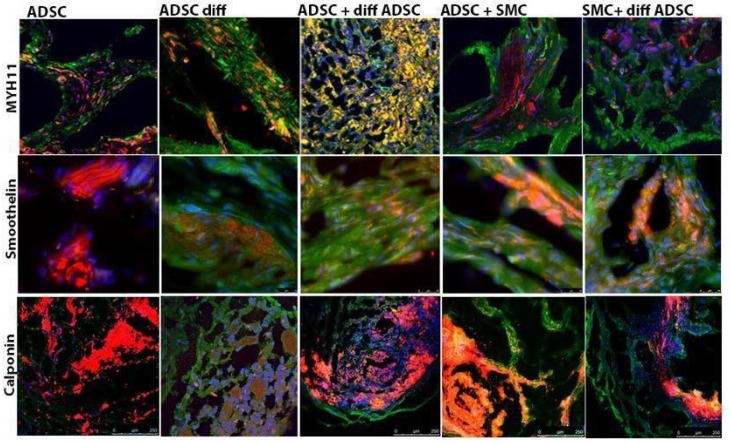

Fig:Immunostaining of engineered tissue with different cell combinations. Tissue sections were immunostained for smooth muscle associated markers MYH11, smoothelin and calponin (green), PKH (red) and nucleus staining (DAPI, blue).

DISCUSSION & CONCLUSIONS: The presented research offers key information on survival and functionality of bioengineered smooth muscle tissue grown using differentiated ADSC in combination with differentiated cells. This approach could help to engineering contractile bladder tissue for future clinical application.

ACKNOWLEDGEMENTS: The authors recognize the research efforts of those who have contributed to this field of study and declare no conflict of interest.

Does cell passage number influences the effect of adipose tissue derived stem cells secretome on neurodifferentiation and axonal growth?

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INTRODUCTION: Mesenchymal like stem cells (MSCs), such as those derived from adipose tissue (ASCs), have already shown to have a positive effect on central nervous system (CNS) cell populations, regarding their viability, proliferation and densities [1]. Such effect has been mostly attributed to soluble factors, as well as vesicles, present in their secretome [2-3]. Yet, little is known about the impact that passaging might have in the secretion profile.

METHODS: Aiming at studying if ASCs passage number influences their secretome, and consequently the neurodifferentiation it induces, human neuroprecursor cell (hNPCs) cultures were incubated with secretomes of P3, P6, P9 and P12 ASCs, collected as conditioned media (CMs). Simultaneously, to evaluate the influence of passaging in axonal growth, an in vitro model of axonal regeneration, based on dorsal root ganglion (DRG) explants, was exposed to CMs collected in the same conditions. As an attempt to characterize the secretome obtained from different passages, a proteomic analysis was performed using mass spectrometry.

RESULTS: Results revealed that the secretome of P3-P12 ASCs induced similar neurodifferentiation profiles of hNPCs (assessed through MAP-2 immunocytochemistry – Figure 1 hNPCs), as well as similar axonal outgrowth from DRG explants (assessed through NF immunocytochemistry – Figure 1 DRGs). The results obtained from proteomic analysis allowed us to identify several proteins, like Clusterin, Pigment epithelium derived factor (PEDF), DJ1, Interleucin-6 (IL-6), Cystatin and Galectin, all of which have already been proved to have a neuroprotector/ /neurodifferentiating role. Further investigation is ongoing to reveal other proteins that may be involved in axonal growth promotion.

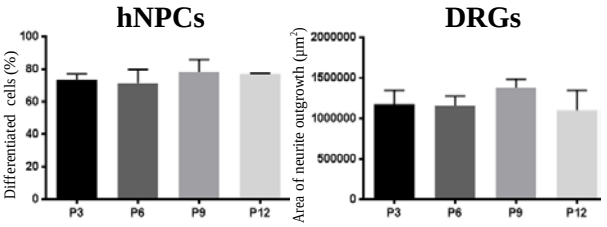

Fig. 1: Neurodifferentiation (hNPCs) and axonal growth (DRGs) outcomes after exposure to secretomes obtained from ASCs in different passages. One-way ANOVA, n=3, average ± SD, p<0,05. No statistically significant differences found.

DISCUSSION & CONCLUSIONS: Our observations lead us to conclude that cellular passaging does not influence significantly ASCs secretome properties concerning their ability to induce neurodifferentiation and axonal growth, as does not alter its composition regarding the identified proteins.

ACKNOWLEDGEMENTS: EU-FP7-Health-2011-collaborative project 278612, Biohybrid - Templates for peripheral nerve regeneration; Santa Casa Neuroscience Awards - Prize Melo e Castro for Spinal Cord Injury Research; Portuguese Foundation for Science and Technology; Canada Research Chairs (LAB) and a SSE Postdoctoral Fellowship (KM Panchalingam).

DISCLOSURE: All authors declare no conflict of interest.

2D and 3D co-culture of bladder-derived smooth muscle and smooth muscle-like adipose-derived stem cells induces smooth muscle microtissue formation

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INTRODUCTION: Tissue engineering aims to provide alternatives to bladder reconstruction to overcome the significant limitations associated with enterocystoplasty, such as metabolic disturbance, urolithiasis, infections and malignancies. Our ultimate goal is to bioengineer contractile bladder tissue and to further improve its vascularization and innervation during and after regeneration. To achieve this we are combining bladder-derived smooth muscle cells (SMCs) and pre-differentiated smooth muscle-like adipose-derived stem cells (pADSCs) in an optimized PEG-based hydrogel scaffold.

METHODS: SMCs and ADSCs were isolated using a standardized protocol and ADSCs were differentiated into pADSCs. All cells were characterized via immunostaining and flow cytometry[1]. SMCs and pADSCs were then mixed in different ratios and co-cultured in 2D and 3D using glass chamber slides or 2% PEG hydrogel scaffolds[2] respectively. Cell viability and proliferation were investigated. Cells were analyzed morphologically and for the expression of the specific SMC-markers calponin, MyH11 and smoothelin using immunofluorescence. Microtissue size and morphology was assessed.

RESULTS: pADSCs and SMCs proliferated significantly faster after 4, 8 and 16 days when co-cultured compared to single cell cultures. After 8 days the co-cultured cells in ratios 1:1 and 1:3 proliferated significantly faster compared to the 1:5 (SMCs:pADSCs) ratio. Cells in 1:1 and 1:3 ratios showed a stable expression of all SMC-markers at a similar level as native SMCs. After 2 weeks of 2D co-culture the 1:1 and 1:3 ratios formed large microtissue structures positive for all SMC markers, whereas the 1:5 ratio showed only minimal microtissue formation and irregular smoothelin expression.

3D cultures of either SMCs or pADSCs in 2% PEG hydrogel showed poor cell survival. No microtissue formation was observed. Upon co-culture at the ratios 1:1, 1:2 and 1:3 (SMCs:pADSCs) cells showed healthy, native morphology and microtissue formation was detected already after 1 week. After 3 weeks of co-culture, formed microtissues exhibited distinct, ratio-dependent, significantly different morphological properties, increased intercellular contact (*Fig.1*) and strong expression of all SMC-markers.

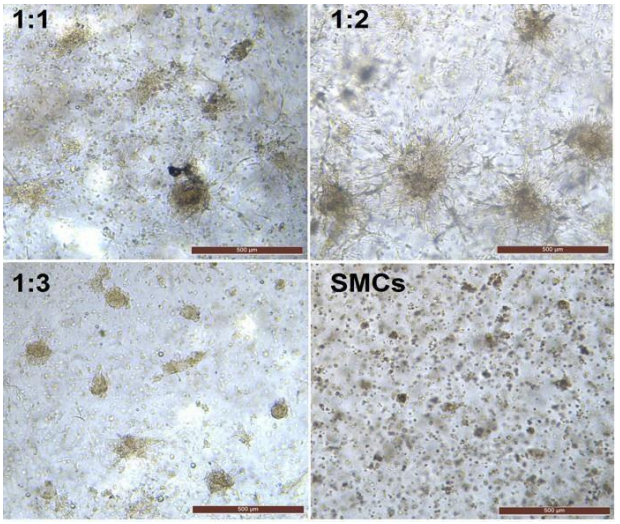

Fig. 1: Morphological assessment of microtissues grown in 2% PEG hydrogel after 3 weeks of culture using brightfield microscopy. Scale bar: 500µm

DISCUSSION & CONCLUSIONS: We have shown that both 2D and 3D co-cultures of SMCs and pADSCs exhibit a beneficial effect on cell proliferation, phenotype stability, microtissue formation and faster cell-cell network development compared to single cell cultures. This effect may help to engineer functional bladder tissue by solving some of the major issues of tissue engineering, namely poor cell survival, proliferation and phenotypic instability.

ACKNOWLEDGEMENTS: The authors gratefully acknowledge funding for this research provided by Fromm Fellowship and Helmut Horten Stiftung.

Mesenchymal stem cells attenuate adriamycin induced nephropathy by diminishing oxidative stress and inflammation

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INTRODUCTION: The Adriamycin (ADR)-induced nephropathy is widely used animal model of progressive chronic renal disease resulting in glomerulosclerosis, podocyte injury and fibrosis. The present study investigated the protective effects and mechanisms of human bone marrow derived-mesenchymal stem cells (MSCs) on the ADR induced nephropathy in rat model.

METHODS: MSCs were characterized by cell surface CD marks as well as differentiation induction into osteoblasts and adipocytes.

hBMSCs were co-cultured with renal tubular epithelial cells or spleenocytes *in vitro* to evaluate effects on oxidative stress related molecules (O_2^-, H_2O_2, malondihydrogenase, 4-HNE, catalase) and NF-kB or inflammation related cytokines (CD4, CD8, IL-12, IL-4, IL-10, INF-γ, COX2, TNF-α) expression.

Male Sprague-Dawley rats were divided into normal control (Normal), ADR+Vehicle (CON) and ADR+MSCs (MSC) groups. Nephropathy was induced by single injection of ADR (4 mg/kg). Three days later, 0.5 ml of saline with or without 2×10^7 MSCs was injected via tail vein for MSC or CON. The rats were sacrificed at 1 week and 6 weeks after ADR injection. Serum level of protein, cholesterol, triglycerides and creatinine were measured. Kidneys were harvested then NF-kB and its mark molecules (p38, p-JNK, pERK) as well as inflammation related cytokine levels were evaluated. Profibrotic molecules (fibronectin, α-SMA, type I collagen) and nephrin expression were also tested. Electron microscopic and light microscopic observations were used for structural analysis.

RESULTS: *In vitro* test, MSCs reduced oxidative stress related molecules, inflammation related cytokines and NF-kB production.

NF-kB and related molecules were dramatically decreased in MSC compared to CON *in vivo* test. ADR treated rats observed increasing inflammation related cytokines, oxidative stress related molecules, and profibrotic molecules but decreased to Normal level in MSC.

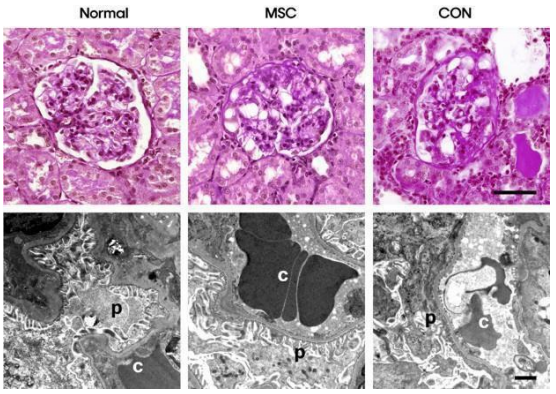

Fig. 1: LM and TEM findings of the glomerulus. Mesangial proliferation and glomerulosclerosis are observed in ADR treated groups but these degenerative changes are prominent in CON than MSC. Fusion of foot processes and loss of slit diaphragms are more evident in CON compare to MSC. p: podocyte, c; capillary.

Mesangial proliferation and glomerulosclerosis are observed in ADR treated groups but these degenerative changes are prominent in CON than MSC. Fusion of foot processes and loss of slit diaphragms are more prominent in CON compare to MSC.

CONCLUSIONS: MSCs protect chronic renal disease by decreasing NF-kB expression but also directly undermine inflammation and oxidative stress.

Preventing anastomotic leakage with adipose tissue-derived stem cell sheets

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INTRODUCTION: Anastomotic leakage is the most feared complication after gastrointestinal surgery. Adipose tissue derived stem cells (ASCs) are promising candidates for promoting wound healing since they are able to secrete many wound healing-promoting factors. Application of ASCs asa cell sheet in other tissues has been shown to promote wound healing even better than ASCs in suspension.The aim of this study was to investigate whether ASC sheetscan promote the healing process of intestinal anastomosis, thereby helping to preventanastomotic leakage.

METHODS: ASCs were isolated from human subcutaneous adipose tissue, seeded at passage3-5in 400,000 cells/cm^2 and cultured for two days. A 5-suture colorectal anastomosis (anastomotic leakage model)[1] was performed in 60 Wistar rats, which were randomly divided into 2 groups; control and treatment withASC sheet transplantation around the anastomosis. Detachment, leakage, abscess formation, adhesion severity and bursting pressure were evaluated on post-operative days (POD) 3 and 7. Picrosirius red for collagen deposition and immuno-histochemical staining for CD3, CD34 and CD163 were performed.

RESULTS: Transplantation ofASC sheets to a colorectal anastomotic wound in rats did not result inperitonitis oran increase in adhesion formation. ASC sheet application reduced leakage score, abscess number and detachment of the anastomosis.On histology, more CD3+ T-cellswere seenafter transplantation of ASC sheets at POD7. Inthe control condition CD163+ inflammatory macrophages decreased in time, whereas macrophage numbers remained unchanged in time in the ASC sheet group.The anastomotic bursting pressure (ABP) was slightly increased in the ASC sheet group. However, no increase of vessel density and collagen deposition were observed in the healing area.

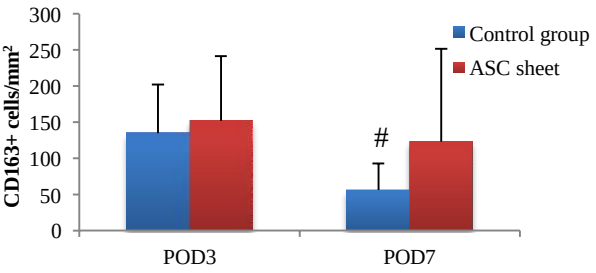

Fig. 1: The number of anti-inflammatory macrophages per mm^2 detected with anti-CD163 in the anastomosis area 3 and 7 days post operation #p<0.05 between POD3 and POD7

Table 1. Intraabdominal observation

POD	Parameters	Control	ASC-sheet
3	Leakage score (0-3)	1.9±0.4	1±0.6*
	ABP (mmHg)	23.2±9	37.5±20
	Abscess no.	1.6±0.5	1±0.7*
	Adhesion no.	4.1±1.4	4±1.6
7	Leakage score (0-3)	1±0.6	0.5±0.8*
	ABP (mmHg)	133.2±73	176.8±56
	Abscess no.	1.2±1.2	0.4±0.6*
	Adhesion no.	5±1.4	5±1.2

*p<0.05

DISCUSSION & CONCLUSIONS: ASC sheets prevent anastomotic leakage in a colorectal anastomotic leakage model as shown by a lower leakage score, less abscess formation, and higher ABP.Anti-inflammatory macrophages (CD163+) might have contributed to this a positive effect. The phenotype of the increased numbers of T-cells (CD3+) is under current investigation.

ACKNOWLEDGEMENTS: P. Sukho is supported by a grant from the Netherlands Fellowship Program (NFP-PhD.12/435).

Improved intestinal adaptation by administration of adipose derived stem cells in experimental short bowel syndrome

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INTRODUCTION: Short bowel syndrome is a highly disabling malabsorptive condition resulting most commonly from extensive intestinal resection and characterized by the inability to maintain protein-energy, fluid, electrolyte or micronutrient balances when on a regular diet [l]. It was shown that the adipose tissue was rich in mesenchymal cells [2]. They have also reported that isolation of stem cells from adipose tissue had less morbidity when compared to the bone marrow. The adipose derived stromal vascular fraction (SVF) cells were indicated to comprise multipotent mesenchymal stem cells and were proven as effective as the cultured mesenchymal stem cells [3, 4]. The aim of the current study was to search the effects of stem cell therapy in rats with short bowel syndrome.
METHODS: Female Sprague Dawley rats with short bowel syndrome were enrolled to the study. There were two study groups; in group 1, only phosphate buffered saline was administered to submucosal space of the residual bowel and group 2 adipose derived stem cells in phosphate buffered saline was administered to submucosal space of the residual bowel. All rats were sacrificed under anesthesia by the 7th day of the experiment. Residual small bowel segments were resected for histopathologic examination. Adipose tissue derived stem cells were labeled with DiI dye. Body weight and small bowel length were also recorded.
RESULTS: Compared to group 1, the length and diameter of the residual small bowel increased significantly in group 2. In group 2, villus height, crypt depth, mucosal thickness, mitosis and the number of goblet cells that known as parameters of intestinal adaptation increased significantly. Rats were sacrificed on the 7th day after SBS operation. All rats were weighed on the SBS operation day and 7 days later. In group 2, weight loss was found to be less than group 1. Stem cells were observed as endothelial cells and was seen among the villous components in the flourescent microscope examination (Figure 1).

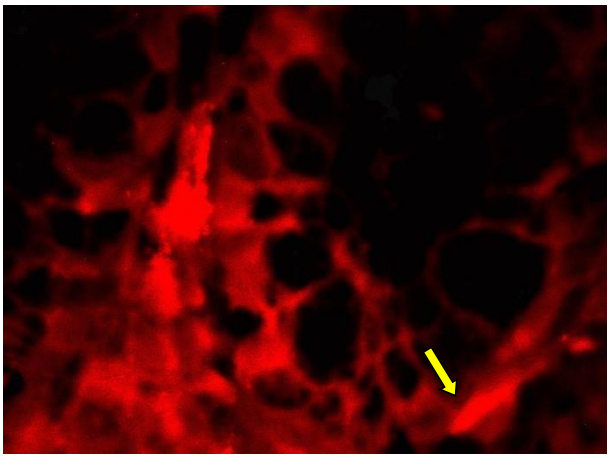

Fig. 1: DiI positive stem cell among intestinal villus cells (shown by arrow).

DISCUSSION & CONCLUSIONS: Adipose derived stem cells have been utilized with low donor site morbidity and with high potential in regenerative medicine. Increased vascularization, villus height, crypt depth and number of goblet cells increased durability at small bowel wall were observed. In this study we observed that stem cells affected positively intestinal adaption in the short bowel syndrome model. Further studies are needed to applying humans and determine the long term effects.

Priming OEC/MSC-laden microspheres to promote neovascularization

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INTRODUCTION: Therapeutic success of bioengineered tissues is highly dependent on appropriate vascularization, in order to avoid ischemia and necrosis and allow for long-term *in vivo* viability. Amongst pro-angiogenic therapies, cell-based approaches of endothelial cells (EC) transplantation are attractive [l], but more effective cell delivery strategies are urgently needed. A "delay" approach, where extended *in vitro* incubation leads to formation of more structured vascular beds, has been shown to correlate with faster and more functional graft-host integration of implanted constructs [2]. Here, injectable microniches were created by co-culturing outgrowth EC (OEC) and mesenchymal stem cells (MSC) inside optimized RGD-modified alginate hydrogels [3]. The effects of growth factors (GF), culture conditions (hypoxia -O_2 vs. normoxia +O_2), and culture time on OEC sprouting were evaluated.

METHODS: OEC were isolated from human umbilical cord blood as previously described [1] and co-cultured MSC (Lonza) within RGD-modified alginate microspheres. The influence of GF (+ / -), culture conditions (-O_2 vs. +O_2) and culture time on OEC ability to spread within alginate microspheres, as well as the importance of pre-maturate the cell-laden microspheres, was evaluated. Image analysis software was used to quantify OEC ability to organize and sprout within alginate microspheres.

RESULTS: The combined stimulus of +O_2+GF contributed for more extensive OEC sprouting (Fig. 1A) in OEC/MSC-laden microspheres, as compared to culture under +O_2-GF, -O_2+GF and -O_2-GF conditions (Fig. 1B). Microspheres cultured for 7 days under +O_2+GF were subsequently transferred to -O_2-GF for an additional week, to simulate an ischemic environment. Under such conditions, OEC sprouting was not reversed. Non-matured (freshly prepared) and pre-matured (+O_2+GF, 7 days) OEC/MSC-laden microspheres were embedded in a fibrin gel and cultured under -O_2-GF for 3 days. While matured cells showed extensive outward migration and sprouting through the fibrin gel, non-matured cells migrated poorly, remaining essentially inside the microspheres. Outward migration quantification shows that maturated cells are able to migrate approximately 10 times more than freshly prepared cells, even under -O_2-GF.

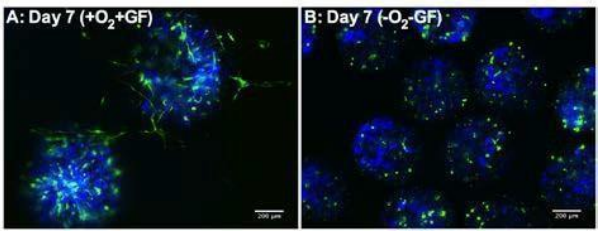

Fig. 1: Culture under +O_2+GF contributed for OEC sprouting in OEC/MSC-laden microspheres. Scale bar: 200 µm. Blue: MSC. Green: OEC.

DISCUSSION & CONCLUSIONS: Combining +O_2+GF to prime OEC/MSC co-cultures in microgel it is critical to enhance OEC sprouting. Additionally, it was disclosed that cell pre-culturing in +O_2+GF leads to an adequate maturation that allows OEC to preserve their organization, even after shifting to -O_2-GF. This is a promising result, as the combination of -O_2-GF recapitulates the in vivo ischemic microenvironment. Thus, the successful combination of OEC/MSC with an injectable biomaterial may be basis for tissue-engineered constructs for multiple applications in the field of regenerative medicine.

ACKNOWLEDGEMENTS: INEB integrates the i3S Research Unit, partially supported by FCT (Portuguese Foundation for Science and Technology). This work is funded by: 1) FCT/MEC via National Funds and co-financed by FEDER via PT2020 Partnership Agreement under the 4293 Unit I&D; 2) FCT project UID/BIM/04293/2013; 3) FCT grant SFRH/BD/94306/2013 (AL Torres); 4) FCT and POPH/ESF (EC) (CC Barrias position FCT Investigator 2013); 5) Silva Lab (Department of Biomedical Engineering, UC-Davis). Authors have no conflicts of interest to disclose.

Therapeutic potential of neural crest-derived multipotent stem cells in an *in vitro* model of ischemic injury of hippocampus

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INTRODUCTION: Adult neural crest-derived multipotent stem cells (NC-MSCs) are showing great promises in treatment of CNS and PNS diseases. The aim is to study the therapeutic potential of NC-MSCs in an *in vitro* model of ischemic injury of hippocampus.

METHODS: Organotypic hippocampal culture was done according to L. Stoppini [1]. The slice oxygen-glucose deprivation (OGD; *in vitro* model of ischemic injury) was performed in gas chamber (10 min, 35°C). Two hours following OGD/reoxygenation, 2.5×10^5 NC-MSCs were overtopped a slice. Adult NC-MSCs were isolated from the bulge of whisker follicle of FVB-Cg-Tg(GFPU) 5Nagy/J GFP mice by M. Sieber-Blum method [2] in our modification [3]. Neurotrophins production was detected by Multi-Neurotrophin *Rapid*™ ELISA kit (Biosensis). NC-MSCs phenotype, clonogenic and multilineage differentiation potential have been determined. Immunohistochemical staining of hippocampal slices was done with GFAP; NeuN, olig-2, Iba-1, nestin, β-III-tubulin and GFP antibodies.

RESULTS: The NC-MSCs were $Sox10^+nestin^+CD44^+CD73^+CD90^+CD117^+CD271^+$ $Sca\text{-}1^+CD45$, had high clonogenicity potential (CFU – 62.8±2.5%), were able to differentiate into multiple cell types (neurons, glial cells, melanocytes, osteoblasts, chondrocytes and adipocytes) and produced the neurotrophins (NGF – 1.2±0.4; BDNF – 2.4±0.6; NT3 – 0.7±0.1; NT4/5 – 2.1±0.2 ng/ml per 10^6 cells over 24h). It has been shown that the OGD led to death of hippocampal CA1 neurons and to reactive gliosis. NC-MSCs transplantation resulted in gliosis reduction and preservation of CA1 neurons quantity. The transplanted NC-MSCs survived during entire period of a study (14 days), were distributed in damaged areas of hippocampal tissue, but did not differentiate into any CNS cell type.

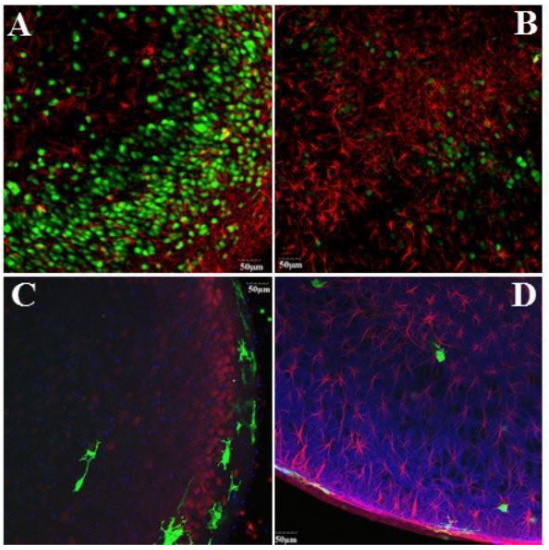

Fig. 1: Immunohistochemical staining of hippocampal slices for $NeuN^+$ neurons (A, B – green, C - red), $GFAP^+$astrocytes (A, B, D - red), olig-2^+ oligodendrocytes (C - blue), β-tubulin-III^+ neurons (D - blue) and GFP^+ NC-MSCs (C, D - green), CA1 area. A - control; B - 14 days after OGD; C-D – 14 days after OGD+NC-MSCs.

Table 1. Quantitative analysis of $NeuN^+$ neurons

Experimental groups	number of slices (n)	number of $NeuN^+$ nuclei
Control slices	n=7	454.0±39.3
Slices after OGD	n=7	197.1±22.2
OGD+NC-MSCs	n=23	468.1±19.2

DISCUSSION & CONCLUSIONS: Our results demonstrated significant neuroprotective effect of NC-MSCs transplantation. It seems to be it was realized via paracrine action without differentiation of NC-MSCs into CNS cell types. It can be perspective way for the ischemic stroke cellular therapy.

Graphene oxide selectively targets cancer stem cells, across multiple tumour types: Non-toxic cancer treatment, via differentiation-based nano-therapy

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INTRODUCTION: Tumour-initiating cells (TICs), a.k.a. cancer stem cells (CSCs), are difficult to eradicate with conventional approaches to cancer treatment, such as chemo-therapy and radiation. As a consequence, the survival of residual CSCs is thought to drive the onset of tumour recurrence, distant metastasis, and drug-resistance, which is a significant clinical problem for the effective treatment of cancer. Thus, novel approaches to cancer therapy are needed urgently, to address this clinical need.

Towards this end, here we have investigated the therapeutic potential of graphene oxide (GO) to target cancer stem cells. [1] Graphene and its derivatives are well-known, relatively inert and potentially non-toxic nano-materials that form stable dispersions in a variety of solvents.

METHODS: GO was prepared by using the Hummer's method with modifications [2]. The effects of GO on the anchorage independent clonal expansion of MCF7 CSCs was assessed using the tumour-sphere assay, a functional assay which directly measures CSCs proliferative expansion. The Cignal Lenti reporter assay (luc) system was chosen for monitoring the activity of several signal transduction pathways in MCF7 cells. The Luciferase Assay System was used on all luciferase reporter MCF7 cells treated with GO.

RESULTS: We show that graphene oxide effectively inhibits tumour-sphere formation in multiple cell lines, across 6 different cancer types, including breast, ovarian, prostate, lung and pancreatic cancers, as well as glioblastoma (brain). In striking contrast, graphene oxide is nontoxic for "bulk" cancer cells (non-stem) and normal fibroblasts (hTERTBJ1).

Mechanistically, we present evidence that GO exerts its striking effects on CSCs by inhibiting several key signal transduction pathways (WNT, Notch and STAT-signalling) and thereby inducing CSC differentiation.

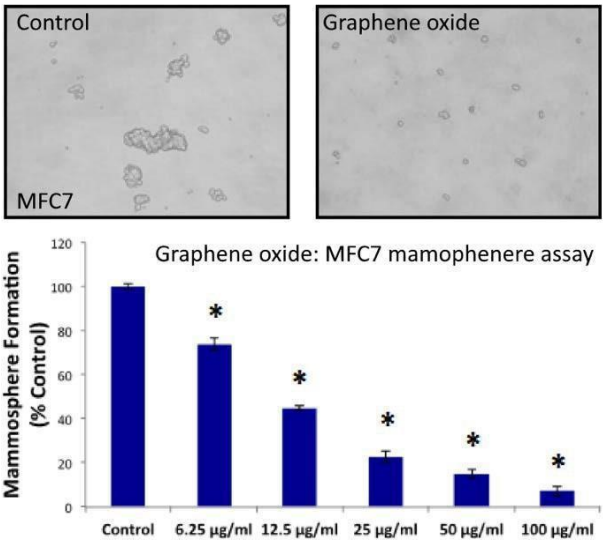

Fig. 1: (Top) Representative images showing that GO (big flakes, 25µg/ml) inhibits the anchorage-independent proliferation of MCF7 breast cancer cells. (Bottom) GO inhibits the anchorage-independent proliferation and mammosphere formation of MCF7 CSCs in a concentration dependent manner.

DISCUSSION & CONCLUSIONS: Our results suggest that GO could directly be used as a therapeutic for targeting CSCs, possibly as a differentiation agent. In this context, we envision that GO could be delivered i.v. or p.o., as a new anti-cancer therapeutic, depending on the location of the tumour. Alternatively, GO flakes could also be used as a lavage solution during surgery, to clear the tumour excision site or the peritoneal cavity (as in ovarian or other peritoneal cancers) of residual CSCs, with the aim of preventing tumour recurrence and distant metastasis, via differentiation-based nano-therapy.

ACKNOWLEDGEMENTS: This work was supported by The University of Manchester and EPSRC (UK).

Platelet-rich plasma anchors adipose stem cells injected in the urinary sphincter in a pudendal nerve-transected rat model of stress urinary incontinence and restores sphincteric function

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INTRODUCTION: Adipose derived stem cells (ADSC) are non immunogenic and have the ability to self-renew and to differentiate into multiple cell types. They have been used to restore urinary sphincter function in animal models. But there is not strong evidence regarding the role of the vehicle that must be used in combination with ADSC to improve effectiveness at the injection site. Our goal is to assess whether platelet rich plasma (PRP) could anchor adipose stem cell (ADSC) in the injection site and promote urethral sphincter restoration in a stress urinary incontinence (SUI) rat model.

METHODS: Thirty five female inbred Wistar rats were used in our study. Animals were divided into seven groups (five animals per group): continent (C), sham (S), PNT (D), PNT+PBS injection (P), PNT+PBS+ADSC injection (PA), PNT+PRP injection (R) and PNT+PRP+ADSC injection (RA). Twenty five females rats underwent bilateral pudendal nerve section (PNT) to induce SUI. ADSCs were purified from fat tissue of a 4-week-old inbred male Wistar rat, labeled CM-Dil and injected into the urinary sphincter in twelve oÂ´clock position with 70 microliltres of PBS or PRP. Four weeks after injection, cystometry was undertaken in all animals and leak point pressure (LPP) measured to assess urethral resistance function. All groups were sacrificed after cystometry, urethra sections were submitted for histology, immunohistochemistry assessment.

RESULTS: LPP was increased significantly in R, RA and PA animals after implantation ($P < 0.01$), but was not different from group C and S. Histological and immunohistochemical examination demonstrated increased numbers of surviving ADSCs increased muscle/collagen ratio as well as increased microvessel density at the injection sites in RA compared to PA animals (CM-Dil +)

DISCUSSION & CONCLUSIONS: PRP may anchor ADSCs into the injection site and potentially improve the histology and function of the urethral sphincter in a SUI rat model.

The arteriovenous loop model – an essential tool for vascularized tissue engineering and angiogenesis studies in human and veterinary medicine

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INTRODUCTION: The blood vessel network is an integral part of our body supplying nutrients, exchanging gases and removing waste products from organs and tissues. Especially in Tissue Engineering and Regenerative Medicine vascularization is mandatory for the survival of artificial or transplanted organs. However, the generation of a functional blood vessel network in engineered tissues or organs remains challenging. Furthermore, there is still the need to gain a better understanding of vascularization processes in an *in vivo* setting which could potentially help to adjust or define new therapeutic approaches.

METHODS: An arteriovenous (AV) loop is created microsurgically at the inner sides of the hind limbs of rats and sheep. In the rat the A. and V. femoralis and a venous interponate serve as loop vessels, in the sheep the A. and V. saphena. The AV loop is embedded into a closed implantation chamber with different matrices, such as different scaffolds for tissue engineering purposes or hydrogels for studying vascularization processes. For stimulation of tissue formation or angiogenesis, cells such as mesenchymal stem cells (MSC), myoblasts, lymphatic endothelial cells (LEC) and growth factors rhBMP-2, bFGF or VEGFA are added to the matrix. Further, motoric nerves can be implanted for functionalization of tissues. Using 3D imaging methods vascularization can be visualized *in vivo* over time (MRI) or after explantation (µCT). Further evaluation methods include (immuno)histology, gene expression analyses, REM, SEM.

RESULTS: A microsurgical angiogenesis model was established in the small and large animal model highly suitable for generating axially vascularized artificial tissue that can be microsurgically transplanted into the defect site after an adequate prevascularization time (Fig. 1a/b). In fact, using MSC and rhBMP-2 vascularized bone tissue could successfully be generated both in sheep and rats. For muscle TE purposes, we developed the rat EPI-loop model, in which the epigastric vein is used for loop creation together with the obturator nerve for neurotization of implanted muscle cells. Besides, the AV loop model provides an excellent tool for studying angiogenesis in an isolated and well controlled *in vivo* setting. A lymphatic network developed in the chamber after implantation of MSC and LEC. Using bFGF and VEGFA blood angiogenesis was efficiently stimulated. In a first clinical case successful *in situ* bone tissue engineering for the therapy of a large bone defect using the AV loop model could be demonstrated [1].

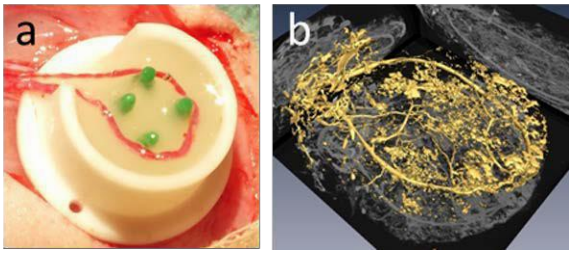

Fig. 1: (a) AV loop model rat, (b) µCT AV loop model sheep

DISCUSSION & CONCLUSIONS: During the last decade we were able to demonstrate the suitability of the AV loop model for vascularized tissue engineering and for angiogenesis and anti-angiogenesis studies. By modulating the chamber architecture, matrix, cells or growth factors it is possible to generate a multitude of individually customized axially vascularized and transplantable tissues useful both for human and veterinary medicine. Further, it is intended to generate a tumor blood vessel network within the chamber as a useful tool for evaluating anti-angiogenic therapies and tumorangiogenesis in principle.

ACKNOWLEDGEMENTS: This study was funded by Staedtler Stiftung, EKFS Stiftung, Baxter GmbH, DFG, IZKF/ELAN/EFI University of Erlangen-Nürnberg (FAU), AO Foundation, Roth Stiftung, Xue Hong and Hans Georg Geis Foundation. We would like to thank PD Dr. A. Hess for his support with µCT/MRI.

Therapeutic potential of mesenchymal stem cells and limbal epithelial stem cells for ocular surface healing

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INTRODUCTION: Damage of ocular surface represents one of the most common causes of impaired vision or even blindness. If the corneal damage is more extensive and the limbal region is involved, the defect can lead to limbal stem cell deficiency. In such cases, corneal transplantation alone is not a sufficient treatment method. Cell therapy, based on transplantation of stem cells, is an optimal treatment. However, if autologus limbal stem cells (LSCs) are not available, other sources of stem cells are tested. In this study, the terapeutic potential of transplanted LSCs and mesenchymal stem cells (MSCs) were compared in a rabbit experimental model of corneal injury.

METHODS: Stem cells were obtained by enzymatic digestion of limbal tissue (LSCs) or isolated from femurs of rabbits (bone marrow-derived MSC – BM-MSC) and from subcutaneous fat (adipose tissue-derived MSC – AD-MSC). The growth of stem cells on plastic or on nanofiber scaffolds was determined by cell viability (WST assay-Roche) and proliferation test. Nanofiber scaffolds were prepared from the biocompatible polymer poly(L-lactic) acid (PLA) by a needleless electrospinning procedure, as we have described elsewhere [1,2]. The differentiation of the cells into adipocytes was confirmed by staining with Oil Red O [3]. The expression of genes in cultured cells or in control and treated corneas was determined by quantitative real-time PCR using a StepOne-Plus real-time PCR system (Thermo Fisher Scientific). Expression of genes for adipocyte-specific markers (ADPC, PPARgama) and for immunoregulatory molecules (IDO-2, Cox-2, iNOS, HGF, TGF-beta, VEGF) were determined by PCR. Statistical significance of results was calculated by Student´s t-test and ANOVA.

RESULTS: In this study we used a model of the alkali-injured ocular surface in rabbits and compared the therapeutic potential of two types of MSCs (AD-MSCs, BM-MSCs) and tissue-specific LSCs. The injury of the cornea with 0.25N NaOH induced damage of the corneal epithelium, an increase in corneal thickness, a strong infiltration with cells of adaptive (T lymphocytes) and innate (iNOS-expressing cells) immunity, an increase in the presence of apoptotic cells (caspase-3^+ cells), neovascularization, and corneal opacity associated with decreased corneal transparency. All these parameters characterizing the ocular injury were decreased in the treated eyes. The nanofiber scaffold itself slightly supported healing and decreased the harmful impact of injury. The treatment of injured eyes with nanofiber scaffold seeded with stem cells significantly decreased all of the harmful manifestations of the injury and improved re-epithelialization of the damaged cornea. The less pronounced therapeutic effects of AD-MSCs in comparison with BM-MSCs or LSCs could be due to the lower differentiation potential of AD-MSCs and to the different spectrum of growth and immunoregulatory factors produced by these cells.

DISCUSSION & CONCLUSION: Our results show that BM-MSCs have comparable therapeutic effect to those of tissue-specific LSCs on the healing of corneal injury. We suggest that an important role in a healing process is represented by the production of a numerous trophic and growth factors that can support the growth of residual corneal epithelial cells and LSCs, and by ability of MSCs to suppress the local inflammatory reaction that could impede the healing process. All of these properties make BM-MSCs a promising candidate cell population for improving ocular surface healing in situations when autologous LSCs are difficult to obtain or are absent.

ACKNOWLEDGEMENTS: This work was supported by Grant 14-125805 from the Grant Agency of the Czech Republic, Grants 889113 and 80815 from the Grant Agency of the Charles University, and Projects Biocev CZ.1.05/1.1.00/02.0109, NPUI: LO1309, UNCE 204013 and SVV 260206.

DISCLOSURES: The authors indicated no potential conflict of interest.

Decellularized xenogenic meniscus as a replacement for meniscal tissue

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INTRODUCTION: Meniscus replacement still represents a challenging problem [1-2]. Currently used synthetic, biologic-synthetic or allogenic materials do not guarantee sufficient security regarding degenerative changes in the knee joint. The aim of this study was the biochemical and *in vitro* characterization of a chemically decellularized allograft.

METHODS: Porcine menisci (pMC) were decellularized by a chemical multistep procedure [3]. Decellularization was assessed histologically, by quantification of DNA and immune-response- provoking α-Gal epitopes using an ELISA inhibition assay. The content of sulfated glycosaminoglycanes (GAG) was analysed by using the 1,9-dimethylmethylene blue assay, the fraction of denatured collagen (w_D) by the hydroxyproline assay. *In vitro* cytotoxicity was defined according to ISO 10993-5 and *in vitro* revitalization with primary bovine meniscus cells (bMFC).

RESULTS: After chemical treatment HE-staining demonstrated that the allografts are cell free. The DNA content was verified to be below the quantification limit of 0.06 ng/mg (see Table 1), the amount of GAG was decreased by 61.37 % and no significant increase of w_D, was observed. The α-Gal epitopes were reduced by a factor more than $2.56 \cdot 10^2$. Biological investigations, in line with ISO-Standards (EN ISO 10993-5), showed no cytotoxic effects, even after sensibilisation of different indicator cells (L929 cells, bMFC and hBMSC).
In vitro seeding experiments of decellularized scaffolds with bMFC demonstrated very good acceptance of the scaffolds, and the capability to revitalize the matrix long-term. After 14 d of cultivation noticeable synthesis of matrix material as well as cell migration could be observed. During the first 42 d the thickness of newly synthesized tissue on the scaffold increased up to approximately 300-400 µm. Moreover, cells migrated along the collagen fibers, up to 400 µm into the tissue. *De novo* synthesis of typical ECM components like aggrecan, collagen type I and type II were identified immunohistochemically.

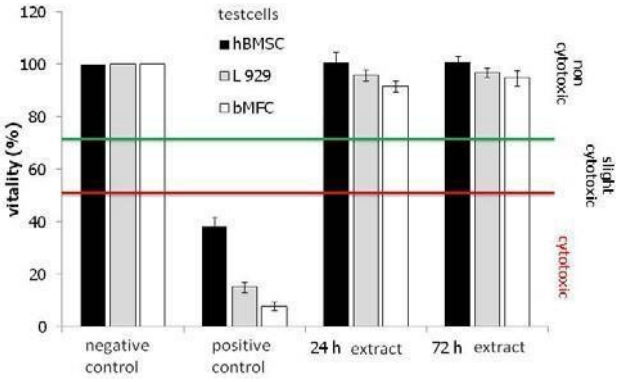

Fig. 1: In vitro cytotoxicity demonstrates a high viability and reflects the noncytotoxic quality of the extracted pMC matrices.

Table 1: Content of sulfated glycosaminoglycanes, DNA and fraction of denatured collagen (w_D)

	native tissue	dezellularized tissue
content of sulfated GAG (µg ·mg^{-1})	11,00 ± 2,65	4,25 ± 0,34
w_D (%)	4,72 ± 0,52	5,15 ± 0,83
DNA content (ng·mg^{-1})	280 ± 110	< 0,06

DISCUSSION & CONCLUSIONS: Non-collagenous components from decellularised pMC have been reduced without significant increase of the fraction of denatured collagen. The *in vitro* investigations proved the meniscus grafts to be non-cytotoxic, suitable chondroconductors, with excellent biological compatibility. Decellularized meniscus grafts therefore provide a very promising alternative to existing materials.

ACKNOWLEDGEMENTS: The authors wish to thank the DFG (German Research Foundation, BR 3817/1-1 and BU 461/21-1) for funding the project.

Comparing the effect of electrospun PLGA nanofibers alignment and RGD peptide conjugation on Marrow Stromal Cells spreading and proliferation

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INTRODUCTION: Bone tissue is composed of mineralized aligned collagen fibers. Therefore, aligned electrospun nanofibers (NF) show great potential as a bone tissue-engineering scaffold. The effect of nanofiber alignment on the rabbit fibroblast adhesion and proliferation was studied and it was demonstrated that higher cell spreading and proliferation was observed on the aligned NF, compared with that on the random NF [1]. Integrin binding sequence of cell adhesive RGD peptide increases cell attachment and spreading [2]. However, the effect of nanofiber alignment and RGD peptide conjugation on cell spreading and proliferation has not been studied. Therefore, the objective of this study is to evaluate the effect of different concentration of RGD conjugation on aligned NF and its effect on cell morphology and proliferation.

METHODS: GRGDC peptide was synthesized on the solid phase and functionalized by the addition of a cysteine residue using a previously described procedure [3]. GRGDC peptide was conjugated to Ac-PLA by Michael addition reaction. PLGA+PLA-RGD conjugate with different RGD peptide concentration was electrospun with the lab made wire setup shown in Fig 1 to fabricate highly aligned NF. Marrow stromal cells (MSCs) were seeded on the RGD peptide conjugated aligned NF, cultured up to 7 days and morphology of the cells were examined by florescent and scanning electron microscopy. Total cell DNA content was measured by using Quant-it Picogreen assay to define the cell proliferation on day 1,3, and 7. To confirm the RGD peptide attachment FITC labeled RGD peptides were conjugated on NF and imaged by fluorescent microscopy.

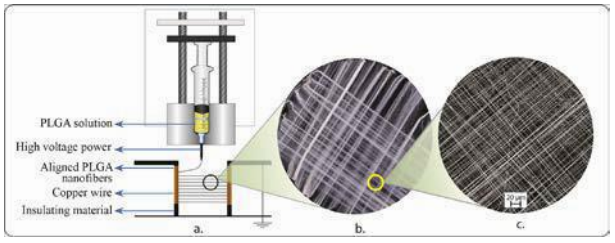

Fig. 1: Schematic diagram electrospinning setup to fabricate aligned NF.

RESULTS: 1% RGD concentration is the highest peptide concentration that can conjugate on the NF, which was confirmed by measuring the FITC labeled RGD peptide concentration by following the technique reported previously [3]. MSCs proliferation on the 1% RGD conjugated NF was significantly higher on day 3 and 7 compared to 0% and 0.5% RGD conjugated NF. Similarly, cell spreading was significantly higher on the 1% RGD peptide conjugated NF.

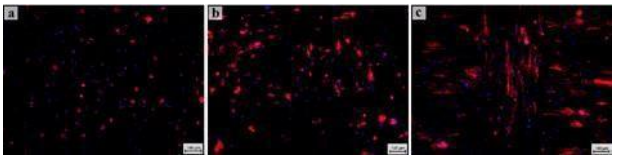

Fig. 2: Fluorescent microscopy images of different concentration of RGD peptide conjugated NF (a) 0%, (b) 0.5%, and (c) 1%. Cell nuclei and cytoskeletal actin are stained with 4,6-diamidino-2-phenylindole (DAPI; blue) and phalloidin (red). Scale bars represent 100µm.

DISCUSSION & CONCLUSIONS: Cell morphology analyses showed that 1% RGD peptide conjugated aligned NF enables higher cell spreading and proliferation. The spreading direction of the cells on RGD conjugated samples was similar as the orientation of the fibers. It was also determined that a 1% RGD concentration was the optimum RGD concentration to maximize cell attachment and spreading to the NF. Also, increasing the RGD concentration enhanced MSCs proliferation on the aligned NF. It was concluded that RGD peptide concentration influences cell spreading more than NF orientation.

2

ACKNOWLEDGEMENTS: This study was financially supported by The Scientific and Technological Research Council of Turkey (TÜBİTAK, 214M268)

A Study on wound healing properties of eucalyptus oil/polyurethane mat

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INTRODUCTION: Eucalyptus oil is derived from eucalyptus leaf, native to Australia and has been reported for its anti-inflammatory properties.[1] In this study, we have designed an efficient anti-bacterial, anti-inflammatory patch/scaffold with wound healing properties using eucalyptus oil (Eu) and polyurethane (PU). The eucalyptus oil/ polyurethane (Eu/PU) scaffolds were fabricated with varied Eu concentrations and its wound healing properties were studied in rabbit dermal fibroblasts as models using MTT assay, SEM, contact angle and FTIR, etc.

METHODS: PU (10 wt.%) solution with 3, 5, 10 wt.% concentration of Eu was used to prepare the scaffolds/mats. Mixed solvent of DMF:THF (1:1) was used to prepare PU polymer solution. Eu drops were added carefully to the polymer solution and stirred for 2 h. Finally, different concentration of Eu/PU solution was poured on the glass dish and dried for 24 h at room temperature.

RESULTS: Eu/PU scaffolds with 3, 5 and 10 wt.% Eu was found to show higher proliferation of rabbit dermal fibroblasts compared to only PU patch. At 5th day of cell culturing, 3 wt.% Eu/PU scaffolds showed remarkable increase among the other experimental groups. The SEM image showed most of the pores of 3 wt.% Eu/PU scaffolds were covered with ECM on 3rd day of culture (Fig 1). At 5th day, morphology of rabbit dermal fibroblast on 3 wt.% Eu/PU patch showed longer connective fibrous bands compared to other experimental groups. The contact angle result showed that with an increase in Eu concentration in scaffolds, the hydrophobicity of the Eu/PU scaffolds increased, which can be ordered as PU < 3%Eu/PU < 5%Eu/PU < 10%Eu/PU.

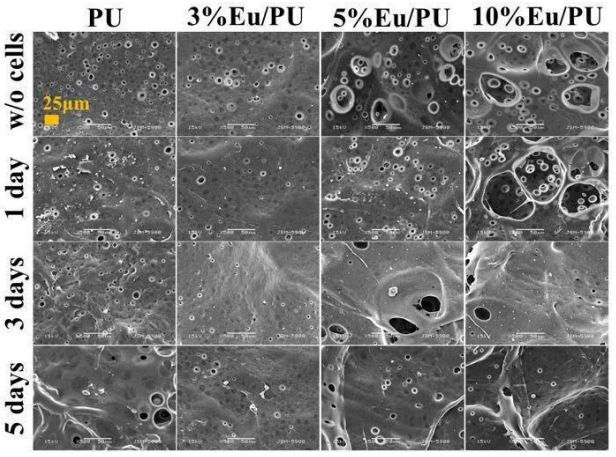

Fig 1. SEM images of cells morphology on polyurethane and (3, 5 & 10 wt %) eucalyptus oil/polyurethane scaffolds.

DISCUSSION & CONCLUSIONS: We have successfully fabricated Eu/PU scaffolds to achieve improved biological characteristics. *In vitro* studies showed that the as-synthesized Eu/PU scaffolds have good cell biocompatibility and cell viability. Further, the morphological and structural analysis showed the 3 wt.% Eu/PU scaffolds can be envisioned as a potential biomaterial for wound dressing and skin regeneration.

ACKNOWLEDGEMENTS: This research was supported by NRF-2012M3A9C6050204 and BK21 PLUS.

Peripheral nerve regeneration by porcine small intestinal submucosa

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INTRODUCTION: The search for a suitable material to guide the nerve regeneration has been a challenging tasks. Many kind of materials including artificial or natural products has been studied for this purpose, despite its limitations and complications. We investigated the possibility of using porcine small intestinal submucosa(SIS), a tissue consisting of nonimmunogenic acellular collagen with many kinds of growth factors, as a natural materials for peripheral nerve guidance channel.

METHODS: Left sciatic nerves were cut 5mm in length in 20 Sprague Dawley Rat. Graft between cut nerve ends were performed with silicone tube (group 1, n=7), silicone tube filled with platelet supernatant(group 2. n=7), dry porcine SIS(group 3, n=6). All rats underwent motor function test and electromyography study on 4th week and 10 weeks after grafting. On 10 weeks, the animals were sacrificed after EMG study and graft including proximal and distal nerve segments were retrieved for histological analysis.

RESULTS: Foot ulcers due to analgesia were fewer in group 3 than group 1 or 2. Run time test for motor function study were 2.67 in group 1, 3.61 in group 2 and 5.92 in group 3. Group 3 animals showed better EMG response for distal motor latency, amplitude compared with group 1 and group 2 animals. Histologically, all graft contained some axons and myelination, but the number of the axon and the degree of myelination was significantly high in group 3 animals than group 1 or 2.

DISCUSSION & CONCLUSIONS: These results show that porcine SIS was excellent option as a natural biomaterial for peripheral nerve regeneration. And more, it could be used as biocompatible barriers covering neural tissue, for example, dural repair, since this material contains many kinds of nerve growth factors.

Potency of duck's feet-derived collagen/poly(lactic-co-glycolic acid)/hydroxyapatite scaffolds for bone tissue engineering

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INTRODUCTION: Poly(lactic-co-glycolic acid) (PLGA) has been widely used in tissue engineering because of its easy synthesis protocols. However, PLGA have some demerit i.e. causing inflammation reaction due to low rate of cell adhesion and acidity of degradation products. On the other hand, collagen possesses about 30% of human protein, main ingredient of ECM and used for cartilage, bone and skin regeneration owing to its biocompatible and biodegradable features. Previously, duck's feet-derived collagen (DC) have showed reduced inflammation reaction when mixed with PLGA [1]. Inspired by those properties of collagen, we have fabricated 80% DC/PLGA/hydroxyapatite (HAp) scaffolds to study its potential for bone tissue engineering. Further, we also studied the as-fabricated scaffold's influence on osteogenesis of bone marrow-derived mesenchymal stem cells

METHODS: DC/PLGA/HAp scaffolds with varied DC wt% (10, 20, 40, 60, 80 wt%) were fabricated via salt leaching method. All samples characteristics were studied by FTIR, compressive strength, porosity and SEM (*Fig. 1*). Also, osteogenic differentiation of rabbit bone marrow mesenchymal stem cells (rBMSCs) cultured in scaffold was evaluated by ALP assay, MTT assay, RT-PCR, SEM.

RESULTS: We found decrease in porosity with an increased DC content in scaffolds. In contrast, compressive strength was increased. *In vitro* studies showed cell proliferation and osteogenesis.

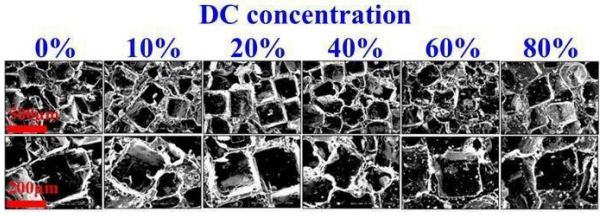

Fig. 1: SEM images of DC/PLGA/HAp scaffolds as a function of DC content.

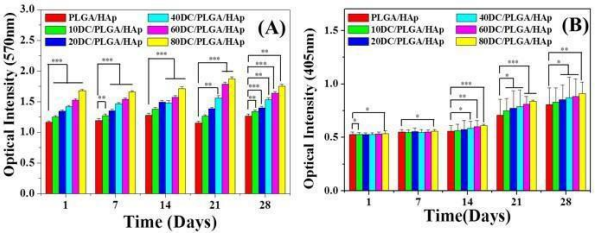

Fig. 2: Osteogenic differentiation and cell proliferation of rBMSCs in DC/PLGA/HAp scaffolds was analysed by MTT(A) and ALP(B) assay after 1, 7, 14, 21, and 28 days.

differentiation were increased with rising DC content. Notably, cell proliferation and oestrogenic differentiation are highest in 80wt% DC/PLGA/HAp scaffold (*Fig. 2*).

DISCUSSION & CONCLUSIONS: This study showed the highest cell proliferation and osteogenesis in 80wt% DC/PLGA/HAp scaffold.

ACKNOWLEDGEMENTS: This research was supported by Bio-industry Technology Development Program(KMFAFF, 112007-05-4- SB010), Technology Development Program(814005-03-2-HD020)

Osteogenic differentiation evaluation of quercetin-functionalized silk fibroin /hydroxyapatite scaffolds

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INTRODUCTION: Silk is a well-studied biomaterial for various biomedical applications owing to its properties biocompatibility and biodegradability [1]. Quercetin (Qtn) is a type of flavonoids, mostly found in onion and apple skin, and has been reported to affect osteogenesis [2]. Hydroxyapatite (HAp) is biodegradable and osteo-conductive biomaterial, widely used in tissue engineering. In order to increase osteogenic differentiation, herein, Qtn-functionalized silk fibroin/HAp scaffold was fabricated for bone regeneration study.

METHODS: For this study, Qtn/SF/HAp scaffold was fabricated with varied Qtn content in the scaffolds i.e. 0, 25, 50 and 100µM via freezing dry methods (*Fig 1*). Physio-chemical characterizations of Qtn/SF/HAp scaffolds were performed using SEM (*Fig 2*), compressive strength, porosity, FTIR, etc. *In vitro* studies (osteogenic differentiation and cell proliferation) on scaffold were evaluated using ALP assay, MTT assay, RT-PCR and SEM.

RESULTS: In this study, cell proliferation was found to decrease with an increased Qtn content in the scaffolds, as shown by ALP, MTT, RT-PCR, and SEM results. In contrast, osteogenic differentiation strength was increased. However, osteogenic differentiation is higher in Qtn/SF/HAp scaffolds than SF/HAp scaffolds. Notably, osteogenic differentiations are highest in 25µM Qtn/SF/HAp scaffold.

Fig. 1: Gross image of Qtn/SF/HAp scaffolds.

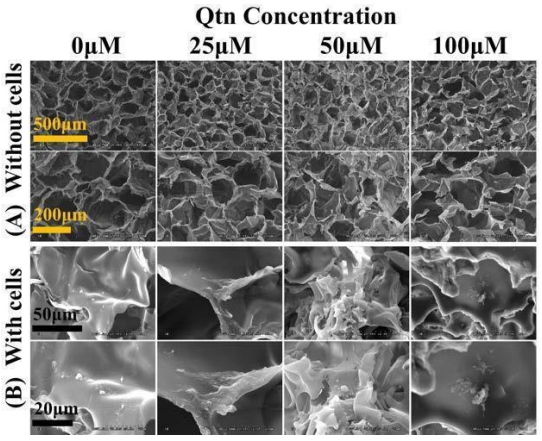

Fig. 2: SEM images of Qtn/SF/HAp scaffolds without (A) and with (B) rBMSCs.

DISCUSSION & CONCLUSIONS: This study suggests that 25µM Qtn/SF/HAp scaffold is highly-effective for osteogenesis, however, it shows lower cell viability than SF/HAp. This is because Qtn inhibits cell proliferation [3]. Collectively, the Qtn/SF/HAp scaffold can be envisioned as a potential material for bone regeneration.

ACKNOWLEDGEMENTS: This research was supported by Bio-industry Technology Development Program (KMFAFF, 112007-05-4- SB010), Technology Development Program(814005-03-2-HD020)

Two year follow-up for bone regeneration of BMP-2/VEGF-immobilized polycaprolactone porous beads using mini-pig model

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INTRODUCTION: Sufficient reconstruction of bony defect caused by fraction, tumor resection, and skeletal deformities is often essential. Recently, autologous transplantation of cortical and cancellous bones have been considered as a gold standard for the bone regeneration, however, donor site morbidity and limited availability of donor materials are still remained as significant challenges in clinical fields. To overcome these limitations, we prepared human periosteum-derived osteoblasts seeded into growth factors (BMP-2 and/or VEGF)-releasing polycaprolactone (PCL)/Pluronic F127 porous beads. The bone regeneration behaviors of the growth factors-immobilized PCL beads were investigated for 2 years using a miniature pig defect model.

METHODS: PCL/Pluronic F127 porous beads were fabricated by an isolated particle-melting method developed by our laboratory [1]. The Pluronic F127 molecules exposed on the pore surfaces of the bead act as an intermediary between the PCL pore surface and heparin that can interact with the growth factors (BMP-2 and/or VEGF). Their morphology, growth factor release behavior, *in vitro* osteogenesis using human periosteum-derived osteoblasts (POs) in the growth factors-immobilized porous beads, and the animal study using mini-pig defect model to investigate the effectiveness of the porous beads for bone regeneration were conducted.

RESULTS: From the growth factor release experiment, it was observed that the growth factors immobilized onto PCL porous beads were continuously released up to 28 days, for both BMP-2 and VEGF. From the *in vitro* osteogenesis using POs as a model cell, the dual growth factors-immobilized porous beads showed the effective osteogenesis of POs. From the *in vivo* animal study for bone regeneration using a mini-pig defect model for two years, it was also observed that the dual growth factors-immobilized porous beads promoted effectively the regeneration of defect bone (Figs. 1 and 2).

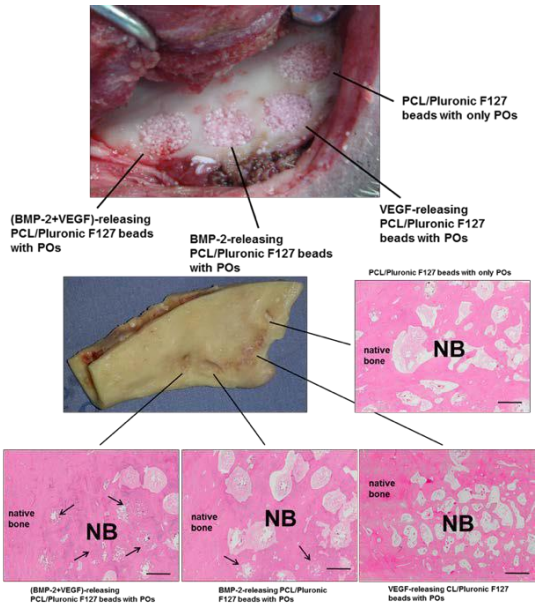

Fig. 1: Histological sections of mandibular bony and surrounding cranial tissue showing the bone regeneration behavior (after two years).

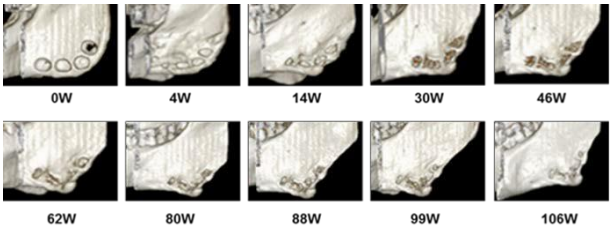

Fig. 2: Computed tomography images of mandibular bony showing the mineralized bone regeneration behavior at different time periods.

DISCUSSION & CONCLUSIONS: Our results suggest that the combination of BMP-2 and VEGF stimulates *in vitro* osteoblastic differentiation of cultured human POs seeded into the PCL/Pluronic F127 porous beads and their long-term *in vivo* bone regeneration.

ACKNOWLEDGEMENT: This research was supported by the National Research Foundation of Korea (NRF-2014R1A2A2A04003979).

The effect of verapamil coated silicone gel sheet in hypertrophic scar

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INTRODUCTION: Hypertrophic scar has emerged as a disease entity. Moreover, it is considered a dermal fibroproliferative lesion rather than an inevitable postoperative outcome. The authors developed a novel treatment based on the topical application of a silicone gel sheet containing calcium channel blocker, verapamil microparticles. The ability of these silicone gel sheets to inhibit hypertrophic scar in a rabbit ear wound model was examined.

METHODS: Ten New Zealand White rabbits with a total of 80 wounds in both ears were used in this study. The rabbits were divided into five groups (control; silicone gel sheet; and silicone gel sheet plus 0.25, 2.5, and 25 mg of verapamil per gram). Histopathologic findings were quantified.

RESULTS: The mean scar elevation index, fibroblast counts, and capillary counts differed significantly among the five groups ($p < 0.05$). The median scar elevation index was significantly lower in the silicone gel sheet plus 2.5 mg of verapamil per gram group than in the silicone gel sheet group (1.2 versus 2.2). The median number of fibroblasts was significantly lower in the silicone gel sheet plus 0.25 mg of verapamil per gram group than in the silicone gel sheet group (172.5 versus 243). In the median number of capillary lumina, there was no significant difference between the silicone gel sheet group and the silicone gel sheet plus 0.25, 2.5, and 25 mg of verapamil per gram groups (28.5, 18, 20, and 18, respectively).

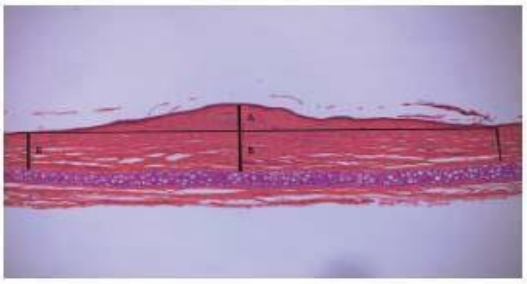

Fig. 1: Scar elevation index. The ratio of total scar area (A plus B) to the area of normal tissue below the hypertrophic scar (B).

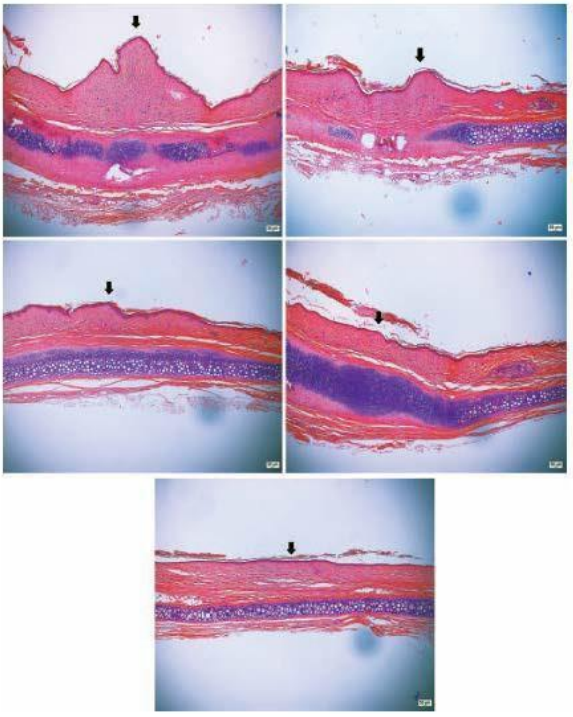

Fig. 2: Histopathologic findings. (above left) Control group. (above right) silicone gel sheet group. (center, left) silicone gel sheet plus 0.25mg of verapamil group. (center, right) silicone gel sheet plus 2.5mg of verapamil group. (below) silicone gel sheet plus 25mg of verapamil group

DISCUSSION & CONCLUSIONS: Topical application of a silicone gel sheet with verapamil microparticles may be a novel, effective treatment method for hypertrophic scar, but its safety and efficacy in humans must be tested in clinical trials.

Si-based bioactive materials for bone formation: the role of osteoclasts

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INTRODUCTION: Silicate is often used as a substituent in calcium phosphate (CaP) based bioactive ceramic/glass (BG). BG possess angiogenic and osteogenic properties are desirable for bone regeneration [1]. Bioglass 45S5 (widely used commercial BG) conditioned medium containing 0.59 mM Si (17ppm) was able to stimulate human osteoblast differentiation [2]. Osteoclasts are the only cell type that recycle damaged bones to allow healthy bone remodelling. The effect of osteoclastic activities to silicate is not fully understood and is essential to tissue engineering construct development. A recent study show soluble silicate inhibits osteoclast formation from RAW264.7 macrophages at 1.8 mM (50ppm) [3]. This could be through the inhibition of ROS (reactive oxygen species) production as reported previously [4]. This study looked into the potential of Si based material on murine osteoclast formation and function, including the possible link between reactive oxygen species generation and osteoclastogenesis.

METHODS: 1. ROS production was measured by CM-H2DCFDA (Molecular ProbesTM) on RAW264.7 cell-line. **2.** RANKL production by Saos-2 cell-line was measured by RANKL ELISA kit (Bosterbio). **3.** Bone morrow mononuclear cells from MF-1 mice, were used for osteoclast formation and function. Cells were cultured under the stimulation of MCSF and RANKL plus ion conditioned media.

RESULTS: ROS production used as an indicator of early inflammatory response in macrophage. Ca produced 40% more ROS compared to Si based material (Figure 1A). RANKL production by Saos-2 showed Ca at 10ppm produced >100% more RANKL compared to Si at (160ppm), (Figure 1B). Si at a concentration of 3 ppm (Figure 2B) showed optimal in osteoclast formation and function, whereas control (Figure 2A) have more osteoclasts apoptosis. However, Si at 30 ppm (Figure 2C) showed inhibition of osteoclastogenesis.

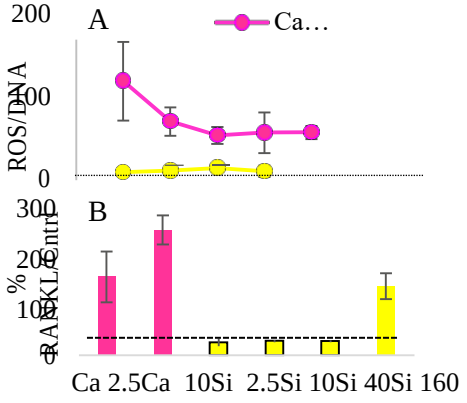

Fig.1 A) ROS production by RAW264.7 Cells.Ca increased ROS production by 80% compared to control. B) RANKL production by Saos-2 (osteoblast like cells) at day 7. Ca at 10ppm showed a 150% increase of RANKL production. Si at 160ppm showed a slight increased RANKL. (Dash-line=control)

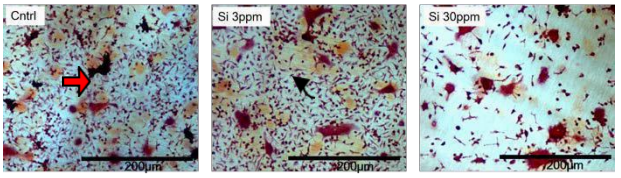

Fig.2: Osteoclasts formation with MF-1 mouse mononuclear cells. Si at 3ppm showed the optimal osteoclast formation and function (brown-resorption pits) compared to Si at 30ppm and control. Red arrow=apoptotic cells, black arrow= resorption, scale bar=200μm.

DISCUSSION & CONCLUSIONS: We showed Si at BG relevant concentration: (30ppm) decreases ROS and osteoclastogenesis compared with Ca. This study could lead to engineering a better bone implant with a lower failure rate.

Bi-phasic silk scaffolds for osteochondral tissue engineering

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INTRODUCTION: The osteochondral interfaces (OCI) are one of the most vulnerable areas in the human body to traumatic injuries and diseases such as osteoarthritis. The OCI consists of bone and cartilage tissues, which have different molecular compositions, cellular organizations, structural and mechanical properties. For successful osteochondral reconstruction, single intact scaffold should be used to support the regeneration of both cartilage and subchondral bone [1,3]. Therefore, this study aims at developing a silk based biphasic scaffold that is porous for the cartilage phase and fiber reinforced for the bone phase, thus mimicking the structure of osteochondral tissues.

METHODS: Silk fibroin solution and degummed fibers from *Bombyx mori* (BM, mulberry) and *Antherea assamensis* (AA, Indian endemic non-mulberry) silk were used to prepare biphasic scaffolds. The scaffolds were characterized for their physical properties such as swelling behavior, degradability, porosity and morphology (using FESEM). *In vitro* compatibility assessment was done using primary chondrocytes (for cartilage) and MG63 cells (for bone). Biochemical assays were performed to estimate ALP activity, DNA, GAG and Collagen content. Finally, gene expression studies were performed using real time PCR.

RESULTS: The results showed that developed scaffolds were biphasic structures with cartilage phase being soft (suitable for chondrocytes) and porous while the bone phase being stiff (suitable for osteocytes) with the reinforced fibers arranged in a mesh like manner. The porous scaffold showed higher swelling and degradation as compared to the fiber reinforced scaffold. The proliferation studies indicated that the porous scaffold supported the growth of chondrocytes, the fiber reinforced scaffold showed enhanced proliferation of osteocytes (MG63 cells) and the biphasic scaffold maintained an intermediate proliferation. The ALP assay showed a low value on day 1 that gradually increased to reach a maximum in case of BM fiber reinforced scaffold on day 14. The gene expression analysis showed that the biphasic scaffolds indicated a ~5 fold increase for both chondrogenic (aggrecan, sox-9) and osteogenic (osteopontin, runx-2) markers.

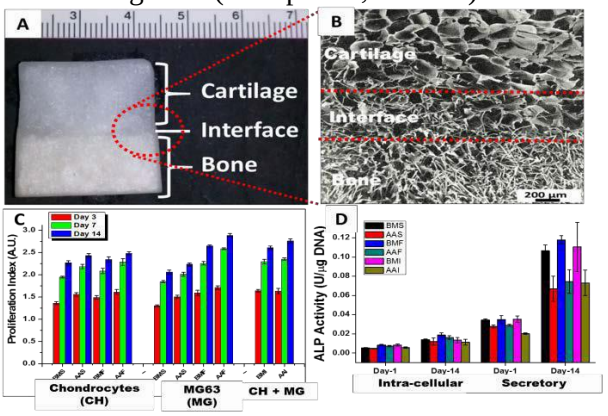

Fig. 1: A) Gross morphology of scaffolds, B) FESEM image, C) cell proliferation assay and D) ALP expression profile of cells on silk scaffolds.

DISCUSSION & CONCLUSIONS: This study reports the development of a silk based biphasic scaffold with a stratified structure composed of two distinct cartilage and bone phases which were well-integrated at a continuous interface. The distinct proliferative behavior of cells on the scaffold may be due to the varied stiffness of the scaffolds, also reported previously [2]. The study showed ALP induction of more than 2 fold and mRNA induction of more than 4 fold, after 14 days of cell culture. This might be explained by the fact that the varied response of cells *in vitro*, depended mainly on the structural or material composition of the scaffold. Thus, the biphasic scaffold showed functionality and it may lead to the development of new strategies for OCI repair and regeneration.

ACKNOWLEDGEMENTS: The authors gratefully acknowledge the financial support from Department of Biotechnology (DBT) and Department of Science and Technology (DST) Government of India and instrument support from CIF, IIT Guwahati.

Novel 3D graphene oxide/alginate hydrogels for nanomedicine applications

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INTRODUCTION:
Hydrogels have been widely used in tissue engineering to mimic biological extracellular matrix thanks to their high water content and controllable degradability. However, they often lack the mechanical support and signaling cues needed to properly guide cells. The opportunity to tune hydrogels grafts mechanical properties in relation to the required physiological needs is expected for the implants success. Graphene and its derivatives have recently emerged as promising materials due to their unique mechanical, physical, chemical proprieties [1]. Although they have not yet been widely used for medical applications, preliminary works suggest that both structural and functional properties of polymeric substrates may be enhanced when combined with graphene oxide (GO) [2]. In this work, reinforced 3D GO/alginate (Alg) hydrogels are presented and the effects of GO inclusion on their mechanical and biological behaviour shown.

METHODS: After preparing a GO nanosheets (Sigma Aldrich) aqueous suspension (1 mg/ml) by ultrasonic treatment, alginate (Manugel GMB, FMC Biopolymer) composite solutions were produced (0, 0.5, 2 wt% GO/Alg). Molds of agarose (1% w/v in CaCl 0,1M) were prepared to allocate GO/Alg solutions and chemically cross-link gels via diffusion (2 hr. at 37 °C). GO/Alg hydrogels were characterized through optical/ atomic force microscopy and FTIR analysis.
Biocompatibility tests were performed embedding 3T3 fibroblasts (8 millions/ml) in the GO/Alg hydrogels; cell viability was evaluated at different time points up to two weeks with Dead/alive kit.
Gels mechanical proprieties were assessed via Dynamic Mechanical- Analysis (DMA) up to 28 days of culture (with and w/o cells) at different time points.
All tests were performed in triplicate and statistical analyisis carried out (Mann–Whitney *U* test, n=9, $p<0,005$).

RESULTS: 3D composite GO/alginate hydrogels were successfully realized with 3 mm of height and 5 mm of diameter. Cell viability tests showed that the presence of GO does not decrease cell viability, confirming absence of toxicity, at least up to 2% wt GO/Alg. For all time points cell viabilitywas statistically higher in presence of GO, while there was no significant difference between 0.5 wt% and 2 wt% GO/Alg (Fig. 1A).
Hydrogels functionalized with GO exhibit an Elastic modulus about 3 fold higher than the Alg control at T0. After an initial decreasing of the Young Modulus for the all GO/Alg samples (t1), possibly due to a partial degradation of alginate, a drastic recovery was observed up to 28 days of culture only for GO functionalized samples. The mechanical features improvement was neither mediated nor triggered by cells activity (Fig. 1B).

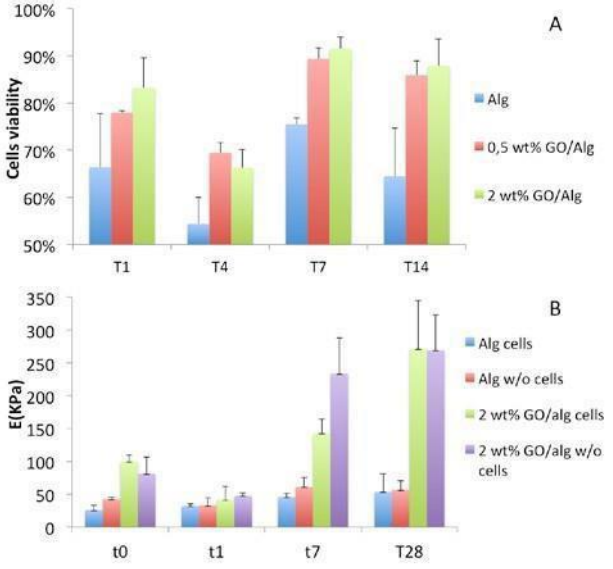

Fig. 1: Cells viability (A) and Young Modulus (B) of hydrogels at different times of culture.

DISCUSSION & CONCLUSIONS: In this work we successfully realized a natural-based 3D hydrogel nano-functionalized with graphene, where both mechanical and biological properties were successfully improved. The delayed stabilization of GO/Alg mechanical proprieties may be due either to a chemical interplay between GO and alginate matrix or to GO self-assembling processes over time. Future developments will be carried out to decouple the chemical and topological role of GO on the results observed up to now. Moreover, functional tests with stem cells will be performed to evaluate the GO effects on cell in vitro differentiation.

Iron-tricalcium phosphate composites define osteoblast morphology and alkaline phosphatase activity

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INTRODUCTION: Develop materials that guide cell responses is a current challenge in regenerative medicine. In the case of bone fracture treatment, osteosynthetic materials require in addition high mechanical strength as well as stiffness similar to bone to prevent stress shielding effect. A new class of biodegradable materials suitable for bone fracture treatment are iron-tricalcium phosphate composites (Fe-TCP) [1]. These composites combine the mechanical strength of iron with the osteoconductivity of calcium phosphates. In addition, Fe-TCP composites are potential biodegradable materials due to the *in vivo* corrosion rate of iron and resorption of tricalcium phosphate. The aim of the present work is to determine the *in vitro* osteoblast response in direct and indirect contract with this new generation of composite materials.

METHODS: Fe-TCP composites (0, 25, 50, 75 and 100 vol.% Fe) were fabricated by spark plasma sintering at 1100 °C during 10 min. Phase composition and microstructure after sintering were determined by x-ray diffraction (XRD) and scanning election microscope (SEM). The mechanical strength was determined by diametral tensile strength (DTS) and compression tests. MG-63 cells were seeded in direct and indirect contact with the composites to test cell adhesion, viability, morphology and differentiation by XTT test, live/dead staining, SEM and alkaline phosphatase (ALP) activity. Cell cultures were performed in static conditions in 24 well plates using DMEM.

RESULTS: Fe-TCP composites were successfully fabricated. XRD shows that the composition corresponds to a mixture of iron and TCP in two crystalline phases, alpha (more soluble) and beta phase. No thermal degradation or chemical reaction between components was observed. This is an advantage against similar biostable composites, where titanium induces the thermal decomposition of hydroxyapatite leading on the formation of CaO. Fe-TCP composites showed mechanical strength (DTS between 30 and 150 MPa) similar to current metallic alloys used to treat bone fractures. In particular the composite with 25 % of iron showed a modulus of elasticity (27 GPa) in the range of bone tissue. Osteoblast in indirect contract with the composites presented different behaviour as function of iron content. The higher the iron content produced a thicker ring of damaged/dead cells after 24 h of culture. However, outside the ring, osteoblast normally proliferate and presented similar viability after 3 days of culture. In direct contact, osteoblast attach and spread on the composite surface, presenting different morphology after 6 h of culture (Fig. 1). At longer times, osteoblast detached from the surface probably due to the accumulation of degradation products in the small volume of the cell culture well (1 ml). In addition, attached osteoblasts presented different ALP activity, showing a maximum activity on composites with 25 to 50 % of iron.

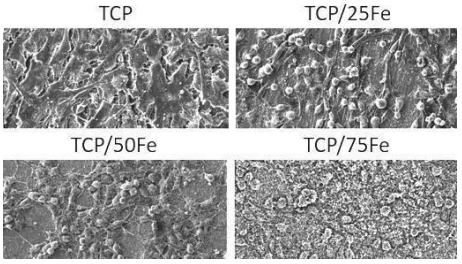

Fig. 1: MG-63 cells in direct contact with Fe-TCP composites after 6 h of culture.

DISCUSSION & CONCLUSIONS: The present results show promising properties of Fe-TCP composites to control the osteoblast morphology and early osteogenic activity. Together with their mechanical properties suitable for treatment of bone fractures, Fe-TCP composites appear as new alternative to biodegradable metals, such magnesium, for the fabrication of temporal osteosynthetic implants.

ACKNOWLEDGEMENTS: To financial support from SoMoPro 3 (co-financed by H2020-MSCA and the South-Moravian Region) and MEYS-CZ under the project CEITEC 2020 (LQ1601). SDT acknowledges CONACYT-SNI (P.1777000).

Free-standing films from hydrophobized hyaluronan as biodegradable implants with anti-adhesive properties

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INTRODUCTION: Hyaluronic acid modified by acyl chains [1] (acyl-HA) is a perspective material for tissue engineering and drug delivery [2]. While native HA dissolves quickly in water, implants from acyl-HA degrade only gradually. Time needed for disintegration of the implant depends on HA molecular weight, acyl chain length and its degree of substitution (DS). When prepared in the form of film [3], acyl-HA can be used as a temporary barrier to prevent unwanted tissue adhesions after surgery.

METHODS: Free-standing films were prepared by casting acyl-HA solutions on a hydrophobized glass substrate in a specialized drying apparatus. Films were characterized by profilometry, electron microscopy, mechanical testing and equilibrium swelling degree in phosphate-buffered saline (PBS). Interaction with cells was studied by cytotoxicity and cellular adhesion assays. Finally, anti-adhesive efficiency of the films was evaluated *in vivo* on WISTAR rats.

RESULTS:

Fig. 1: Photo of free-standing film from acyl-HA and its cross-section imaged by cryo-SEM.

Homogenous films of acyl-HA about 15 µm thick were obtained by solution casting; thickness variations within the film area (10×8 cm) were below 10 % (Fig. 1). Substrate side of the films was very smooth with roughness under 30 nm. Equilibrium swelling degree between 500 % and 1800 % was observed. The Young´s modulus decreased from GPa to MPa upon swelling, however, the strain at break increased due to the plasticizing effect exerted by the aqueous environment, so the hydrated films were able to withstand greater strains before breaking. Films retained their integrity in PBS for up to several months; the time needed for disintegration correlates with their DS and swelling degree. Films were not cytotoxic *in vitro* (Fig. 2). *In vivo* test proved that the films prevent adhesions of tissues in abdominal space of rats. After 30 days, 12 out of 15 rats in the tested group were found without significant adhesions while in the control group severe intergrowth of incised tissues was observed.

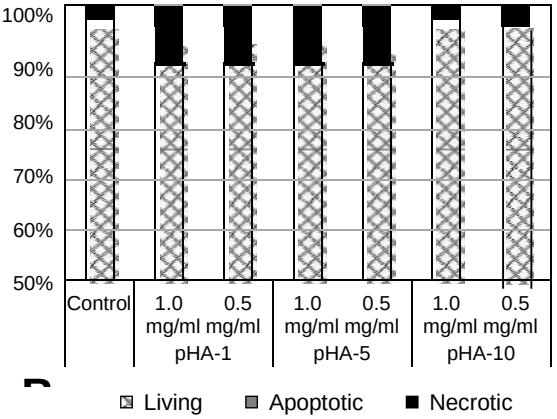

Fig. 2: Direct contact cytotoxicity assay for acyl-HA films - 3T3 cell viability after 72 hours.

DISCUSSION & CONCLUSIONS: Free-standing films of acyl-HA are effective as an anti-adhesion barrier in abdominal surgery. Their mechanical properties in wet state are sufficient for manipulation during the surgery. The durability of the film implant can be adjusted by the degree of substitution of the acyl-HA.

Non-mulberry silk fibroin based nerve guide biomaterials for neural tissue engineering

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INTRODUCTION: Around 2.8% of the trauma patients are affected by peripheral nerve injury throughout the world. Among various types of nerve injury the defect sizing gap of 1-2 cm or more between two stumps require guidance strategy. This uses suitable artificial nerve bridging biomaterial containing the regenerative potential of the autograft. This strategy can replace the complexity and uncertainty of autograft or commercially available nerve protecting wraps [1]. Biomaterials derived from natural sources are versatile in nature and well accepted in the *in-vivo* system. Silk, being a natural biopolymer, is proved as biocompatible biomaterial [2, 3]. The slow biodegradability, mechanical stability and dielectric properties of the fibroin based biomaterial can be exploited in the clinical repair of peripheral nerve injuries.

METHODS: In this study, we prepared nerve guide conduits by electrospinning technique using fibroins from both mulberry (*Bombyx mori*) and non-mulberry Indian tropical tasar (*Antheraea mylitta*) species combined with polyethylene oxide and collagen using genipin. Biophysical properties of the fabricated electrospun mats were evaluated using SEM, FTIR, and structural integrity in terms of protein leaching, tensile strength and TGA studies. The cytocompatibility of the materials was assessed by immunocytochemistry and fluorescence microscopy using human Schwann-like cell line (sNF 94.3). The cellular functionality was analyzed employing quantitative RT-PCR analysis of the level of expression of neural-specific genes (S-100, GFAP, and P75).

RESULTS: The compositions incubated in 0.5% w/v alcoholic genipin solution show minimum protein leaching from the matrices. The SEM images reveal that mats are porous in nature and fiber diameter ranges from 300 – 600 nm. The FTIR study confirms the formation of new bonds between protein and other components. The TGA result indicates that the matrices become more stable. The crosslinked mats can withstand more load than pure protein mats. From immunofluorescence and confocal studies, it is observed that the cells grown on equal volume blend of fibroin/collagen show better actin morphology with maximum S-100 (the protein responsible for nerve regeneration) expression. Nearly 2 fold increase in gene expression is observed for S100 and P75 genes on this matrix composition compared to the pure matrices.

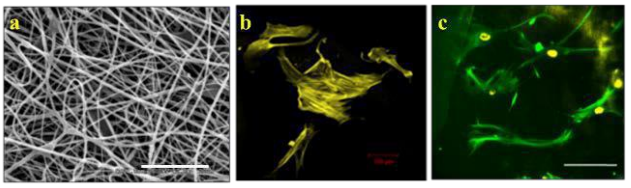

Fig. 1: (a) Scanning electron microscopic image of the electrospun mat. Scale bar = 20µm. Fluorescence images of (b) TRITC-phalloidin stained actin cytoskeleton and (c) anti S-100α antibody - FITC-labelled goat anti-rabbit IgG antibody stained Schwann cells grown on fibroin-collagen matrix. Scale bar = 100µm.

DISCUSSION & CONCLUSIONS: The investigation shows that non-mulberry fibroin and collagen enhance Schwann like cell adhesion and growth compared to mulberry and glass surface. Blend of fibroin and collagen (1:1) helps better cell attachment than the pure and other blends (fibroin:collagen = 3:1 and 9:1). The fabricated conduits have advantageous effects for preparing nerve guide conduits for better Schwann cell regeneration. They may be functionalized using nerve growth factors before the surgery for *in vivo* peripheral nerve injury model.

ACKNOWLEDGEMENTS: This work is supported by UKIERI-British Council, Department of Biotechnology and Indian Council of Medical Research, Govt. of India.

DISCLOSURES: Authors have nothing to disclose.

Integrating biochemical and topographical cues to direct stem cell fate

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INTRODUCTION: The ideal tissue engineering strategy for ligament (or tendon) regeneration should recapitulate the bone – calcified cartilage – fibrocartilage – ligament interface. Combinations of biochemical gradients [1], multiple cells types [2] and different materials and/or architectures [3] have previously been used for such interface tissue engineering, with limited success. Electrospun fibres are commonly used as biomaterials for ligament and tendon engineering, but directing mesenchymal stem cell (MSC) differentiation on such scaffolds remains a challenge. Here we explore how combinations of topographical (fibre alignment) and bioactive (growth factors) cues regulate MSC differentiation towards either a calcified cartilage, fibro-cartilaginous or ligamentous phenotype. By integrating substrate topography and bioactive cues, we demonstrated that it is possible to guide MSCs to deposit an organized ligament-like extracellular matrix or a cartilaginous template to engineer the bone – ligament interface.

METHODS: Sheets of polycaprolactone (PCL, MW 70 – 90 kDa) electrospun microfibres were collected on a mandrel rotating at 1000 rpm / 200 rpm to produce aligned / randomly-aligned fibres. Cell – laden electrospun mats were cultured in media supplemented with either connective tissue growth factor (CTGF) or transforming growth factor β3 (TGFβ3). At day 10, actin cytoskeleton, vinculin, and gene expression were analysed. At day 20 biochemical and immunofluorescent assays were performed.

RESULTS: Cells remained viable and proliferated on both aligned and random (non-aligned) scaffolds. Aligned and randomly aligned substrates guided the orientation of MSCs (Fig.1); this cell morphology was maintained over time. In terms of collagen production, no significant difference was found between MSCs on aligned or random substrates, suggesting that the underling substrate does not affect biosynthesis. Cells supplemented with connective tissue growth factor (CTGF) elongated on the fibres, irrespective of fibre orientation, a result that was not seen on random fibres in the absence of CTGF. CTGF – treated cells expressed ligamentous hallmarks: increased *col I*, *col III* and tenomodulin expression with respect to untreated control, with minimal expression of the chondrogenic markers *col II*, aggrecan and *sox-9*. Upon supplementation with TGFβ3, MSCs increased their expression of the chondrogenic genes aggrecan, *sox-9* and *col II*, especially on aligned substrates which promoted increased cellular aggregation. Sequential application of TGFβ3 followed by CTGF promoted increased expression of both *col I* and *col II*, indicative of fibrochondrogenic differentiation.

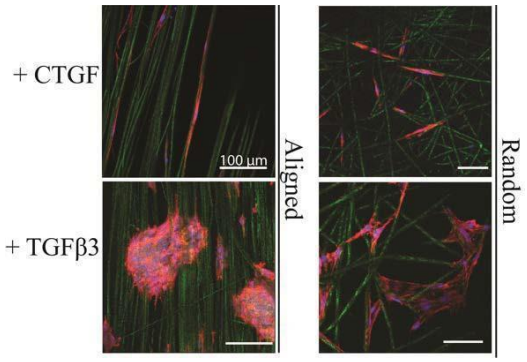

Fig. 1: F-actin (red) and DAPI (blue) staining.

DISCUSSION & CONCLUSIONS: This study demonstrated that the appropriate combination of bioactive and topographical cues can be used to direct MSC differentiation towards either a ligament or a fibrocartilage-like phenotype. Aligned microfiber sheets can serve as a template to guide MSC differentiation and the deposition of a unidirectional organized extracellular matrix by resident cells. This concept can be applied to fibre bundle by spatially immobilizing these bioactive signals along the length of the construct to engineer the interface from bone to ligament.

Biocompatible cross-linking of hyaluronic acid using transglutaminase

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INTRODUCTION: Injectable hydrogels are advantageous in minimally invasive application of tissue engineering scaffolds. Transglutaminase (TG) catalyzed reactions can be employed for *in situ* cross-linking of proteins providing a nature-mimicking fabrication method of network formation.[1] To date, cell-compatible and chemoselective TG-mediated cross-linking was demonstrated only for synthetic polymers.[2] In the present work, we employed for the first time a TG-catalyzed reaction for the *in situ* cross-linking of hyaluronan (HA) macromolecules.

METHODS: HA was modified with glutamine acceptor substrate derived from *N*-terminus of α_2-plasmin inhibitor providing HA-*Gln* derivative. 8-arm PEG macromers bearing the lysine donor peptide (PEG-*Lys*) was synthesized according to published procedure.[2] HA-*Gln* and PEG-*Lys* were dissolved in deionized water. Tris buffer containing $CaCl_2$ and thrombin-activated factor XIIIa were added to the mixture. All contents were homogenized and the hydrogel was formed within 3 to 6 minutes.

RESULTS: Hyaluronic acid (HA), a major natural component of extracellular matrix, was first modified with 2-dithiopyridyl groups (HA-SSPy) according to our previously reported protocol.[3] Glutamine acceptor peptide (Ac-NQEQVSPLERCG-NH_2) containing cysteine aminoacid was attached to HA-SSPy in the course of thiol-disulfide exchange reaction. The obtained HA-*Gln* derivative exhibited substrate properties for factor XIIIa when it was formulated with PEG cross-linker terminated with lysine donor peptide (PEG-*Lys*, Figure 1). This gel experiment indicated the formation of amide bonds between amine group of lysine of PEG-*Lys* and the carboxamide group of glutamine residue of HA-*Gln*. Mechanical properties of the obtained hydrogel was tuned by changing the concentration of HA and PEG components as well as the ratio between glutamine acceptor and lysine donor functional amino acid residues on the components.

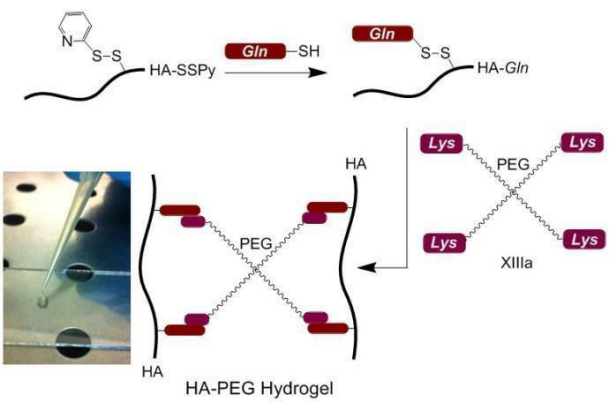

Fig. 1: Preparation of HA-Gln substrate from hyaluronic acid modified with 2-dithiopyridyl (SSPy) groups and formation of HA-PEG hydrogel upon action of factor XIIIa on HA-Gln and PEG-Lys.

DISCUSSION & CONCLUSIONS: *In situ* cross-linking of hyaluronic acid in the presence of living cells was achieved using transglutaminase (TG) catalyzed reaction. The elaborated method of preparation of HA substrate for TG can be applied for fabrication of hydrogels containing another glycosaminoglycans as well as for orthogonal combination of TG chemistry with chemoselective bioconjugation schemes.

ACKNOWLEDGEMENTS: This work was supported by EU FP7 project Biodesign.

VEGF-associated clay gels promotes in vitro angiogenesis: potential tissue engineering application for chronic wounds

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INTRODUCTION: Biological agents including Vascular Endothelial Growth factors (VEGF) have been investigated as agents that improve wound healing [1], yet delivery in an active economical form remains a significant clinical challenge [2]. Here we tested whether Laponite, a synthetic smectite clay biomaterial [3], can localise bioactive VEGF to stimulate *in vitro* angiogenesis through; i) adsorption of VEGF to Laponite gel surfaces and ii) incorporation of VEGF within Laponite gels.

METHODS: 3% Laponite hydrogels containing fibronectin was used to coat the wells of a 48-well plate. Aqueous $rhVEGF_{165}$ (0.04 µg/ml) was added to Laponite hydrogel surfaces and incubated for 2 hours at 37°C. Aqueous $rhVEGF_{165}$ was then removed and Laponite surfaces washed with PBS (adsorbed VEGF gels). Separately, $rhVEGF_{165}$ was pre-mixed (0.04-5.00 µg/ml) with Laponite hydrogels prior to well coating (incorporated VEGF gels). In addition, Laponite hydrogel surfaces absent from VEGF treatment were used as control surfaces. Plain growth medium was added to all Laponite-VEGF surfaces and to half of the control surfaces (negative control); all remaining control surfaces were treated with growth medium mixed with $rhVEGF_{165}$ (0.04 µg/ml) (positive control). Human umbilical vein endothelial cells (HUVECs) were seeded at 2.5 x 10^4 cells/well on all Laponite surfaces and incubated at 37°C for 18 hours. Microscope images were taken and tubule networks quantified by image analysis.

RESULTS: VEGF-mediated stimulation of HUVEC tubules was successfully established and identified on Laponite hydrogel surfaces (*Fig. 1*). It was shown that adsorbed VEGF gels significantly increased the formation of tubules (p = <0.001). HUVEC tubule formation was also significantly increased when cultured on incorporated VEGF gels containing 1.00-5.00 µg/ml VEGF (*Fig. 2*, p = <0.01 to <0.0001).

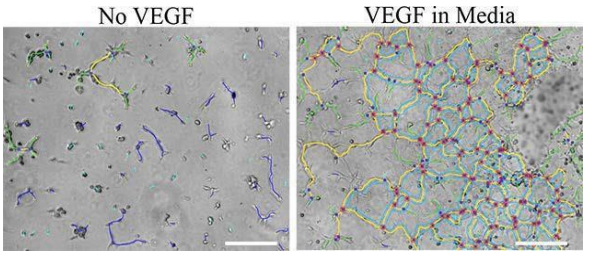

Fig. 1. VEGF-mediated stimulation of HUVEC tubules on Laponite hydrogels. Left: Control; Right: 0.04 µg/ml VEGF in media. Scale=500 µM.

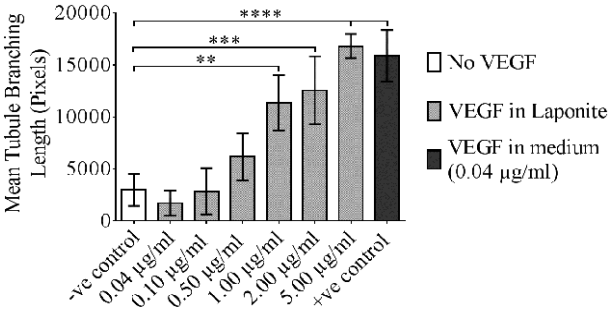

*Fig.2. Quantification of HUVEC tubules at different VEGF-Laponite conditions. **, ***, **** denotes p = <0.01, <0.001 & <0.0001 respectively.*

DISCUSSION & CONCLUSIONS: We have shown the potential for Laponite hydrogels in retaining biologically active VEGF to promote angiogenesis *in vitro*. We also established that >1 µg/ml VEGF was required to elicit a significant biological response when pre-mixed within 3% Laponite hydrogels. The use of these clays could have significant clinical potential to localise proangiogenic VEGF to chronic wounds. This would require further study using a wound healing model in healthy and diabetic mice.

ACKNOWLEDGEMENTS: Thanks to Tizard, Grundy Educational Trust, Novo Nordisk and the EPSRC for providing funding for this project.

Collagen-based scaffold as a new tool for 3D neural stem cell culture

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INTRODUCTION: The promising potential of Neural Stem Cells (NSCs) in tissue engineering applications is due to their high regenerative capacity in promoting functional recovery in the central nervous system diseases and injuries. Despite their tremendous potential, current methods to culture NSCs are limited. In fact adherent 2D cultures are greatly simplified versus the *in vivo* microenvironment. A porous collagen-based scaffold was here investigated as an innovative tool in 3D NSC culture.

METHODS: The polymeric scaffolds (3.00 mm high, 4.00 mm diameter) were developed starting from type I collagen by supra-molecularly assembling process performed in biomimetic conditions upon pH variation. The performances of the final 3D scaffolds in wet condition were modulated performing a cross-linking process using 1,4-butanediol diglycidyl ether (BDDGE) (Figure 1A). NSCs were isolated from the subventricular zone (SVZ) of 8-week-old male C57BL/6 mice, expanded by mechanical dissociation in vitro and cultured in NeuroCult basal medium with NeuroCult proliferation supplements (Stem Cell Technologies), heparin 0.002%, bFGF 10 ng/ml, EGF 20 ng/ml and PenStrep [l]. Scaffolds were placed one per well in a 24-well plate and a drop of 35 µl containing 8.0×10^4 NSCs was seeded on the centre of the upper sample surface allowing cell attachment for 20 min in the incubator, before adding into each well 1 ml of differentiation medium (NeuroCult basal medium supplemented with NeuroCult differentiation supplements, Stem Cell Technologies). A standard differentiation protocol on matrigel coating was used as control. 7 days after cell culture, cell viability was assessed with Live&Dead assay. Immunofluorescence and Western blot were performed.

RESULTS: 3D and 2D cell cultures were analysed for cell viability with the Live/Dead assay based on the simultaneous determination of live and dead cells with two probes. A very high ratio of viable cells was seen with no significant differences among 3D and 2D (Figure 1B, C). The microtubule-associated protein 2 (MAP2) family proteins is an abundant group of cytoskeletal components, which are predominantly expressed in neurons. Positive results for MAP2 were found in 3D cell culture both in immunofluorescence and western blot analysis (Figure 1D, E).

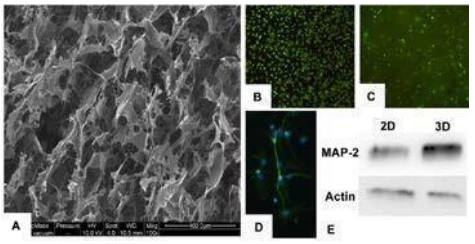

Fig. 1: A SEM Image of the 3D scaffold. B,C Live&Dead assay of 2D and 3D respectively (live cells in green, dead cells in red). D Immunofluorescence image of 3D NSC culture (MAP2 in green, DAPI in blue). E Western blot bands.

DISCUSSION & CONCLUSIONS: In contrast to traditional 2D culturing systems, in which cells typically grow as monolayer sheets adhered to a matrix, 3D cultures create an environment that enables cells to contact and interact in all dimensions with the scaffold and other cells in culture. This preliminary study showed the great potential of collagen-based scaffold for 3D neural stem cell culture. After 7 days of culture NSCs colonized the whole scaffold, and a marker of neuronal differentiation was well expressed. The establishment of 3D NSC culture model is essential for a prediction of cell behavior in a more physiological condition, resulting in a great advancement in central nervous system diseases and injury studies.

ACKNOWLEDGEMENTS:
This work has received support from the Italian Multiple Sclerosis Association, the Italian Ministry of Health and the ERC.

Fabrication of duck's feet collagen-silk hybrid biomaterial for tissue engineering

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INTRODUCTION: We extracted collagen from duck's feet for application in scaffold biomaterial. Also, we fabricated duck's feet collagen/silk hybrid scaffold for the purpose of modifying the degradation rate of duck's feet collagen.

METHODS: A simple method using acid had been employed to extract collagen from duck's feet. Duck's feet collagen (DFC) and duck's feet collagen/silk (DFC/S) hybrid scaffolds were prepared using freeze drying method.

RESULTS:

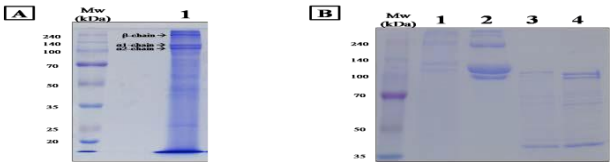

Fig. 1: A)SDS-PAGE of DFC (line 1) shows that DFC contained two main α-chains (α1 and α2) and one β- chain. B) Collagenase reaction of duck's feet and porcine collagen for 3 hours did not showed collagen type I band. Lane 1:DFC, 2: porcine collagen (PC), 3:DFC+collagenase, 4: PC+collagenase.

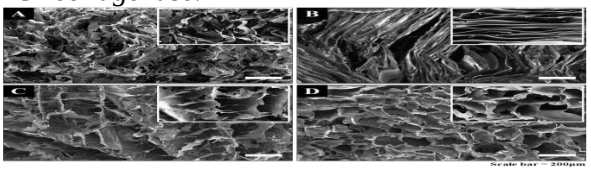

Fig. 2 The cross-sectional SEM images of the scaffolds.(A)PC, (B)S, (C) DFC, (D)DFC/S. The distribution of pores shape showed more homogeneity in the DFC/S hybrid scaffold than other scaffolds.

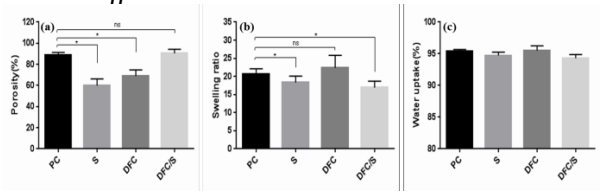

*Fig. 3(A) Porosity, (B) swelling ratio, and (C) water uptake of the scaffolds. Error bars represent mean ± SD for n = 3. *p< 0.05.*

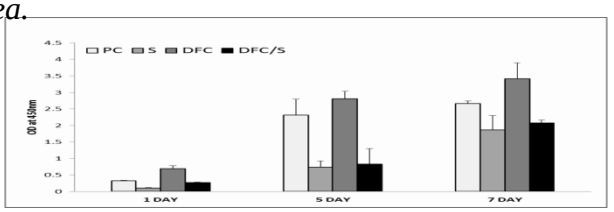

Fig. 4 Cell viability results for the scaffolds after culturingNIH3T3 fibroblasts. DFC represents high cell growth than the PC and S. DFC/S scaffold showed higher cell viability compared than that of silk scaffold.

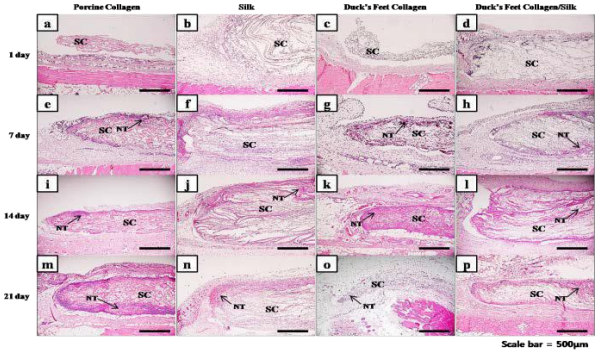

Fig. 5 H&E staining of cross sections of implanted scaffolds. The scale bar indicates 500 μm.

DISCUSSION & CONCLUSIONS: Extracted collagen from duck's feet is biocompatible and resembles collagen extracted from porcine which is commercially used. Addition of silk to fabricate a DFC/S hybrid scaffold could enhance the biostability of DFC scaffold. DFC/S scaffold increased the cell viability compared to silk alone. Animal studies also showed that DFC/S scaffold was more biocompatible than silk alone and more biostable than duck's feet or porcine collagen alone. Additionally, DFC/S hybrid scaffold had high porosity, cell infiltration andproliferation.

DISCLOSURES: Conflict of interest: none

Fabrication of fluorescent silk materials utilized in biotechnological and medical applications

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INTRODUCTION: Silk fibroin (SF) is a natural polymer widely used and studied for diverse applications in the biomedical field. Recently, genetically modified silks, particularly fluorescent SF fibers, were reported to have been produced from transgenic silkworms. However, they are currently limited to textile manufacturing. To expand the use of transgenic silkworms for biomedical applications, a solution form of fluorescent SF needed to be developed. Here, we describe a novel method of preparing a fluorescent SF solution and demonstrate long-term fluorescent function up to one year after subcutaneous insertion. We also show that fluorescent SF labeled p53 antibodies clearly identify HeLa cells, indicating the applicability of fluorescent SF to cancer detection and bio-imaging. Furthermore, we demonstrate the intraoperative use of fluorescent SF in an animal model to detect a small esophageal perforation (0.5 mm). This study suggests how fluorescent SF biomaterials can be applied in biotechnology and clinical medicine.

METHODS: Preparation of fluorescent silk fibroin solution. The fluorescent cocoons were heated overnight at 60 °C in an aqueous solution of 3% NaHCO3 with alcalase (1.5 ml/L) and then washed with distilled water several times to remove the glue-like sericin proteins. Subsequently, the extracted silk was dissolved in a 9.5 M LiBr solution with 1 mM DTT at 40 °C. Then, this solution was filtered through a miracloth and dialyzed with distilled water for 2 days to remove the salt. The final concentration of the aqueous silk fibroin solution was 4 wt. %. The SF solutions were stored at 4 °C before use to avoid premature precipitation or freeze-dried to obtain regenerated silk fibroin.

RESULTS: We can produce fluorescent SF solution as film, gel, membrane, powder and porous sponge forms. In addition, the fluorescent silk fibroin that results from the transgenic cocoons can be modified by conjugating it to other proteins such as antibodies, enzymes, or tumor markers. Therefore, fluorescent SF can be applied as a bioimaging tool or a controlled release drug targeting system in clinical medicine.

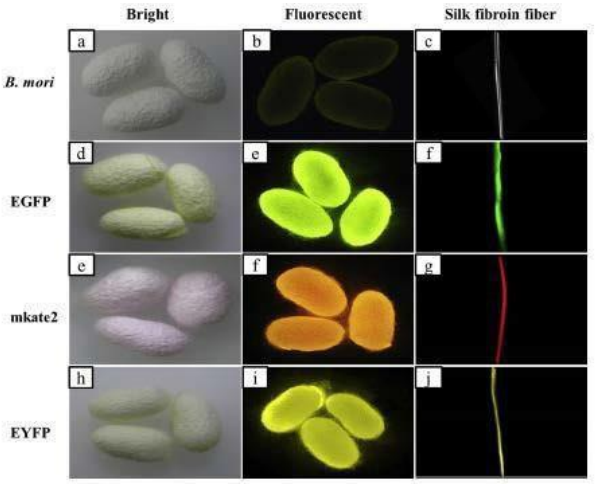

Fig. 1: Multicolored fluorescent cocoons and silk fibroin fibers.

DISCUSSION & CONCLUSIONS: There are numerous potential clinical applications for fluorescent SF. We demonstrate the visual inspection of esophageal perforation using EGFP fluorescent SF solution to image the surgical field intraoperatively in an animal model. Using this as a prototype, fluorescent SF solution can shift the paradigm of surgical inspection by enabling localization of a variety GI fistula lesions.

ACKNOWLEDGEMENTS: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (NRF-2015R1A4A1041631), Republic of Korea..

Physical changes during *in vitro* degradation of biodegradable PGLA woven band for application fecal diverting device

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INTRODUCTION: Anastomotic leakage is the most serious complication of bowel surgery, especially low rectal anastomosis. Various factors contribute to anastomotic leakage. Residual fecal matter might be a critical cause of anastomotic leakage and that preoperative bowel preparation can reduce the incidence of anastomotic leakage. The fecal diverting device (FDD) was fabricated for fecal diversion from the proximal colon above the anastomosis to outside the anus for protecting the rectal anastomosis. The goal of this study was to develop a biodegradable poly (glycolic-*co*-lactic acid) (PGLA) band that enable to easily removed from the body using weaving technology for FDD and then investigate its biodegradation behavior *in vitro*.

METHODS: USP 3/0 PGLA braided multi-filament was used as warp-thread and USP 4/0 was used as weft-thread without dyeing. PGLA band was weaved plain using narrow fabric weaving loom and the width of band was 9 ± 1 mm. Biodegradation behaviour of PGLA band in simulated intestinal fluid pH 6.8 [USP 26, phosphate-buffered saline (PBS)] was observed by incubation in an electrical thermostatic oscillation tank at 37 ± 0.5 °C for 8 weeks. The PBS with a volume at 100 mL was put into each bottle and maintained at 37 ± 0.5 °C and was changed with new solution weekly to keep pH at 6.8 ± 0.2. Eight samples were tested for getting each experimental data point.

RESULTS: The surface morphologies of PGLA band samples degraded in PBS solution were monitored at 0 to 8 weeks. Figure 1 shows that the surface of PGLA band at 7 weeks is more degraded than 3 weeks. As shown in table 1, tensile strength of PGLA bands decreased rapidly from 3 to 6 weeks. During the first 3 weeks of incubation, the weight of PGLA bands changed slowly, which decreased gradually after degraded for 4 weeks.

DISCUSSION & CONCLUSIONS: The *in vitro* degradation behavior of biodegradable PGLA band was investigated in this study. During the 8 weeks of degradation the tensile strength and weight of PGLA band were decreased.

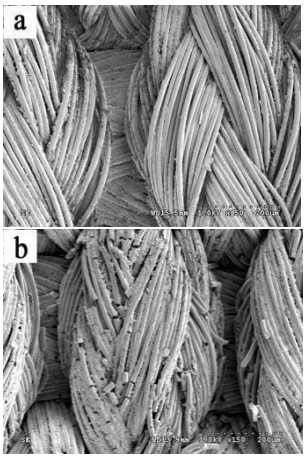

Fig. 1: The morphology changes of PGLA bands in vitro degradation: where (a), (b) photographs are bands degraded at 3 and 7 weeks.

Table 1. Tensile strength retentions and weight loss comparison of bands in vitro degradation.

Weeks	Tensile strength retention (%)	Weight loss (%)
1	100.0	0.1
2	99.5	0.2
3	61.0	0.3
4	27.5	4.6
5	10.3	9.3
6	1.7	22.2
7	0.6	25.1
8	0.2	29.5

ACKNOWLEDGEMENTS: This research was financially supported by the "Advanced medical new material (fiber) development program" through the Ministry of Trade, Industry & Energy (MOTIE) and Korea Institute for Advancement of Technology (KIAT) (R0003394).

Osteogenic differentiation of human dental pulp stem cells using in an injectable *in vivo*-forming hydrogel

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INTRODUCTION: Recently, human dental pulp stem cells (hDPSCs) have drawn attention as a promising source of adult stem cells s which is the subject of considerable research. Among several scaffolds, injectable in vivo-forming hydrogels appear to be a promising scaffold, because they can easily incorporate various cells and biological factors such as growth factors simply by mixing and then can generate complicated scaffold geometries in vivo. In this study, hDPSCs were examined as a source for bone tissue engineering using an in vivo-forming hydrogel [1].

METHODS: Fresh hDPSCs were isolated from third molar teeth that had been extracted from healthy. Osteogenic induction of hDPSCs was confirmed by Alizarin Red S (ARS), von Kossa (VK), and alkaline phosphatase (ALP) staining at 1, 2, 3, and 4 weeks. Methoxy polyethylene glycol-polycaprolactone block copolymer (MC) diblock copolymer (750–2400 g/mol) was prepared as reported previously [2]. The MC solutions for in vitro osteogenic differentiation were prepared by mixing the appropriate components of hDPSCs and osteogenic factors. The solution of hDPSCs-loaded MC hydrogel either without or with OF was subcutaneously injected into rats. Differentiated osteoblasts of hDPSCs-loaded MC hydrogel were identified by ARS and VK staining and were found to exhibit characteristic expression of genes like osteonectin, osteopontin, and osteocalcin at different time points.

RESULTS: The hDPSCs are easily isolated from extracted teeth that are discarded during dental surgery. The stemness of harvested hDPSCs indicated their relative tolerance during in vitro culture. The solution of hDPSCs and a MC block copolymer was easily prepared and formed in vivo hydrogels after animal injection. The evidence of in vivo osteogenic differentiation of hDPSCs embedded in an injectable in vivo-forming hydrogel was confirmed by micro-CT, histological staining, and gene expression. Differentiated osteoblasts in in vivo-forming hydrogels were also identified by ARS and VK staining and were found to exhibit characteristic expression of genes like osteonectin, osteopontin, and osteocalcin at different time points.

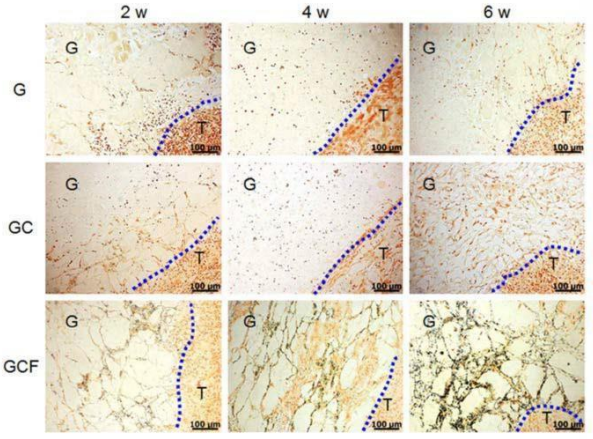

Fig. 1: Von Kossa-stained histological sections removed from rats receiving G, GC, or GCF after 2, 4, and 6 weeks (magnification 200×, scale bars represent 100 μm).

DISCUSSION & CONCLUSIONS: The hDPSCs are easily harvested as a simple and applicable stem cell source for osteogenic differentiation. In particular, hDPSCs in in vivo-forming MC hydrogel with OF can successfully differentiate into osteogenic cells. We emphasized that hDPSCs embedded in an in vivo-forming PC hydrogel may provide benefits as a non-invasive formulation for bone tissue engineering applications.

ACKNOWLEDGEMENTS: This study was supported by a grant from a Basic Science Research Program (2013R1A2A2A04014200 and 2014M3A9E5073700) and Priority Research Centers Program (2010-0028294) through the National Research Foundation of Korea (NRF) funded by the Ministry of Education..

Fabrication of tissue engineered intervertebral disc using enable 3D plotting and scaffold-free technologies

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INTRODUCTION: Intervertebral disc (IVD) mainly consists of Annulus fibrosus (AF) and nucleus pulposus (NP), playing a role of distributing a mechanical load on vertebral body[1]. The goal for regenerating disk tissue is to achieve anatomic morphology and restoration of biological function. Toward this goal, we have previously used lamellar silk scaffolds for AF tissue regeneration and have shown that silk-fibrin/HA composite gels can serve as chondrocyte delivery biomaterials. In the present work, the goal was to design a biphasic scaffold system to support both AF and NP morphologies and cells. We, therefore, utilized a 3D printing system using natural polymer for the AF mimicked structure and a scaffold-free system using NP cells for the engineered NP tissue. We determined whether the biphasic structure, consisting of 3D plotted scaffolds (to mimic the AF) and scaffold-free construct (to mimic the NP), could support the architecture and cell functions of IVD tissue.

METHODS: In this study, decellularized extracellular matrix powder from porcine cartilage and type 1 collagen composite bioink utilized for AF tissue 3D plotting. Chondrocytes were isolated from new-zealand white young (2weeks) rabbit's femurs. Twice passaged $8x10^6$ chondrocytes were seed on AF Scaffold and cultured for 2 weeks. Scaffold-free construct for NP tissue was fabricate by high-density cell culture method. Briefly, the cell membranes fabricated by high cell density culture were centrifuged in 50ml tube at 2000G for 20 mins. After 2 week *in vitro* culture, IVD were fabricated by inserting scaffold-free construct to AF mimicked scaffold's inner site and cultured for 2 more weeks.

RESULTS: To mimic the AF structure, 60° stripe pattern were plotted on the round stick. The prepared 3D plotted scaffold appeared the 5mm inner-diameter and 10mm outer-diameter. Live/dead staining and SEM analysis proved the good cell viability and response on the 3D plotted AF scaffold.

The histological staining showed the newly synthesized type I collagens and glycos-aminoglycans (GAG), charactering the AF tissue. Scaffold-free construct also actively synthesized GAG and type II collagen, the main components of NP. After combine the two types of engineered tissues, The IVD mimicked biphasic tissue showed good integration.

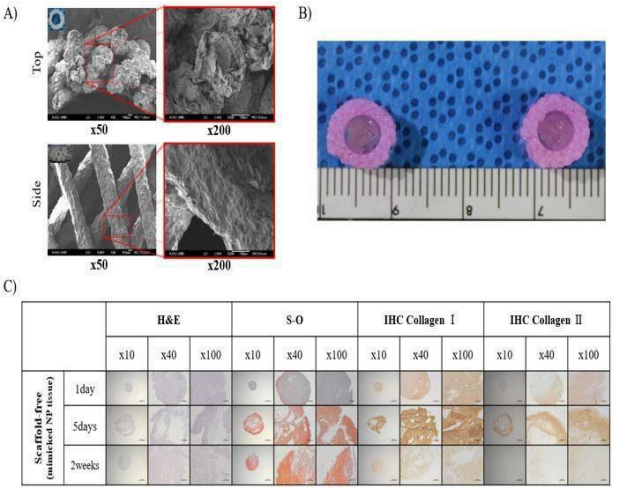

Fig. 1: A) SEM images of 3D plotted AF scaffold, B) Gross image of mimicking IVD, C) Histology of NP scaffold free construct.

DISCUSSION & CONCLUSIONS: This study describes the fabrication of a biphasic IVD structure by using enable techniques of tissue engineering. This fabricated biphasic construct would be used as a model system for the study of the native IVD tissue. In the future, it may have the potential to replace the damaged IVD in the future.

ACKNOWLEDGEMENTS: This research is supported by the NRF-2013R1A1A1011040 project and Korea Health Technology R&D Project (HI14C1632) by Ministry of Health and Welfare.

Manufacture of SLM-enabled electrospun synthetic niches for regenerative medicine

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INTRODUCTION: Stem cells have the ability to self-renew almost indefinitely, presenting a significant opportunity in regenerative medicine. If a synthetic niche environment could be prepared that maintained a mesenchymal stromal cell population, this would have potential in the manufacture of medical devices or scaffolds that could enhance local bone tissue regeneration. The behaviour of stem cells is highly complex, but it is influenced - at least in part - by their defined 3D environments or so-called niches. These play a key role in maintaining stemness whilst directing differentiation when required. This research aims to fabricate and study artificial stem cell niches to ultimately deliver innovative biomaterial devices with the ability of directing or controling aspects of stem cell behaviour. Previous work from the group reported the design and manufacture of artificial niche-equipped membranes for corneal healing[1]. The aim of this research is to translate this knowledge to the area of musculoskeletal tissue regeneration.

METHODS: Patterned stainless steel collectors were design via CAD software and manufactured using 3D printing techniques (selective laser melting, SLM). The collectors were electrospun onto to produce biodegradable scaffolds imprinted with the negative pattern. SEM was used to obtain images of the scaffold and the artificial stem cell niches. Primary rat Mesenchymal stromal cells (MSC) were obtained via cell extraction from the tibias of 4 Whister rats. MSC cells were seeded onto the electrospun scaffolds for 7 days. Presto-blue was used to determine cellular viability at days 1, 4 and 7. Confocal imaging of FITC phalloidin and DAPI stained samples were obtained in a z-ztack database in 10 µm slices at x20 magnification 0.3 NA. Samples were prepared for scanning electron microscopy (SEM) using Hexamethyldisilazane and imaged at multiple magnifications. Samples were fixed, embedded in cryo-medium and sectioned on a cryotome. Sectioned scaffolds were stained using H&E and imaged on an optical microscope.

RESULTS: Well-defined artifical 3D niche environments were successfully incorporated into electrospun scaffolds using template technology. Niches of different sizes were observed using SEM, and these matched the size and depth predicted by the original collector designs. PrestoBlue indicated an increase in cellular metabolic activity over 7 days. Confocal z-stacks enabled the imaging of 3D cell and extracellular matrix conformations within the niche. Moreover, MSCs appeared to have fully infiltrated the synthetic niches.

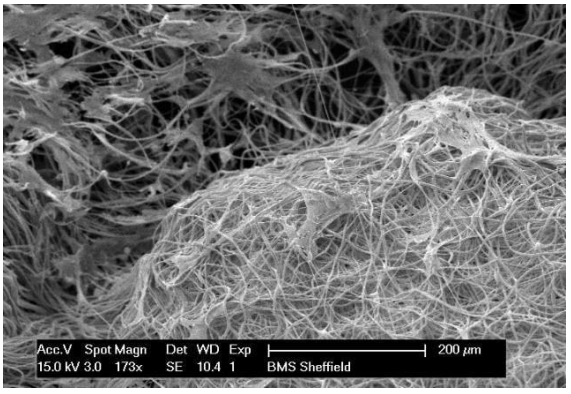

Fig. 1: SEM photomicrograph of rat MSCs within an artificial stem cell niche made from electrospun fibres following 5 days of culture.

DISCUSSION & CONCLUSIONS: The inclusion of additive manufacturing as part of the fabrication process allows the creation of intricate structures that can be designed to mimic certain aspects of the native stem cell niche. MSCs were identified in the niches after culture and cell proliferation was observed over 7 days. The production of these electrospun versatile microfabricated tools offer great potential in regenerative medicine, not only in the area of musculoskeletal regeneration as exemplified here, but in other fields including brain and skin in which several groups at Sheffield are engaged in pioneering research.

ACKNOWLEDGEMENTS: We acknowledge the Women Academic Returners' Programme (WARP) for funding part of this work and MeDe Innovation (the UK EPSRC Centre for Innovative Manufacturing in Medical Devices, grant number EP/K029592/1).

An allogeneic bioengineered myocardial graft limits infarct size and improves cardiac function: pre-clinical study in the porcine myocardial infarction model

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INTRODUCTION: Cardiac tissue engineering combines scaffolds and cells, aiming to renew myocardium and restore cardiac function after myocardial infarction (MI). In this context, decellularized myocardial extracellular matrix provides a physiological cardiac ultrastructure, mechanical properties and local matrix milieu, resembling native myocardium, which drives cell attachment, survival, proliferation, migration and differentiation [1-4].

Herein, we developed a scalable bioengineered myocardial graft (EMG) and evaluated its impact on cardiac function and scar dynamics in the swine MI model.

METHODS: EMG, comprising a decellularized porcine myocardial scaffold repopulated with GFP-labeled porcine adipose tissue-derived progenitor cells (pATDPCs), was engrafted to the myocardial surface of allogeneic wild-type swine after MI induction. Animals were sacrificed one month post-MI. Cardiac function was assessed using magnetic resonance imaging. Infarct size, scar healing, and vascularization were evaluated using quantitative morphometry and histopathology.

RESULTS: Compared to control-MI animals, EMG-treated animals exhibited significantly higher left ventricular ejection fraction (LVEF) at sacrifice relative to baseline (3.3±2.9 vs. −8.0±4.3; P=0.047), and significant improvement from 48 h post-MI to 1 month (50.8±2.6% vs. 61.3±3.1%; P=0.001). Relative to control-MI animals, EMG-treated animals had a 68% smaller infarct size (2.5±0.6% vs. 7.6±2.3%; P=0.048) and significantly lower type I/III collagen ratio (2.3±0.7 vs. 13.0±6.9; P=0.032) than control-MI. GFP+ pATDPCs migrated to underlying ischemic myocardium and expressed cardiac lineage markers (GATA4, MEF2, SERCA2, connexin43, and cardiac troponin I) and endothelial makers (IsoB4 and CD31). Finally, EMG prompted increased infarct myocardium vascularization, and newly formed functional blood vessels connecting EMG to host myocardium.

DISCUSSION & CONCLUSIONS: In the pre-clinical swine MI model, the newly developed EMG improved cardiac function and injured myocardium neovascularization, reduced infarct size, and limited fibrosis progression. This scalable allogeneic EMG is ready for clinical translation.

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ACKNOWLEDGEMENTS: This work has been funded by grants from the Ministerio de Ciencia e Innovación (SAF2014-59892-R); Proyectos de investigación en salud (AES 2014; PI14/0168); Redes Temáticas de investigación Cooperativa en Salud (RETICS): Red Cardiovascular RD12/0042/0047 y Red de Terapia Celular - TerCel RD12/0019/0029 as part of the Plan Nacional de I+D+I and cofunded by ISCIII-Sudirección General de Evaluación y el Fondo Europeo de Desarrollo Regional (FEDER); Fundació la Marató de TV3 (122232 and 201502 30 31); Beca de Recerca Bàsicade l'Acadèmiade Ciències Mèdiques i de la Salut de Catalunya i de Balears 2015; Beca d'Investigació Bàsica de la Societat Catalana de Cardiologia 2015; Generalitat de Catalunya (SGR 2014); and Sociedad Española de Cardiología.

DISCLOSURES: The authors have nothing to disclose

Engineering a capillary bed model for dermal bioavailability measurement for refinement of human health risk assessments

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INTRODUCTION: Skin penetration studies *in vitro* are used to estimate bioavailability via the dermal route. However, current skin penetration models *in vitro* do not incorporate any measure of capillary bed function, and as such do not reproduce physiological conditions: They do not consider the resistance to flow posed by the blood vessels, and the position of the skin vasculature *in vivo* is much closer to the skin surface than that achieved *in vitro*. We propose a novel strategy to mimic skin vascularisation using hollow fibres fabricated with biocompatible materials.

METHODS: Hollow fibres were fabricated by wet spinning as described elsewhere [1]. Casting solutions were formulated using various *PolymerX* (*PX*) fibres (a new membrane material developed in- house) with different levels of porosity and surface polarity (*Fig. 1*). *PX* Micro-hollow fibres were also fabricated using a syringe needle [2] (*Fig. 2*) in order to be threaded through the dermoepidermal junction. Initially, a novel skin penetration flow cell was designed, including four hollow fibres of approximately 1 cm length underneath dermatomed human skin from three different donors. Permeation of radiolabelled [1-methyl-^{14}C] Caffeine and [Ring-U-^{14}C]-2,4-Dinitrochlorobenzene (DNCB) were tested (doses of approximately 32 µg cm^{-2}, and 494 µg cm^{-2} respectively) and analysed via liquid scintillation counting.

RESULTS: *Fig. 1* and *Fig. 2* show SEM micrographs of a *PX* hollow fibre and a *PX* micro-hollow fibre respectively. The porous structure can be seen to have a desirable asymmetric morphology with interconnected pores. The permeation rates of both caffeine and DNCB observed through the porous outer surface of the fibres into their lumen demonstrate the suitability of the membranes for the design of a capillary bed bioreactor for skin pseudo-vascularisation. Consequently, the fluxes in the skin penetration cells with and without hollow fibres were determined to elucidate the effect of this pseudo-vascularisation. The fluxes were compared to confirm the fact that caffeine, for example, permeates faster *in vivo* than *in vitro* according to the literature [3].

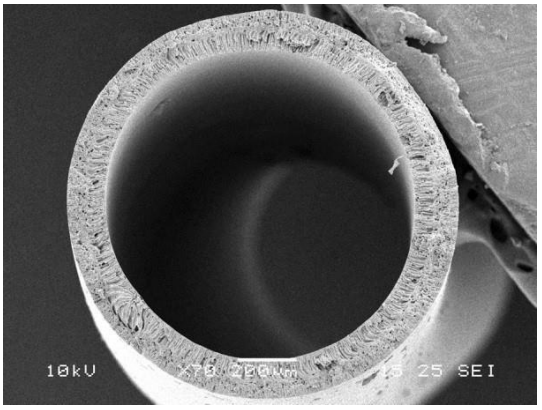

Fig. 1: SEM micrograph of PX hollow fibre (Magnification X70).

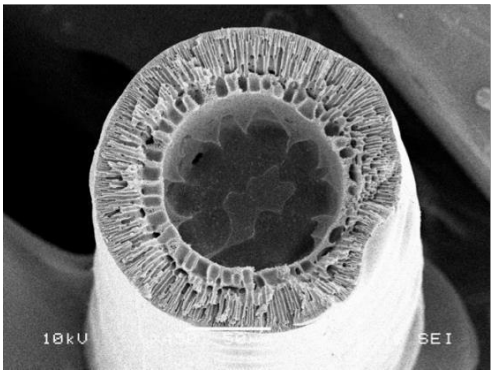

Fig. 2: SEM micrograph of PX micro-hollow fibre (Magnification X450).

DISCUSSION & CONCLUSIONS: Caffeine and DNCB permeate through the hollow fibres, demonstrating the suitability of the membranes for the design of a capillary bed bioreactor for skin pseudo-vascularisation. The next step in the design of a capillary bed bioreactor is the incorporation of the porous micro-hollow fibres (via microsurgical suture) into the epidermal-dermal junction.

ACKNOWLEDGEMENTS: We thank InnovateUK, NC3Rs and ESPRC for funding.

Functionalization of electrospun polyester fibers with calcium–binding bisphosphonate groups for reinforcement of calcium phosphate cements

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INTRODUCTION: Calcium phosphate cements (CPCs) are promising candidate materials for replacement and regeneration of hard tissue. In addition to their osteocompatibility, they can be injected and/or molded into bone defects. However, their brittleness prohibits their use in load-bearing sites. The use of polymeric fibers as reinforcing agents was shown to improve the mechanical properties of CPCs [1]. Currently, the efficacy of fiber reinforcement is still limited due to the hydrophobicity of commonly used fibers, resulting into poor adhesion between the fibers and the ceramic matrix. To improve this adhesion, bisphosphonates (BP) can be instrumental since they bind strongly to calcium ions in hydroxyapatite. Herein, we present a novel method to functionalize commonly used polymeric fibers with these calcium-binding BP groups.

METHODS: Sub-micron fibers were produced by electrospinning a 4 wt% solution of poly-L-lactic acid (PLLA) in hexafluoro-2-propanol at 20 kV and a flow rate of 5µL/min. By using aminolysis for different time periods [2], the fiber meshes were chemically fragmented and functionalized with amine groups. SEM was used to investigate the morphology of samples before and after the aminolysis procedure. The TNBS assay was used to quantitatively determine the amount of $-NH_2$ groups on the aminolyzed PLLA fibers.

Subsequently, we investigated if the aminolyzed polyester fibers could be further functionalized with BP groups through a Michael addition reaction. For this purpose, the Michael acceptor, acrylamidoalkyl bisphosphonate (AcrBP), was synthesized by acrylating alendronic acid using acryloyl chloride in aqueous NaOH [3]. Briefly, the reaction took place between the newly synthesized AcrBP and the $–NH_2$ groups present on the sub-micron fibers, in HEPES buffer at pH 7. The amount of covalently linked AcrBP was quantified by determining the phosphorous amount using ICP-OES.

RESULTS: SEM images revealed that continuous PLLA meshes were progressively cut into much shorter sub-micron fibers with increasing aminolysis time from 2 hours to 6 hours (Fig. 1).

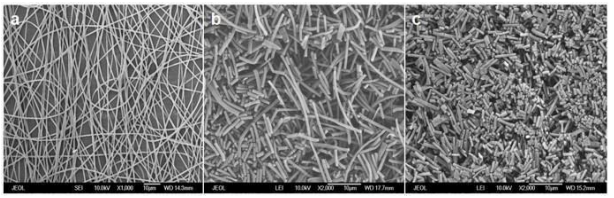

Fig. 1: SEM images of PLLA fibers for different aminolysis times: a. pristine electrospun mesh; b. 4 hours; c. 6 hours.

Following conjugation of AcrBP, the amount of BP could be tuned by controlling the duration of aminolysis time between 2h and 6h (Fig. 2).

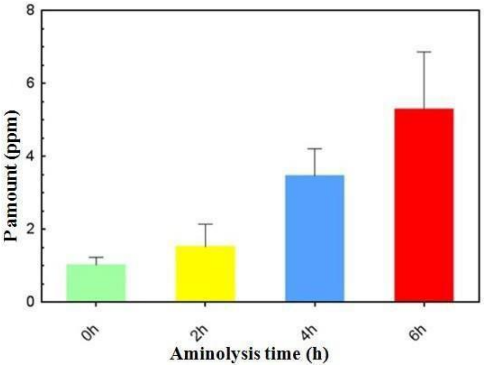

Fig. 2: Detection of phosphorous on BP fibers as a function of aminolysis time.

CONCLUSIONS: PLLA sub-micron fibers were successfully functionalized with calcium-binding BP groups by aminolysis of electrospun fiber meshes and subsequent Michael addition reaction between AcrBP and aminolyzed PLLA fibers.

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ACKNOWLEDGEMENTS: Research supported by the Dutch Technology Foundation STW.

Hierarchical ionic-doped scaffolds for osteochondral tissue engineering

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INTRODUCTION: The repair of osteochondral (OC) defects has been increasing owing the high number of trauma related injuries or osteoarthritis. Although current clinical options are effective for the treatment of the OC defects, new therapeutic options are necessary for the complete regeneration of the damaged articular cartilage[1]. Three-dimensional hierarchical scaffolds structured with several levels of organization can provide enhanced mechanical performance and allow suitable transduction of the mechanical stimuli to the cellular level. Hierarchical scaffolds composed of silk fibroin and calcium phosphates incorporating different ions (eg. Sr, Zn, Mn) for the repair/regeneration of cartilage and bone are herein developed. These scaffolds presented great bioresorbability and osteointegration, and high mechanical strength[2]. The presence of these ions in the scaffolds can stimulate osteogenesis and angiogenesis, and to tune not only the host healing response at the site of injury to facilitate the tissue repair, but also to mimic native OC tissue organization with the ultimate goal of achieving a fully integrated and functional engineered tissue[3].

METHODS: Ionic-doped calcium phosphate (CaP) powders were prepared by aqueous precipitation, followed by heat treatments at 800-1000 ºC and milling. Silk fibroin (SF) concentrated at 16 wt.% was extracted from *Bombyx mori* cocoons. Hierarchical scaffolds were produced through supercritical fluids processing.
XRD, FTIR, SEM and µ-CT techniques were employed to characterize the starting powders and scaffolds. The degradation behaviour and the mechanical strength of the scaffolds, after immersion in PBS, were also determined. *In vitro* cell viability and proliferation was assessed during 14 days. Osteogenic differentiation using human adipose-derived stem cells (hASCs) from fat pad were evaluated during 28 days of cell culture.

RESULTS: XRD patterns of the scaffolds showed the characteristic peaks of silk-II structure and β-tricalcium phosphate (TCP). The scaffolds presented macroporosity highly interconnected and microporosity with sizes around 500 µm, and 1-10 µm, respectively, with TCP powder well distributed
throughout the scaffolds. The degradation behaviour showed that the scaffolds SF/doped TCP exhibit less degradation when compared with the SF scaffolds alone. In the same manner, the measured mechanical strengths showed that the ionic-doped scaffolds exhibit higher strength than SF scaffolds within the range of values for cancellous bone (2-12 MPa). Zn-doped scaffolds improved proliferation, while Mn- and Sr-doped structures presented greater osteogenic potential. The combination of SrZn in the doped scaffolds led to an average influence on cell proliferation and osteogenesis when compared with single ions.

DISCUSSION & CONCLUSIONS: In the present study, hierarchical scaffolds incorporating different ions were successfully developed using a green technology, with promising applications in OC tissue engineering. The scaffolds presented a suitable pore size and porosity, and high interconnectivity suitable for promoting cell attachment and proliferation. The low degradation rate and improved mechanical properties can be controlled by the presence of doping ions. In vitro assays using hASCs presented different responses on cell proliferation/differentiation when varying the doping agents.

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ACKNOWLEDGEMENTS: The authors would like to thank to the financial support from the Portuguese Foundation for Science and Technology for the fellowship grants of S.Pina (SFRH/BPD/108763/2015), R.Canadas (SFRH/BD/92565/2013), and to the distinction attributed to J.M. Oliveira under the Investigator FCT program (IF/00423/2012).

Fish skin collagen for adipose stem cell cultures – biocompatibility evaluation

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INTRODUCTION: Fish collagen is a novel biomaterial for cell culture and scaffold construction with some advantages over conventionally used bovine or porcine collagens [1-3]. Collagen from skin of silvery carp *(Hypophtalmichtys Molitrix)* or African sharptooth catfish *(Clarias gariepinus)* was extracted, purified and biocompatibility tested against rat or human adipose stem cells (ASC). Cell proliferation potential and differentiation capability into osteo- and chondrogenetic lineages were evaluated and compared to conventional collagen (commercially available collagen-coated surfaces) or collagen-free cell culture system.

METHODS: Silvery carp or African sharptooth catfish skins were mechanically purified, washed and collagen was extracted with lactic acid. Resulting extract was filtered through polyester filters and lyophililized. Sterile collagen was used to cover bottom of 6-well culture plates in concentrations 0.001%, 0.002% or 0.01%. Collagen content on plastic surfaces was measured using semi-quantitative Sirius Red/Fast Green Collagen Staining Kit.. Commercially purchased bovine- or porcine collagen-coated plates, and uncoated culture plates were used as controls. Rat or human ASCs were enzymatically extracted from adipose tissue, in vitro cultured until 3rd passage and seeded on collagen or control plastic surface using cell concentrations od $3x10^3$/ml or $1x10^4$/ml. Cell numbers, morphology and doubling time on collagen and in control cultures were evaluated, as well as doubling time, CFU-F frequency and adipogenic or osteogenic differentiation, measured by Oil Red and Alizarin Red respectively. Differentiation potential of ASCs grown on collagen or control plastic surfaces were measured by colorimetric analysis of stain intensity.

RESULTS: **A**dhesiveness, viability and morphology of ASCs cultured on collagen surfaces did not differ significantly when compared to control cultures, as well as the frequencies of CFU-F colonies. Both human and rat ASCs expressed significantly better proliferative potential when cultured on fish collagen in comparison with commercially available bovine, porcine or rat tail collagen - coated plates. There was no significant difference of all parameters tested when compared data from cultures on surfaces coated with collagen from silvery carp skin or African sharptooth catfish skin collagen. Adipogenic differentiation of human or rat ASCs on fish collagen did not differ significantly from controls, osteogenic differentiation was best supported by fish collagen in rat ASC cultures, whereas human ASCs showed no difference in osteogenic capability on collagen-coated plastics when compared to control plastic surfaces. In general, cell growth, proliferation and differentiation were optimal when ASCs were cultured on 0.002% fish skin collagen, and slightly inferior on 0.001% or 0.01% collagen-coated plastics.

DISCUSSION & CONCLUSIONS: Recently, bovine or porcine collagen is used for the construction of scaffolds ("empty" or colonized by stem or progenitor cells) for clinical implantations for regenerative medicine purposes. Our data suggest the superiority of fish skin collagen over collagens extracted from "conventional" sources like bovine, porcine or rat tissues. These advantages consist of better growth and differentiation rate of ASCs on fish collagen when compared to mammalian collagens. Other advantage is overcoming the risk of zoonoses like bovine spongiform encephalopathy, which are substantial limitation when considering the clinical applications. It may be concluded that collagen extracted from fish skin is a novel biomaterial important not only for in vitro applications, but also for regenerative medicine purposes (scaffold construction).

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ACKNOWLEDGMENTS: Study was supported by the National Center for Research and Development (STRATEGMED 1/233224/10/NCBR/2014, project START).

Novel three-dimensional hydrogel model to study extracellular matrix remodelling of aortic valves related to the development of aortic stenosis

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INTRODUCTION: Aortic valve stenosis is the most common valve disease in the western world and the final stage of calcific aortic valve disease (CAVD). It is well known that trans-differentiation of valvular interstitial cells (VICs) induced by pathophysiological stimuli plays an important role in this disease. Nevertheless, most biochemical studies are conducted using classical two-dimensional (2D) cell culture techniques missing the complex network and signalling of extracellular matrix proteins[1,2,3]. The aim of this study was to establish a novel, innovative hydrogel of aortic valve extracellular matrix (ECM) for three-dimenstional (3D) cultivation with high similarity to in vivo conditions to reveal new aspects about CAVD.

METHODS: Ovine aortic valves were decellularized by a detergents based protocol using sodium dodecyl sulphate, deoxycholate acid and sodium azide. Afterwards, aortic valve ECM was lyophilized and mechanically homogenized in a pepsin/HCL solution. After adjusting for pH, the resulting hydrogel was mixed with collagen type I and VICs and incubated for one hour at 37°C. Subsequently culture medium was added (DMEM with 0.5% FCS). The resulting 3D cultures were cultivated 7 days and treated with either control medium, calcification medium (containing 1.5mM calcium chloride ($CaCl_2$) in combination with 10mM β-glycerol phosphate (βGP) or 5ng/ml transforming growth factor β (TGFβ). Proliferation and ECM remodelling were analysed by haematoxylin eosin (HE) and Movat pentachrome staining, respectively. Identical cell culture conditions were used for classical 2D VIC cultures on plastic dishes with or without 1% gelatine coating. Medium supernatants were collected on day 7. Matrix metalloproteinase 2 (MMP2) activity was determined using gel zymography. Collagen content was assessed by in gel fluorescence staining.

RESULTS: After evaluating different homogenization techniques, combining lyophilisation, mechanic homogenisation and enzymatic digestion proved to be the most effective protocol to obtain a homogenous gel. The stability of this homogenate could be further supported by adding collagen type I, the most abundant ECM protein of the native aortic valve. TGFβ enhanced proliferation of VICs within the hydrogel and induced the formation of a prominent cell layer on the peripheral area of the hydrogel compared to control conditions. Movat pentachrome staining underlined the described effects on ECM remodeling.

In 2D experiments, VICs cultured on cell culture plastic showed minor effects on MMP2 activity and collagen accumulation after treatment with calcification medium. In contrast, when the cells were cultured on gelatine coating, calcification medium significantly upregulated MMP2 activity (1.8 fold; n=3) and decreased collagen accumulation (0.3 fold; n=3). On the other hand, TGFβ had more prominent effects on cells cultured on plastic instead on gelatine coating. TGFβ decreased MMP2 activity and enhanced collagen accumulation (4.2 fold compared to control, n=3;) only in VICs cultured on plastic.

DISCUSSION & CONCLUSIONS: These results underline the tremendous effect of ECM proteins on VIC signalling. In 2D experiments we could show that the effect of pathophysiological stimuli depends on surface treatment. The established hydrogel of decellularised aortic valves is an innovative approach to study the role of VIC signalling *in vitro* and to achieve knowledge on the pathomechanisms of CAVD more in detail.

ACKNOWLEDGEMENTS: We would like to acknowledge the technical support of Gisela Müller and Milan Lukic.

Evaluation of the potential of fucoidan-based microparticles for diabetes treatment

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INTRODUCTION: Marine organisms have in their constitution materials with a wide range of properties and characteristics inspiring their application within the biomedical field. One important example is fucoidan (Fu), an underexploited sulfated polysaccharide extracted from the cell wall of the brown seaweeds, with high solubility in water[1]. Fucoidan is composed of L-fucose and glucuronic acid including sulfate groups and has important bioactive properties such as antioxidative, anticoagulant, anticancer and in the reduction of blood glucose[1,2]. In this work, the biomedical potential of fucoidan was assessed by processing modified fucoidan (MFu) into microparticles by photocrosslinking using superhydrophobic surfaces and visible light[3,4]. Biological performance on the developed constructs using human pancreatic beta cells is currently under investigation.

METHODS: To design the materials structures, fucoidan was modified by methacrylation reaction[3]. Briefly, Fu aqueous solution 4% w/v was mixed with methacrylated anhydride (MA) in volume of 12% v/v at 50ºC to react for 6h. Further, MFu particles with and without insulin (0.5% w/v) were produced by pipetting a solution of 5% MFu v/v with triethanolamine and eosin-y (photoinitiators) onto superhydrophobic surfaces[4] (Fig. 1A) and then photocrosslinking using visible light[4]. MFu and developed particles were characterized using ^{1}HNMR, turbidimetry and SEM to assess their chemistry and morphology, respectively. Moreover, the insulin release was evaluated in phosphate buffered saline (PBS) solution at pH 7and simulated intestinal fluid (SIF) at pH 5. The ability of the developed materials to support adhesion and proliferation of cells was assessed by suspension culture of human pancreatic cells 1.1B4 ($3.5x10^5$ cells/ml) in contact with MFu microparticles during up to 7days.

RESULTS: The chemical modification performed on Fu was confirmed by the presence of vinyl and additional methyl peaks in the ^{1}HNMR of modified fucoidan, not present in Fu spectrum. Methacrylated fucoidan was obtained with a methacrylation degree of 17%. The produced fucoidan particles have round shape and average diameter of 1.53 mm (Fig. 1B). The insulin release in PBS and SIF demonstrate that the particles can release insulin in a sustained manner under the studied period. It seems that the insulin release is slower for SIF (pH5, Fig. 1C), than for PBS. The biological tests regarding the culture of pancreatic beta cells demonstrate that cells show a round-like shape and tend to form pseudo-islets during the culture period studied (Fig. 1D).

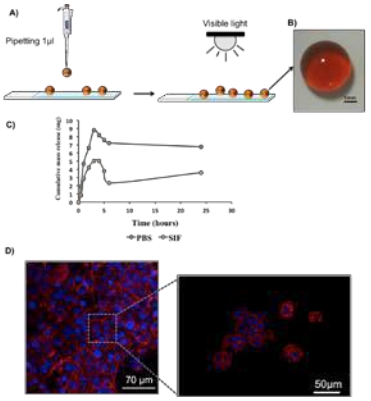

Fig. 1: A) Scheme of modified fucoidan particles production, B) modified fucoidan-particles, C) insulin release profile and D) confocal microscopy images of 1.1B4 cells seeded onto MFu after 7 days in culture; staining of actin (red) and nuclei (blue).

DISCUSSION & CONCLUSIONS: This work demonstrates the successful production of fucoidan-based-microparticles through the methacrylation of fucoidan, using visible light and superhydrophobic surfaces. The covalent crosslinking methacrylated fucoidan through visible light represents a promising method to obtain biocompatible fucoidan particles with a uniform round shape. The obtained insulin release profiles are sensitive to different pH (pH7 and pH5), mimicking the normal physiological pathway for insulin release. Furthermore, the results suggest these systems could be used for treatment of type I diabetes mellitus as they sustain beta cells viability and proliferation. The response also suggested, that the MFu particles could be a good candidate as drug delivery vehicles for the diabetes mellitustreatment.

ACKNOWLEDGEMENTS: This work was partially funded by projects 0687_NOVOMAR_1_P (POCTEP),CarbPol_u_Algae(EXPL/MARBIO/0165/2013), ComplexiTE(ERC-2012-ADG 20120216-321266). Portuguese Foundation for Science and Technology is also gratefully acknowledged for doctoral grants of L. Reys and N. Oliveira and post-doctoral grants of S.S. Silva and D. Soares da Costa.

The effect of two fatty acids on the antibacterial properties of calcium phosphate cements

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INTRODUCTION: Local delivery of antibiotics from acrylic bone cements has been frequently employed for prophylactic purposes or to treat postoperative infections. A recent *in vitro* study found that local delivery of fatty acids from acrylic bone cements could also give a certain inhibiting effect on bacterial growth [1], possibly presenting an alternative to some of the currently used antibiotics. However, any potential effect of their incorporation into resorbable and more biocompatible bone cements remains to be assessed. In this study, fatty acids were incorporated into brushite and hydroxyapatite cements with the aim of producing antibacterial calcium phosphate based bone cements.

METHODS: Brushite cements (BRU) were prepared by mixing 54wt% MCPM powder, 44wt% β-TCP powder and 2wt% sodium pyrophosphate. The powders were mixed with distilled water at a L/P ratio of 0.32 mL/g [2]. Hydroxyapatite cements (HA) were prepared by mixing α-TCP powder with an aqueous solution containing 2.5 wt% sodium phosphate at a L/P ratio of 0.4 mL/g [3]. Linoleic (LA) and ricinoleic (RA) acids were added to the liquid phase of the cement at a concentration of 0, 10, 20, 50, 100 and 200 mg/mL, after determination of the minimum inhibitory concentration (MIC) of each fatty acid. The antibacterial effects were evaluated against *Staphylococcus aureus* (SA, Culture Collection University of Göteborg, 15915, Sweden). Discs of 10 mm diameter by 3 mm height were seeded for 2h in a suspension of SA (10^6 CFU/mL) at 37°C. Then, the bacterial suspension was homogenized, submitted to serial dilutions and plated in agar plates for bacterial counting. For each test, freshly mixed pastes were used.

RESULTS: The addition of RA into the HA formulations did not allow for obtaining a workable paste and no further tests were done with HA cements containing RA. Moreover, control HA specimens did not show any antibacterial effects and allowed the growth of bacteria (Fig. 1). However, all the concentrations tested for LA inhibited to a similar extent the proliferation of SA in HA cements. The control brushite cements showed intrinsic antibacterial properties. The addition of LA and RA into the brushite formulation did not have a significant inhibition effect on bacterial growth (Fig. 1).

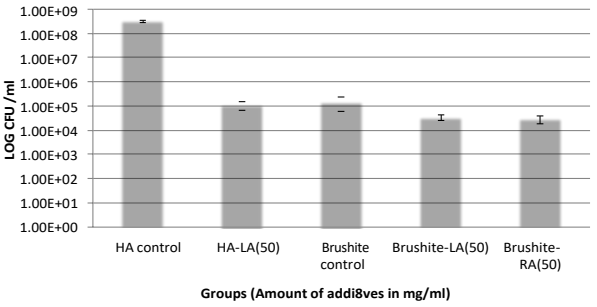

Fig. 1: Effect of fatty acids on SA proliferation when incorporated into the formulation of CaP cements.

DISCUSSION & CONCLUSIONS: This study demonstrated that antibacterial properties could be achieved for calcium phosphate cements through the incorporation of fatty acids. The antibacterial actions of fatty acids have been demonstrated previously, and are usually attributed to the length and degree of unsaturation of the fatty acids. Furthermore, brushite cements were found to have an intrinsic antibacterial effect, likely due to the acidic reaction taking place during setting.

ACKNOWLEDGEMENTS: Funding includes STINT (IG2011-2047) and MAT2012-38438-C03 (cofunded through European Regional Development Funds).

Design and analysis of hydrogel-based devices as reservoir systems for *in situ* cell and drug release

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INTRODUCTION: Ischemic heart disease is the main problem within cardiovascular diseases as well as the leading cause of mortality in many European countries [1]. A natural mimic hydrogel construct acting as a reservoir system to deliver multiple biomolecules to targeted ischemic sites should represent a promising and minimally invasive strategy. The aim of the current research was to propose an approach in developing a multilayer hydrogel based on collagen/collagen–gelatin nanoparticles (GNPs)/collagen-low molecular weight hyaluronic acid (LMWHA). The viscoelastic properties as well as the morphological and biological features of the different devices were preliminary analyzed.

METHODS: Three-layer systems were developed integrating a conventional method and an electrospray-based technique. Each system consisted of a lower collagen layer, an upper collagen or collagen-LMWHA layer and a middle GNP-loaded collagen layer. Small amplitude oscillatory shear tests were performed on all the developed systems and the linear viscoelastic behaviour was investigated. All the tests were carried out at 37°C, using a rheometer (Gemini, Bohlin Instruments). The viscosity as a function of the shear rate was evaluated through steady state shear measurements at 37°C in a wide range of shear rate (0.01 – 100 s^{-1}). GNPs were produced using a two-step desolvation technique, and the encapsulation of different amounts of bovine serum albumin (BSA) was studied. Scanning Electron Microscopy (SEM) was performed to evaluate the morphological features of collagen-based networks and of both unmodified GNPs and BSA-loaded GNPs. Cell viability and proliferation were assessed by using the MTT assay.

RESULTS: Results from small amplitude shear tests evidenced that G' values were always higher than G'' ones in the frequency range investigated. Steady state shear measurements highlighted a shear thinning behaviour for all the proposed materials, as the viscosity decreased with increasing the shear rate. The nanoencapsulation process affected the GNPs depending on the amount of the entrapped BSA. The average mean size for the GNPs was about 170 ± 36 nm. SEM analysis showed the influence of GNPs on the structural/morphological features of the collagen-based network (i.e., fibrils diameter). MTT assay showed no toxic effects in terms of cell adhesion and proliferation for all the systems studied.

DISCUSSION & CONCLUSIONS: A multilayer composite hydrogel [2] was developed. All the materials/systems generally showed a gel-like and shear thinning behavior, as well as appropriate and tunable mechanical/rheological and structural/functional properties. The presence of LMWHA influenced the behaviour of the systems. Both GNPs and BSA-loaded GNPs were optimized in terms of shape and size. The proposed materials/systems can be also easily injected and are able to support the adhesion and proliferation of hMSCs. Furthermore, the presence of LMWHA seems to improve cell viability.

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ACKNOWLEDGEMENTS: This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement number 317304.

Synthesis, structural and micromechanical properties of 3D hyaluronic acid/collagen based cryogel scaffolds for tissue engineering

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INTRODUCTION: Based on [1] we have used cryogelation to fabricate macroporous scaffolds from hyaluronic acid (HA) and hybrid scaffolds HA/collagen using ethylene glycol diglycidyl ether as chemical crosslinker. We have investigated structural, macro-mechanical and for the first time local viscoelastic properties of the matrices as well as viscous properties of the pores using Multiple Particle Tracking (MPT) microrheology.

METHODS: MPT has been performed by monitoring the Brownian motion of inert microspheres that were evenly distributed in the pores or in the network. The resulting trajectories have been transformed into mean square displacement (MSD) traces from which local viscoelastic moduli G´ and G´´ were extracted. Macro-mechanical properties were characterized by rotational rheometry and uniaxial compression tests. Pore size and matrix thickness were obtained by confocal laser scanning microscopy (CLSM).

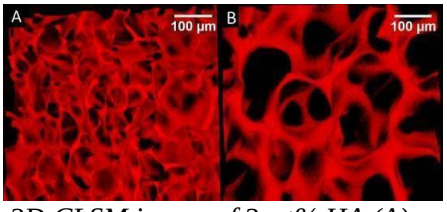

Fig. 1: 3D CLSM image of 3 wt% HA (A) and 3 wt% HA / 0.1 wt% collagen (B) cryogel.

RESULTS: Cryogels are characterized by large interconnected pores of size ~50-200 µm, pore wall thickness ~5-30 µm depending on the collagen content (Fig. 1) and swell instantaneously when immersed in water. We have shown how pH value, pH exposure time, freezing temperature, polymerization time but also cryogel composition influence their properties: swelling capacity is increased by 20 % when 0.1 wt% collagen is added. The Young´s modulus in the hydrated state Ehyd increases from 1.8 to 3.5 kPa when collagen content increases from 0 to 0.3 wt%, respectively. At the same time, the elastic plateau modulus G0 increases from ~1000 to 3000 Pa. For MPT measurements performed in the pores (Fig. 2A), MSD tracers vary almost linearly with time indicating that the motion of the particles is purely diffusive and that the microenvironment surrounding the particles responds like a homogeneous viscous liquid. From the averaged MSDs, we have determined a pore viscosity η_{MPT} = 0.9±0.2 mPas. For MPT measurements in the HA matrix (Fig. 2B), all MSDs exhibit almost no time dependence (slope ≈0).

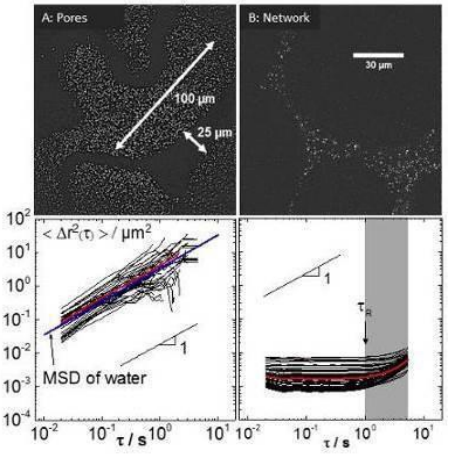

Fig. 2: Upper row: microscope image of PS particles, 0.5 µm diameter, dispersed in water filled pores (A), entrapped in the swollen network (B); lower row: corresponding MSDs.

This indicates that particles are highly constrained by the surrounding fluid which is consistent with an elastic trapping of tracer particles in a gel-like network. At long times, the slope of the MSDs traces increase indicating slow viscous diffusion of the beads corresponding to the relaxation time τ_R of HA chains in the matrix.

DISCUSSION & CONCLUSIONS: The characterization of local matrix elasticity, relaxation time of HA chains and the degree of heterogeneity are crucial for the development of new tissue engineering scaffolds and will help to understand more specifically how local matrix viscoelasticity affects cell cultivation.

Pericardial matrix derived hydrogels functionalized with multiwalled carbon nanotubes for cardiac applications

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INTRODUCTION: Engineering biocompatible scaffolds for culturing cells, possessing inherent electrical conductivity is greatly needed for cardiac applications. We have developed a new composite injectable scaffold from pericardial matrix functionalized with multi-walled carbon nanotubes and investigated the cardiomyocyte response to these gels. HL-1 cells cultured in decellularized tissue derived scaffold with MWNTs showed higher viability and improved response in comparison with normal cell culture and decellularized matrix alone.

METHODS: Sheep pericardium was harvested, decellularized and digested to obtain a thermos-sensitive gel [1]. Carboxylate functionalized MWNTs were modified with hydrazide groups and dispersed with pericardial matrix (PM) at 0.5 wt% (PMNT) and incubated at 37 °C to induce gelation. The hydrazide functionalization of MWCNTs significantly improved its stability and dispersion properties. HL-1 cardiomyocyte cells were cultured on these gels (PM and PMNT gels) and evaluated for viability, expression of connexin43 (Cx-43), expression of α-actinin and beating properties of the cells investigated [2] using AB assay, immunostaining and Fluo-4 monitoring, respectively. Rheological properties of the gels have been studied in the frequency sweep of 1 to 100 Rad/s and 5% strain.

RESULTS: Incorporation of MWNTs in the PM gels resulted in mild stiffening of the gels (Table 1).

Table1. Rheological properties

Sample	*G' (Pa)*	*G'' (Pa)*
PM	194.9±42.57	125.35±15.63
PMNT	247.8±49.21	108.05±15.77

HL-1 cells cultured on PMNT showed higher viability and Cx-43 expression displaying superior electrical conduction between cells. The expression of α-actinin was higher on PM gels alone. Presence of the MWNTs in the PMNT gels caused an increase in the beating frequency, which was twice faster than those on the PM alone.

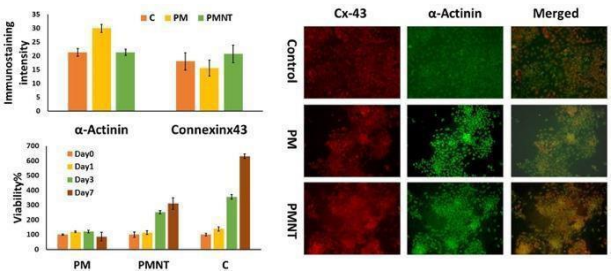

Fig. 1: viability and biomarker expression of HL-1 cells.

DISCUSSION & CONCLUSIONS: Hydrazide modified MWNT resulted in improved stability and elevated stiffness. In addition, the PMNT gels promoted good viability and cell beating properties (Fig. 2), suggesting electrical conduction between cells. Our study suggests that PMNTs are excellent scaffolds for culturing cardiomyocytes, which can be used for cardiac applications.

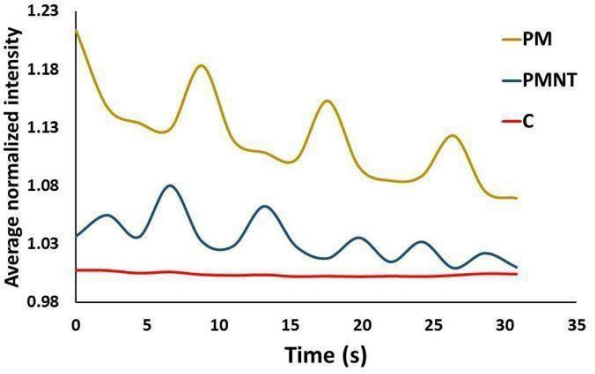

Fig. 2: beating properties of the HL-1 cells.

Positive interaction between the amniotic epithelial stem cells and electrospun poly(lactide-coglycolide), poly(ε-caprolactone), poly(lactic acid)

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INTRODUCTION: Amniotic epithelial stem cells (AECs) retain great potential in cell-based regenerative medicine for their plasticity, immune tolerance and anti-inflammatory properties, thus, ideal for allo and xeno-transplantation settings [1,2]. Their performance would be increased by creating AECs engineered scaffolds allowing the tissue regeneration, mechanical and structural preservation. PLGA, PCL, and PLA nonwovens electrospun scaffolds were tested for oAECs' biocompatibility. In particular, it was studied the correlation between biopolymer characteristics and AECs' attachment, spatial organization and proliferation.

METHODS: PCL, PLA and PLGA scaffolds were prepared by electrospinning using the apparatus EC-CLI by IME Technologies (The Netherlands). The physical and morphological characterization was performed by: X-ray diffraction (Bruker D8), Scanning Electron Microscopy (SEM; ZEISS, EVO), Contact-Angle (DataPhysics Instruments GmbH).
Ovine AECs (oAECs) were isolated by enzymatic digestion and cultured for 48h on all analyzed scaffolds. It was assessed cell adhesion (SEM), survival (using membranes, PKH26, and nuclei, Hoechst33342, vital dyes), spatial organization, proliferation index (detecting and quantifying Ki-67), and DNA quantification (Qubit™ DNA HS Assay, Life Technologies, Thermo Fisher Scientific Inc.). All data were obtained in triplicate and evaluated by T-test ($p<0.05$).

RESULTS: All produced cylindrical fibres were randomly orientated with a mean pore size of about 6 μm for PLA, 8 μm for PCL and 14 μm for PLGA. Structural analysis showed that electrospun PLA and PLGA scaffolds were prevalently amorphous, whereas PCL scaffold showed a discrete level of crystallinity. Contact angle analyses showed samples similar wettability. In fact, regardless the type of polymer, they ranged between 125 and 136°. oAECs seeded on PLA, PCL and PLGA spread within the whole surface and especially around the microfibres. Although, the type of biomaterial influenced oAECs spatial distribution efficiency, and among them PLGA showed the best result. In fact, oAECs coated almost all microfibers and no nuclear pyknosis was observed. Cell proliferation index was significantly higher in PLGA (about 20%) compared to PCL and PLA (about 15 and 10%, respectively: $p<0.05$). DNA quantification demonstrated a different oAECs growth rate, in fact DNA quantity was significantly higher in PLGA (350.66±1.17pg/μl) compared to PCL and PLA (248±1.44pg/μl and 198.66±1.31pg/μl, respectively: $p<0.05$).

DISCUSSION & CONCLUSIONS: these results demonstrate that oAECs and electrospun PCL, PLA and PLGA scaffolds are biocompatible. This positive interaction can be attributed to the geometrical features, including scaffold pore size and biopolymer properties which support oAECs cell adhesion, spatial organization, survival and proliferation. Indeed, the samples having higher percentage of higher pore size (i.e. PLGA) exhibited the highest cell viability in terms of cell migration and proliferation. Thus, hydrophilic nature of these biopolymers seems to mediates the first contact with cells, whereas pore size distribution appears to have an important positive effect on cell diffusion and activity. In conclusion, the investigated biocompatible electrospun matrices, in particular PLGA, represent promising bioscaffolds in AECs-based regenerative medicine.

ACKNOWLEDGEMENTS: We thank Maura Turriani for her valuable technical assistance.

Aligned pristine carbon nanotubes: a promising scaffold for *in vitro* screening of pancreatic cancer

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INTRODUCTION: Translational research between nanotechnology and tumour biology has an increasing interest both for *ex vivo* and *in vivo* applications. Carbon nanotubes (CNTs) due to their biophysical features show great potential for promoting cell growth and proliferation and are able to provide an extracellular environment similar to *in vivo* [1-2]. CNTs are widely used as cancer tissue engineering (CTE) scaffolds in a variety of cancers such as breast, prostate cancers, glioma and lymphoma. However, pancreatic cancer which is a highly lethal disease (the 5-year survival rate is only 6%) with poor prognosis has been rarely investigated on nanosubstrates, which could be a promising *in vitro* tool for disease screening. The aim of this work is to monitor the evolution, i.e., growth, spacial arrangement and extracellular matrix production, of pancreatic cancer cells on fully synthetic, unfunctionalised CNT sheets.

METHODS: Aligned, multi-walled nanotube substrates drawn from a CNT forest as provided from University of Texas at Dallas [3] were used as cell culture scaffolds. The aerogel CNT sheets were attached to circular or square glass coverslips (Thermo Scientific, UK) via capillary pressure or using a rim of polydimethylsiloxane (PDMS) around the glass for better adhesion and all substrates were sterilised in a UV ozone cleaner. Three human pancreatic adenocarcinoma cell lines, i.e., ASPC-1, BxPC-3 and PANC-1 were cultured in 6- or 12-well plates with or without the CNT scaffolds. Biocompatibility of the CNT-based culture surface was assessed via controlling the metabolic activity of all the pancreatic cell lines with the MTS assay (Promega, USA). Raman spectroscopy, optical and scanning electron microscopy as well as confocal microscopy were used for evaluating morphological and adhesion characteristics, as well as cell-scaffold interactions. Production and distribution of laminin, i.e., an extracellular matrix protein naturally secreted by pancreatic cells, was monitored with an immunofluorescence assay (Abcam, UK).

RESULTS: ASPC-1, BxPC-3 and PANC-1 pancreatic cells can grow and proliferate on CNT scaffolds for at least 8 days. Different kinetics were observed for different cell lines, i.e., for ASPC-1 the highest proliferation rate was observed. High level of cell attachment and alignment towards the nanotubes was clearly exhibited via Raman and electron microscopy. Laminin and collagen were formulated by the pancreatic cells on the CNTs demonstrating the *in vitro* creation of an *in vivo* tissue microenvironment and maintenance of the cell phenotype.

DISCUSSION & CONCLUSIONS: We developed a biocompatible cell culture platform for *in vitro* studies of pancreatic cancer, based on pristine, aligned carbon nanotubes. Our findings show that human pancreatic cancer cells behave *in vivo*-like on our fully synthetic CNT scaffold. The latter is of great importance when studying pancreatic cancer *ex vivo* for the development of efficient treatment therapies. Moreover, the biocompatibility of the CNTs points the possibility of their application on the design of targeted drug delivery therapies.

ACKNOWLEDGEMENTS: This work was supported by the Surrey Impact Acceleration Awards (IAA) grant no. 2015/ KN9149C and grant no. 2015/K031562/1 +G060878 as well as an EPSRC-IAA grant no. 2016/ RN0281J.

Sequential synthesis of modular poly(ethylene glycol)—peptide hydrogels for nanoscale control over extracellular matrix features

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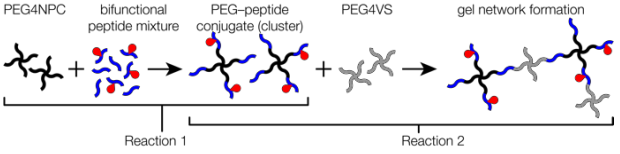
INTRODUCTION: Stem cell behaviour and fate is influenced not only by mobile soluble signals, such as growth factors, but also by immobile characteristics of extracellular matrix (ECM) [1]. For example, on two-dimensional substrates, matrix stiffness [1], degradability [1,2] and cell adhesion ligand presentation [1–3] are all implicated in directing mesenchymal stem cell (MSC) differentiation via mechanotransductive processes. The shift over the last decade to three-dimensional (3D) cell culture models such as hydrogels has played an important role in elucidating the effect of stiffness under more *in vivo*-like conditions, better representing the dimensionality of most tissues. However, the effects of degradability and biomolecule presentation in 3D remain poorly understood. In the present research, modular poly(ethylene glycol) (PEG)–peptide hydrogels with an unprecedented degree of control over these factors have been developed. These materials allow each factor to be investigated either independently or in combination with others, minimising the effect of varying one factor from affecting other factors.

METHODS: Hydrogels consisting of homo-functionalised four-arm PEG–nitrophenyl carbonate (PEG4NPC, synthesised as described in [4]) and PEG–vinyl sulfone (PEG4VS, JenKem Technology USA) – were sequentially cross-linked with custom-synthesised hetero-bifunctional peptides (Peptide Protein Research Ltd. UK) containing N-terminal Lys and C-terminal Cys residues. Peptides were linear (non-functionalised or containing a cleavable matrix metalloproteinase site) or T-shaped (containing branched adhesion ligands or growth factors). Stiffness was controlled by varying PEG molecular weight. Peptide mixtures of various ratios were first conjugated to PEG4NPC in phosphate buffered saline at pH 8.5. These conjugates were then cross-linked with PEG4VS under the same conditions to form hydrogels with varying properties.

RESULTS: Hydrogels with various stiffness, degradability and biomolecule presentation were successfully synthesised via this approach.

DISCUSSION & CONCLUSIONS: Synthetic PEG hydrogels are ideal for studying the effects of ECM properties on stem cell differentiation, due to the bioinertness, non-immunogenicity and low protein adsorption of PEG. The use of multi-arm PEGs cross-linked via branched biomolecule-presenting peptides enables an unprecedented degree of control over individual or combined matrix properties at the nanoscale. Although diverse and highly innovative PEG hydrogels have been in use for over a decade [2], incorporated biomolecules such as cell adhesion ligands or growth factors have typically been attached to PEGs as "pendants", preventing cross-link formation at those sites. This has a knock-on effect on other matrix properties, e.g. reduction of stiffness due to reduced cross-linking. The use of T-shaped peptide cross-linkers with biomolecules attached via branches largely overcomes this issue, enabling variation of one or multiple properties with minimal effects on others. Furthermore, the sequential nature of the reactions enables pre-formation of "clusters" – PEG4NPC with biomolecule-presenting peptides conjugated at desired ratios – which are then cross-linked via PEG4VS, forming a dendrimer-inspired hydrogel network. These materials are now being used to study the effects of matrix stiffness, degradability and biomolecule on differentiation of encapsulated MSC in 3D via gene and protein analyses.



ACKNOWLEDGEMENTS: Nothing to disclose. The authors wish to acknowledge OP Varghese for advice and Jane & Aatos Erkko Foundation, The Company of Biologists, Journal of Cell Science and Royal Society of Chemistry for funding.

Antibacterial effects of equine mesenchymal stem cells

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INTRODUCTION: Mesenchymal stem/stromal cells (MSCs) are multipotent cells implicated in tissue regeneration and immune modulation, thus holding great potential for clinical therapeutic applications in humans and animals. MSC transplantation is widely used for musculoskeletal repair in horses [1] and there is a desire to further expand the therapeutic use of these cells in veterinary medicine. Recently, MSCs of human and murine origin have been shown to exhibit antibacterial effects both *in vitro* and *in vivo* [2, 3]. This is of particular interest in light of the continuing development of antibiotic resistance amongst human and animal pathogens and the need for alternative antimicrobial treatments. Thus, investigating the immunomodulatory and antibacterial effects of equine MSCs is highly relevant.

METHODS: The antibacterial effects of equine bone marrow (BM)-derived MSCs and Fibroblasts against Gram positive bacteria were determined by infecting each cell type in culture with live *S. aureus* and quantifying colony forming units (CFU)/ml 6 hours later. RT-qPCR was used to assess responses in antimicrobial peptide gene expression of the infected cells. Statistical analysis of results was performed using t-test. Separately, BM-MSCs were also cultured and stimulated with purified lipopolysaccharide (LPS) for 24 hours, after which changes in gene expression were analysed using RT-qPCR.

RESULTS: Bacterial numbers were significantly reduced (2.7-fold, $p < 0.05$) after *S. aureus* infection of BM-MSCs compared to Fibroblasts. However, there were no detectable differences in expression of the measured antimicrobial peptide genes after bacterial infection. Treatment of BM-MSCs with LPS induced a 50-fold increase in the expression of the cytokine IL-6, as well as increases in the expression of the antimicrobials Indolamine 2,3-dioxygenase (IDO; 2.8-fold) and Lipocalin-2 (LCN; 1.4-fold).

DISCUSSION & CONCLUSIONS: Similar to reported data for human MSCs, equine BM-MSCs inhibited growth of *S. aureus* in culture, suggesting a direct antibacterial effect, although there were no associated changes in expression of antimicrobial peptide genes. However, BM-MSCs were responsive to LPS stimulation, as demonstrated by upregulation of genes with immune function, suggesting that equine MSCs may possess antibacterial activity against Gram-negative bacteria. We are currently testing the effects of MSCs from bone marrow and other sources on clinically relevant Gram-negative bacteria such as *E. coli*. In addition, we aim to investigate the effects of equine MSCs on the bactericidal activity of immune effector cells, evidence of which has already been provided for human MSCs [4].

Ultimately, our results will provide a first step towards understanding the antibacterial effects of equine MSCs and make way for expansion of current cell-based therapies to combat antimicrobial resistance

ACKNOWLEDGEMENTS: This study has been funded by the Horse Betting Levy Board, UK.

Characterization of equine mesenchymal stromal cells from non-invasive sources

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INTRODUCTION: Mesenchymal stromal cells (MSC) have been extensively studied for their promising capabilities in regenerative medicine. In general, most cell-based therapies consist of the use of autologous MSC. Nevertheless, the low immunogenicity of MSC – indicated by the absence of the expression of major histocompatibility complex (MHC) class II and co-stimulatory molecules CD40, CD80 and CD86 – and their immunosuppressive capacities, enable their use in clinical allogeneic applications [1]. An allogeneic source would provide an off-the-shelf, more standardized and readily available product without the inherent lag period associated with isolation and expansion of autologous MSC [2,3]. Although bone marrow is the best known source for isolating equine MSC, alternative sources such as umbilical cord blood (UCB), umbilical cord matrix (UCM) and peripheral blood (PB) have been reported also.

METHODS: Equine MSC were isolated from 6 mares (PB) and their foals (UCB & UCM) at parturition. Following parameters were analyzed: (i) success rate of isolation, (ii) proliferation capacity, (iii) tri-lineage differentiation ability, (iv) immunophenotypical protein and (v) immunomodulatory mRNA profiles. Linear regression models were fit to determine the association between the source of MSC (UCB, UCM, PB) and (i) the moment of first observation, (ii) the moment of first passage, (iii) cell proliferation data, (iv) the expression of markers related to cell immunogenicity, and (v) the mRNA profile of immunomodulatory factors, except for hepatocyte growth factor (HGF) as no normal distribution could be obtained for the latter variable. To evaluate the association between the source of MSC and the mRNA expression of HGF, the non-parametric Kruskal-Wallis test was performed instead.

RESULTS: While equine MSC could be isolated from all the UCB and PB samples, isolation from UCM was only successful in 2 samples. Proliferation data showed that equine MSC from all three sources could be easily expanded, although UCB-derived MSC appeared significantly faster in culture than PB- or UCM-derived MSC. Equine MSC from both UCB and PB could be differentiated towards the osteo-, chondro- and adipogenic lineage, in contrast to UCM-derived MSC where only chondro- and adipogenic differentiation could be confirmed. Regardless of the source, equine MSC expressed the immunomodulatory genes CD40, CD80, HGF and TGF-β. In contrast, no mRNA expression was found for CD86, IDO and TNFα.

DISCUSSION & CONCLUSIONS: Our data strengthen recent findings that inherent differences exist between MSC from different tissues, as suggested for human MSC. Combining all the observations in this present study, we propose UCB as the most promising non-invasive alternative source for MSC and UCM as the least feasible source due to high contamination risks. Moreover, our data indicate that UCB-derived MSC could be suited for allogeneic use, although their immunogenicity potential needs to be addressed in more detail in future studies.

ACKNOWLEDGEMENTS: We gratefully acknowledge Isabel Lemahieu, Petra Van Damme, and Kristel Demeyere for their excellent technical assistance. Jurgen De Craene has performed the histological stainings. Guy Wouters is thanked for his useful advice in handling the UCM cultures.

DISCLOSURE: authors have nothing to disclose.

First-in-man safety and efficacy of the adipose graft transposition procedure (AGTP) in patients with a myocardial scar

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INTRODUCTION: The present study evaluates the safety and efficacy of the Adipose Graft Transposition Procedure (AGTP) as a biological regenerative innovation for patients with a chronic myocardial scar. The adipose tissue surrounding the heart and pericardium may serve as an autologous biological matrix for salvaging this injured myocardium, as experiments in porcine models have suggested [1-2]. **METHODS:** This prospective, randomized single-centre controlled study included 10 patients with established chronic transmural myocardial scars. Candidates for myocardial revascularization were randomly allocated into two treatment groups. In the control arm (n=5), the revascularizable area was treated with CABG and the non-revascularizable area was left untouched. Patients in the AGTP-treated arm (n=5) were treated with CABG and the non-revascularizable area was covered by a biological adipose graft. The primary endpoint was the appearance of adverse effects derived from the procedure including hospital admissions and death, and 24-hour Holter monitoring arrhythmias at baseline, 1 week, and 3 and 12 months. Secondary endpoints of efficacy were assessed by cardiac MRI. **RESULTS:** No differences in safety were observed between groups in terms of clinical or arrhythmic events. On follow-up MRI testing, participants in the AGTP-treated arm showed a borderline smaller left ventricular end systolic volume (LVESV; p=0.09) and necrosis ratio (p=0.06). The AGTP-treated patient with the largest necrotic area and most dilated chambers experienced a noted improvement in necrotic mass size (-10.8%), and ventricular volumes (LVEDV: -55.2 mL and LVESV: -37.8mL at one year follow-up) after inferior AGTP.

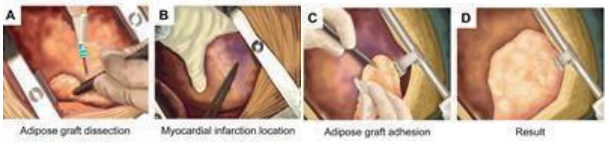

Fig. 1: Schematic illustration of the Adipose Graft Transposition Procedure (AGTP). Pericardial adipose tissue is dissected to create the graft (A). After partial pericardiectomy, MI is located (B) and the pericardial adipose graft is transposed onto the infarct area and glued on healthy edges (C), covering the ischaemic myocardium (D).

Table 1. Mean MRI values at baseline, three months and one year follow-up.

	AGTP-treated			Control		
	Baseline	3 mo.	12 mo.	Baseline	3 mo.	12 mo.
Necrosis mass, gr	33.9	32.9	30.0	21.3	21.6	17.6
Necrosis ratio, %	22.3	20.1	19.3	14.3	14.7	16.4
LVEF, %	41	43	47	42	47	46
LVESV, mL	139.6	131.9	114.9	127.8	99.2	104.6
LVEDV, mL	219.8	218.0	202.4	212.2	187.7	194.6
CO, L/min	5.0	5.5	5.8	5.1	5.9	5.5
SV, mL	80.2	87.3	87.5	84.4	88.5	90.1

DISCUSSION & CONCLUSIONS: Our results indicate that AGTP is safe and may be efficacious in selected patients. Further studies are needed to confirm its clinical value. (ClinicalTrials.org NCT01473433, AdiFlap Trial).

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ACKNOWLEDGEMENTS:
SAF2014-59892, JCI-2012-14025, Fundació La MARATÓ de TV3, Red TerCel (RD06/0010/0016, RD12/0019/0029, RD12/0019/0034), RIC (RD RD12/0042/0027 & RD12/0042/0047), FIS PI14/01682, and FEDER.

Isolation of mesenchymal stem cells from ovine placenta cotyledons

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INTRODUCTION: Sheep are a well-established animal model for musculoskeletal research. In regenerative medicine they are increasingly used as large animal models, which are a legal requirement for licensing of future treatments. Mesenchymal stem cells (MSC) are a frequently used tool in regenerative medicine. However, harvesting MSC may be associated with invasive collection procedures. Therefore, MSC sources accessible using minimal invasive techniques have moved into the focus of interest. In human medicine it was shown that MSC reside in a vascular niche in placenta chorionic villi [1]. Ruminants have a cotyledonary placenta. Instead of having a single large area of contact between the maternal and fetal vascular systems, these animals have numerous smaller placentae built from cotyledons (the fetal side of the placenta) and caruncles (the maternal side of the placenta) together forming so called placentomes. In the presented study ovine placenta cotyledons were identified to be a minimal invasive source of MSCs in sheep.

METHODS: To isolate MSCs the ovine afterbirth was collected upon full term delivery and the cotyledons were dissected. After collagenase digestion, filtration through a cell strainer and Ficoll density gradient centrifugation the obtained mononuclear cell fraction was plated on an adherent culture dish and cultured with DMEM low glucose, 10% FCS, 1% L-Glutamine, 1%Pen/Strep and Amphotericin at 37° and 5 % CO2. Medium was changed twice weekly. Passaging was performed upon 80-90% confluence.

RESULTS: The obtained cells showed the minimally required criteria for the identification of MSCs: Spindle shaped morphology, adherence to plastic, colony formation and *in vitro* differentiation into the mesenchymal lineages bone and cartilage, but showed a lack of adipogenic differentiation potential. The cells were positive for the surface markers CD44, CD166 and CD29 and negative for CD45 and CD31. (1)

DISCUSSION & CONCLUSIONS: Ovine placenta cotyledons are suggested to be an abundantly available, minimal invasive source of MSCs in sheep. The lack of adipogenic differentiation may be due to the fetal origin of the cotyledon. Adipogenic differentiation potential was recently shown to be reduced or delayed in fetal MSCs, resulting in strong impairment of adipogenesis [2]. Further investigation to characterize the obtained cells and to evaluate their regenerative effect *in vivo* will need to be carried out.

ACKNOWLEDGEMENTS: Thank you to the LFG Vetmeduni Vienna, for providing the sheep.

Isolation of adipose tissue growth factors in operating room for wound treatment - a clinical pilot study

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INTRODUCTION: Soft tissue defects and chronic wounds are a medical and economic challenge to health care system. Even 1-3 % of health care expenses are spent on wound care as current treatments lack efficacy. Impaired vascularization is the major obstacle in tissue regeneration. In previous studies, we have isolated a wide number of growth factors and cytokines from adipose tissue and showed that this adipose tissue extract (ATE) induces adipogenesis and angiogenesis *in vitro* [1] and when implanted *in vivo*, stimulates extensive neovascularization and adipose tissue accumulation [2]. In the current study, we transferred the preparation protocol of ATE from laboratory to operating room to be used autologously under surgery and performed a clinical pilot study with autologous ATE in treatment of superficial wounds in skin graft donor-sites.

METHODS: ATE was extracted from human adipose liposuction samples and tested for protein and growth factor yield with distinct variables including incubation time (15, 30 and 45 minutes), temperature (room temperature vs. 37°C) and filter types (2 different 0.2 µm polyethylene sulfone (PES) and 2 cellulose acetate membranes applicable for clinical use).

RESULTS: Adequate amount of protein (<200 µg/ml) was obtained in short incubation times of ATE. No difference in the incubation temperatures was observed. Sufficient protein yield could be obtained with all filters, however, the filter material or surface area of the filter may have impact on the protein concentration and therefore on the feasibility of the ATE production procedure. The variation in the growth factor and cytokine results were in concordance with our previous studies and the variation in growth factor and protein concentration between the samples was lower in OR compared to the laboratory. When applied clinically into superficial wounds, from day 3 onwards greater wound re-epithelization was seen compared to control, especially from wound appendages, and cleaner wound bed. At days 7 and 10 re-epithelialization was greater or almost complete in ATE group with wounds that had stronger epithelial coverage and better aspect in terms of color and moisture and free from debris. (Fig 1).

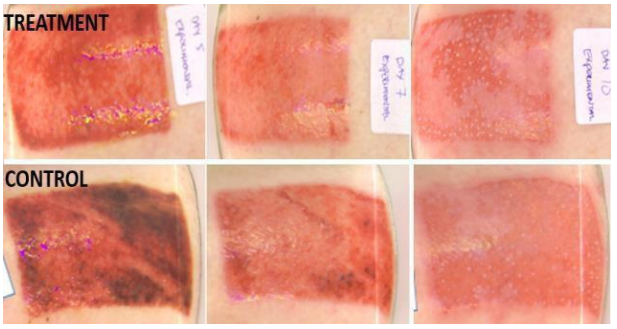

Fig. 1: Wound healing in skin graft donor site. Treatment group (ATE) and control group compared at days 5,7 and 10 after initial treatment.

DISCUSSION & CONCLUSIONS: ATE is an appealing agent for cell promotion, proliferation, migration and differentiation. Any clinical scenario in need of tissue volume loss, wound healing and adequate vascularization would benefit from ATE. The production method was successfully transferred to operation room, from bench to bedside, and is now ready for the clinical trials. The protocol optimization showed that sterile ATE can be produced in the OR with an easy method. This OR ready protocol enables the onset of clinical trials. The simplicity of the product makes it a more attractive choice for clinicians when compared to currently used products.

ACKNOWLEDGEMENTS: The Finnish Funding Agency for Technology and Innovation, Science Center of Pirkanmaa Hospital District

A refined and clinically more relevant, preclinical osteochondral defect model in rabbits

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INTRODUCTION: An osteochondral defect in the femoral trochlear groove of the rabbit is a well described single site defect animal model to study cartilage regeneration and repair[1].As orthopaedic research continues to advance, it is important for commonly available preclinical models to keep pace with the current clinical standard of care. However, current preclinical models of osteochondral defects in rabbits are all performed via open arthrotomies, diverging from clinical practice. In this study we aim to demonstrate the feasibility to create such defects in the rabbit *in vivo* and to investigate, if healing of an untreated defect is affected by the surgical approach. Additionally, it is hypothesized that the use of arthroscopy will reduce the animals burden representing an refinement of the model[2].

METHODS: Eight skeletally mature female New Zealand White Rabbits were used for this study. In 4 rabbits (group 1) an osteochondral defect was created in the medial trochlear ridge of the femur minimal invasively, with arthroscopic technique. In the remaining 4 rabbits (group 2) the femoropatellar joint was approached via an open arthrotomy and a defect of the same size and depth as in group 1 was created. All rabbits received pain medication following the same protocol, and were group housed immediately after surgery for the whole follow up period of 6 weeks. Short-, intermediate-, and long-term effect of the surgical procedure on general condition and activity of the rabbits were monitored. Activity was measured daily during the first postoperative week, followed by three times weekly 2, 3, 4, and 6 weeks after surgery. Movement of each rabbit was monitored with a tracking system (Ethovision, Noldus). After euthanasia and evaluation of the operated stifle joint using the macroscopic cartilage repair assessment ICRS, the distal femora were immediately fixed and later processed for histology.

RESULTS: Creation of a standardized defect was possible in 7 out of 8 rabbits. In one rabbit of group 1 the guiding pin slipped out during drilling leading to a misshaped defect. All rabbits recovered well from surgery and showed no signs of pain or distress in the post-operative period. One rabbit of group 1 was lost during the study period because of respiratory problems. One rabbit of group 2 developed a wound dehiscence and needed additional treatment for one week. Clinically and on video evaluation was no difference in behaviour between the two groups. Statistical evaluation from the activity tracking is pending. In the macroscopic cartilage repair assessment group 1 reached an average score of 8.66 (SD 2.52), which correlates with grade II; nearly normal repair. Group 2 reached an average score of 5.5 (SD 2.08), correlating with group III; abnormal. The slides for histology are currently in preparation.

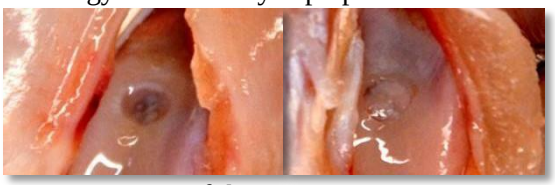

Fig. 1: Images of the macroscopic assessment of cartilage repair – left side a rabbit of the arthrotomy group with an overall abnormal repair, right side a rabbit of the arthroscopic group with nearly normal appearance.

DISCUSSION & CONCLUSIONS: A standardized osteochondral defect in the trochlear ridge of the femur can be created arthroscopically in rabbits. With the same pain medication protocol, both rabbit groups showed normal behaviour after surgery. The macroscopic cartilage repair assessment ICRS showed a better overall repair in the arthroscopic group. It will be interesting to see if histology supports this finding.

ACKNOWLEDGEMENTS: The study was financed by the AO Trauma Research Commission (AOTRC).

Semi-synthetic material design and implementation for chemo-sensitivity testing and diagnostics cancer drug screening

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INTRODUCTION: Despite the considerable advances in cancer treatment approaches and detection techniques [1-4], drug resistance remains a substantial obstacle. In order to improve patient-specific chemotherapy treatment and provide input to optimize the patient's first-line regimen, there is urgency in developing reliable, time saving, and cost-effective screening tools. In preliminary studies performed in our lab, a strategy of designing extracellular matrix (ECM) biomaterial analogs made from PEG-fibrinogen (PF) hydrogels was employed to enhance the outgrowth of tumor metastatic cells for chemo-sensitivity testing. Our first objective was to validate the encapsulating PF hydrogel using artificial tumour biopsies made from human breast adenocarcinoma cells (MDA-MB-231/Mcherry), and normal dermal human fibroblasts (NHDF/GFP). This objective was met by designing a PF hydrogel composition that accelerates MDA-231 cell invasion into the matrix, and discourages mesenchymal cell invasion such that fibroblasts lag behind. Cells that express fluorescent proteins can be monitored in situ, thus identifying migration patterns and reducing effects of mesenchymal-epithelial transition (MET) [5]. A second aim was to examine the inhibitory effects of anticancer drugs, Doxorubicin and Cisplatin on the cell outgrowth in this tumour growth model.

METHODS: Droplets of PEG-fibrinogen solution containing both cell types were cross-linked on a super hydrophobic surface, producing cellularized hydrogel beads. After a two-day incubation in cell culture medium, the cellularized hydrogel beads were placed in a secondary PEG-fibrinogen solution, then cross-linked to form a 'gel in gel' construct. The constructs were then incubated in medium (with or w/o anticancer drugs) and monitored for six days. For the anticancer drug screening experiments, each construct was documented with digital images using a phase contrast microscope, while the fluorescent hydrogel beads were monitored using a time lapse confocal microscope.

RESULTS: Validation entailed monitoring co-culture hydrogel constructs for 6 days using time-lapse microscopy. A dramatic outgrowth of MDA 231 cells (red) over the NHDF cells was observed (Fig 1). In drug resistance experiments, the cells migration was clearly inhibited by the anticancer drugs Doxorubicin, Cisplatin and the combination of both (data not shown).

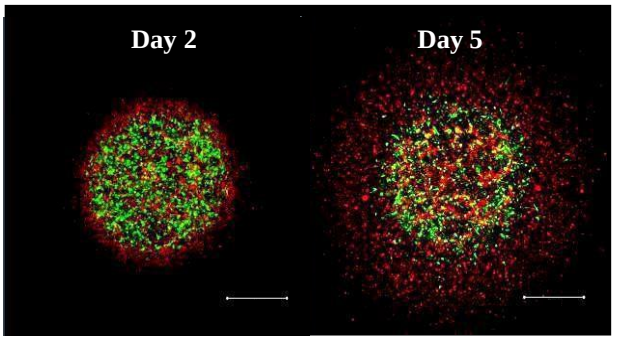

Fig. 1: Cellular outgrowth (3D) at day 1 and 5 from hydrogel beads containing co-cultures of MDA-231/ Mcherry and NHDF/GFP cells. Scale bar=500µm.

DISCUSSION & CONCLUSIONS: The study validated a gel matrix composition that favours the preferential invasion and outgrowth of the MDA-231 tumour cells over mesenchymal cells (fibroblasts), eliminating any unspecific migration of fibroblast or MET effects that can alter the drug screening results. Furthermore, the anticancer drug experiments reinforced the relevance of this technique as a screening tool for chemotherapy drugs.

Fluorescence-based 3D model for drug screening against colorectal cancer

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INTRODUCTION: Two-dimensional (2D) culture models have been widely used for high-throughput screening (HTS) in drug selection process [1]. However, it has been criticized that 2D cultures induced a cell behavior that differs from *in vivo* situation, resulting in an insufficient model for drug development [2]. More recently, multicellular 3D models have become a useful tool for overcoming the traditional 2D monolayers limitations, as they provide a more complete understanding of interactions between tumor cells and its stroma [3].
Nevertheless, existing drug screening models fail to distinguish and quantify the drug response of both, stromal and tumor cells cultured together.
Herein, we report a novel fluorescence-based 3D model of colorectal cancer suitable for HTS drug discovery that employs enhanced green fluorescent protein (eGFP)-labelled tumor cell lines, allowing the quantification of tumor cell viability whether or not other cell types are present.

METHODS: In this study we used human colorectal cancer cell line DLD-1, previously labelled with eGFP, and human adipose derived stem cells (ADSCs) cultured in 96 well plates in three different conditions: *i*, standard monolayer (2D) of DLD-1 cells, *ii*, a 3D model of DLD-1 grown as tumor spheroids, obtained by hanging drop method, embedded in a collagen I matrix (2.3 mg/ml), and *iii*, DLD-1 spheroids co-cultured with ADSCs, both embedded in the same collagen matrix. Cultures were exposed to increasing concentrations (1-30 µM) of 5-fluorouracil, oxaliplatin, irinotecan or curcumin. To analyze the influence of culture conditions in the response of tumor cells to these chemotherapeutic agents, we measure the fluorescence intensity (FI) of eGPF emitted by viable cells.

RESULTS: eGFP expressing DLD-1 cells displayed linear correlation between the number of seeded cells and the emitted FI, with similar sensitivity to the MTT assay. Neither cell proliferation rate nor the sensitivity to anticancer drugs was altered by the expression of eGFP. The results demonstrated that there is a significant difference in survival response to all these anti-cancer agents, being the tumor spheroids more resistant to these drugs than the 2D monolayer (Fig 1). Finally, in a preliminary experiment, we found that the co-culture of ADSCs with DLD-1 spheroids reduces tumor cell proliferation.

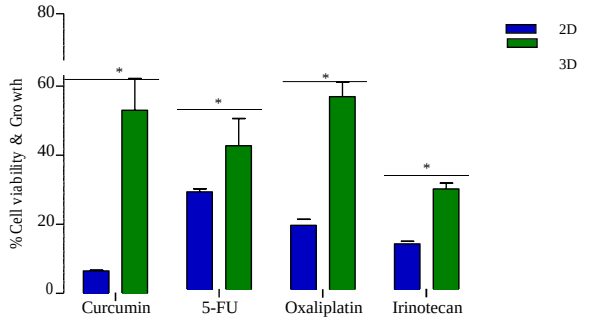

*Fig. 1: Effect of anti-cancer agents (curcumin, 5-fluorouracil, oxaliplatin, or irinotecan) on cell viability in 2D and 3D cultures after 7 days of drug treatment (30 µM). Values represent averages ± SD (*P<0.001).*

DISCUSSION & CONCLUSIONS: The use of fluorescence to assess the impact of drugs on tumor cells proliferation and viability has proved to be as sensitive as MTT. Besides, this method does not require purchase and application of a susbtrate, which makes it technically simple as well as cost and time effective. Finally, this fluorescence-based 3D model of colorectal cancer provides useful information to improve the selection of promising drugs in preclinical stages, which shows the potential of this 3D model to reduce the rate of failure of clinicaltrials.

ACKNOWLEDGEMENTS: This work has been partially supported by Fundación Jesus Gangoiti Barrera.

High-throughput screening of distinct biomaterials for bone repair; techniques to reduce animal use in screening of biomaterials for regenerative medicine

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INTRODUCTION: Biomaterial research has generated a multitude of novel biomaterials in the search for the optimal material for tissue-regeneration. These biomaterials require thorough *in vivo* assessment prior to clinical evaluation and trials. We are committed to the refinement, replacement and reduction of animals, with the aim to relieve the burden of *in vivo* research by using high-throughput screening models as replacement and reduction for *in vivo* studies. Herein, we describe a facile high-throughput material-screening assay, examining an array of novel biomaterials using µCT analysis of an ex vivo chick femoral defect, cultured on the chick chorioallantoic membrane (CAM) ([1].

METHODS: A range of selected (five) biomaterials from the EU Biodesign consortium group were mixed with and without BMP-2 (2 ng/implant), subsequently the materials were implanted into 1 mm chick femur defects isolated from day 18 embryonic chick femurs. The femurs were scanned using a Skyscan 1176 prior to CAM culture. Samples were harvested after 8 days and assessed via µCT using the same scan settings. The biomaterials were evaluated by using the pre scans as controls for the post scans, thereby assessing the bone forming capacity of each individual material with and without BMP-2.

RESULTS: All biomaterials were biocompatible with good cell infiltration. A number of biomaterials exhibited excellent biocompatibility and, critically, demonstrated enhanced volumes of bone formation. These biomaterials were considered suitable for further *in vivo* evaluation.

DISCUSSION & CONCLUSIONS: These studies demonstrate the potential of this high-throughput *ex vivo* uCT chick femoral CAM assay for screening of biomaterial characteristics and bone forming potential. This model offers a facile approach to identify the most promising biomaterials for further, more invasive *in vivo* evaluation whilst addressing the need for reduced animal experimentation.

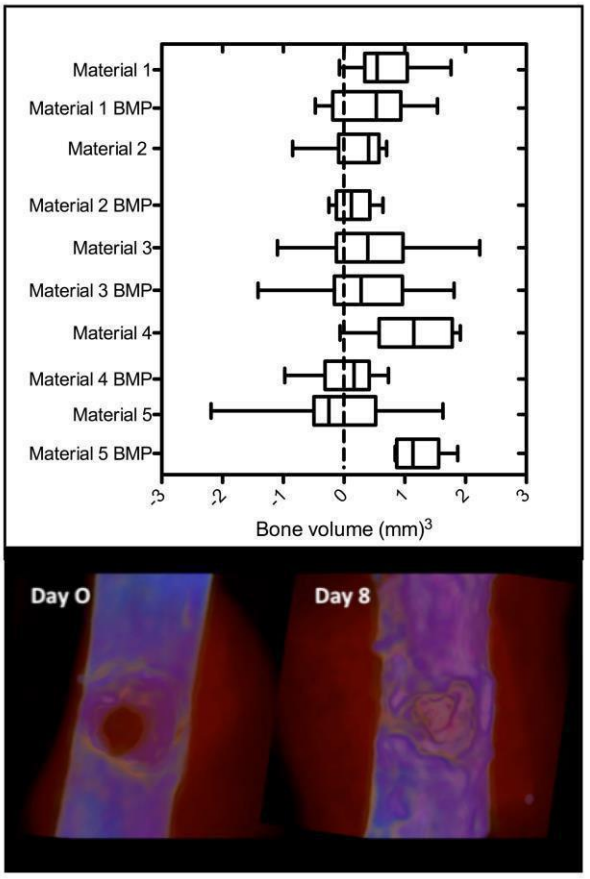

Fig. 1: Variation in bone volume between pre-and post-CAM culture at 8 days (mean ± min/max whiskers). Five biomaterials were evaluated. The biomaterials were applied with and without BMP-2 and implanted into chick femoral defects, analysed pre and post CAM culture using µCT and histology. Lower image shows material 1 +BMP-2 in false colour as a representative of some of the distinct biomaterials that formed bone throughout the sample.

ACKNOWLEDGEMENTS: We acknowledge the provision of materials from members of EU Biodesign – without whom this work would not have been possible (Jöns Hilborn, Alicia El Haj, Martin Stoddart, Dror Seliktar, Håkan Engqvist, Matthias Lutolf).

Assessment of uroprotective effects of pomegranate in cyclophosphamide induced hemorrhagic cystitis: an experimental study in rats

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INTRODUCTION: High antioxidant properties of pomegranate as well as its anti-inflammatory effects can be beneficial in prevention and alleviation of cyclophosphamide-induced Hemorrhagic Cystitis (HC), a fatal yet unpredictable and poor managed phenomenon. We aim to investigate effect of Dietary Pomegranate extracts and juice on alleviating HC episodes.

METHODS: Sixteen Sprague–Dawley rats were divided into 4 groups: Group 1 was the negative control group (sham). In the other 3 groups HC was induced by intraperitoneal administration of a single dose of cyclophosphamide (150 mg/kg). Rats of Group 2 were the positive control receiving only cyclophosphamide. In group 3 rats received Mesna (2-mercaptoethane sulfonate sodium) with a dose of 30 mg/kg suspended in 2 ml of N/S in 3 separate doses (30 minutes prior to cyclophosphamide injection, one dose after 4 hours and the last dose after 8 hours). Rats in Group 4have been receiving a 14-day special diet consisting of Pomegranate juice plus peel extracts (2 cc/day) prior to HC induction. In order to assess protective effect of pomegranate against HC, Urodynamic studies were performed in all rats 48 hours after HC induction. Rats were euthanized afterwards and autopsies were taken from their bladders for histopathological evaluations.

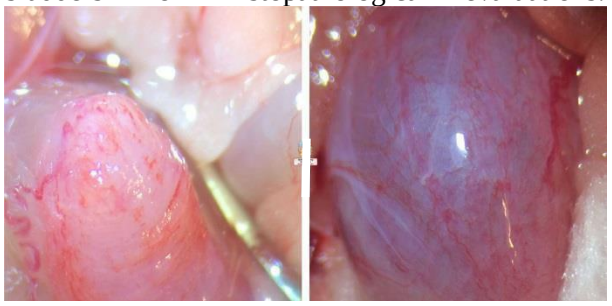

Fig. 1: Hemorrhagic cystitis model in a rat with telengectatic vessels

RESULTS Analysis of urodynamic findings revealed that mean detrusor pressure in Group 4(24.50±7.18cmH_2O) was significantly lower comparing to both positive control group and group 3(43.25±3.59 and 27.75±8.42cmH_2O respectively, p value<0.05) .Mean end-filling pressure in group 4(17.00±7.74) was also lower comparing to group 2 and group 3(14.75±1.89ml/cmH_2O and10.00±4.96ml/cmH_2O), but it was not statistically significant. Histopathological characteristics of cyclophosphamide-induced HC including edema, vascular dilation and hemorrhage, Degeneration, desquamation and destruction of urothelium were markedly decreased in rats of group 4.

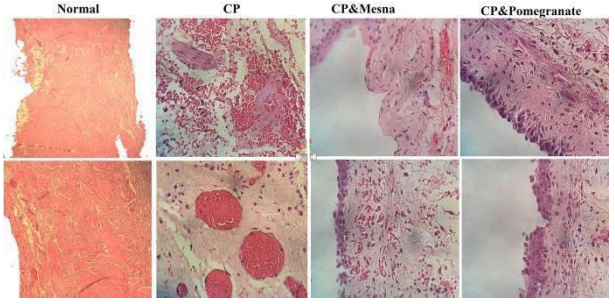

Fig2:. Hematoxylin and eosin staining of control, cyclophosphamide, cyclophosphamide-Mesna and cyclophosphamide-Pomegranate groups.

DISCUSSION & CONCLUSIONS: Hematopoietic cell transplantation recipients who receive cyclophosphamide as part of their conditioning regimen might benefit from Administration of pomegranate-derived antioxidants along with Mesna to decrease severity of HC and its sequels.

DISCLOSURES: Authors declare no conflict of interest

Three-dimensional spheroid culture as a suitable model to study radiation response of salivary epithelial cells

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INTRODUCTION: Radiation treatment often leads to irreversible damage to normal salivary glands (SGs) because of their proximity to head and neck cancers. Optimization of the in vitro model of irradiation (IR)-induced SG damage is warranted to investigate pathophysiology and monitor treatment outcome. Here, we present a three-dimensional (3D) cell culture model to investigate the impact of IR on SGs and the mechanisms underlying IR-induced structural and functional changes.

METHODS & RESULTS: Human parotid epithelial cells (hPECs) were obtained from human parotid glands and plated on either plastic plates or Matrigel. A number of acinar-like 3D spheroids were assembled on Matrigel. After irradiation at 10 and 20Gy, morphological changes of the cells in 2D monolayers and 3D spheroids were observed. The 3D spheroids showed more radioresistance relative to the 2D monolayers. However, as the structural integrity of the 3D spheroids was destroyed by IR, the salivary epithelial and tight junction-related maker genes and proteins showed a greater decrease in the spheroids than 2D monolayer cells, and this occurred proportionally with radiation dosage. Moreover, IR-inflicted 3D spheroids exhibited a loss of epithelial polarity and acinar-specific cellular functions that enable secretion of α-amylase in response to Ca^{2+} agonists.

DISCUSSION & CONCLUSIONS: These results suggest that the 3D spheroid culture of hPECs provides a better model for biological insight into IR damage to SGs and serves as an improved tool for exploration of radiobiology and mode of action of new therapies for prevention or regeneration of SGs.

1

ACKNOWLEDGEMENTS: This research was supported by grants from the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and future Planning (2015R1A2A2A01006528), the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI) funded by the Ministry of Health & Welfare, Republic of Korea (HI15C2807), and an Inha Research Grant

Maintenance of primary human colorectal cancer microenvironment by culture in 3D perfusion-based bioreactor

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INTRODUCTION: Interactions between cancer and non-malignant cells critically affect development, progression and drug responsiveness of human cancers. However, drug screenings are still conducted on 2D tumor cell monocultures or on tumor xenografts generated in immunodeficient animals. These systems do not allow the preservation of the peculiar microenvironment of patients' tumors. The development of novel in vitro methods maintaining the complexity of in vivo tissues is urgently required.

Direct perfusion of media through scaffolds and/or tissues allows active delivery of nutrient in complex systems. By continuously removing spent media and replacing it with new media, nutrient levels are maintained for optimal growing conditions and cell waste products are removed. Moreover perfusion reduces mass transfer limitations, particularly in the central part of the scaffold, thus promoting cellular proliferation and viability [1].

We have investigated the use of a perfusion-based bioreactor for culture of primary colorectal cancer (CRC) samples in vitro.

METHODS: Freshly excised CRC specimens were cut into fragments, inserted between two collagen type I scaffolds in a "sandwich format" and then cultured for up to 10 days in a perfused-based system (U-CUP, Cellek Biotech AG) or under non-perfused conditions. Maintenance of tumor architecture was evaluated by histo-morphological analysis following hematoxilin & eosin staining. Viability of tumor, stromal and tumor-infiltrating immune cells was assessed by immunofluorescence, upon EpCAM, Vimentin, and CD45 staining, respectively. Tumor cell proliferation was evaluated upon EpCAM and Ki67 staining. In addition, the expression of EpCAM, CD90 and CD45 markers was analyzed by flow cytometry on single cell suspensions obtained upon scaffold digestion examples above.

RESULTS: We observed that after 10 days the original tissue architecture was partially maintained in CRC tissues cultured in perfused U-CUP, whereas it was completely lost in non-perfused cultures. Importantly, in U-CUP cultures percentages of proliferating tumor cells (up to 20%) were comparable to those detected in freshly isolated tumor tissues. Phenotypic analysis by flow cytometry confirmed that U-CUP expanded tissues maintained epithelial (up to 41.25%), stromal (up to 13.84%) and hematopoietic cells (up 45.60%) in proportions similar to the original tissues (68.01%, 4.74%, 26.44% respectively).

CONCLUSIONS: Taken together, our results indicate that culture of primary tumor fragments within U-CUP can be successfully achieved over a short-time period allowing the preservation of the diverse cellular components of the CRC specimens. These ex-vivo in vitro tissues might mirror features of the original tumor more effectively than 2D or 3D static cultures, and of xenografts, thus possibly representing useful tools for drug testing and for the evaluation of sensitivity to chemotherapies for personalized medicine.

Primary breast cancer culture in a perfusion-based bioreactor suitable for in vitro testing of immune blockade therapy

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INTRODUCTION: Interaction between cancer cells and immune system critically affects development, progression and treatment of human malignancies. Two-dimensional (2D) in vitro culture systems and in vivo animal models are the primary tools used to test cancer cell response to drugs but they are not suited for the development of immune-mediated therapies. Here we present an innovative method to culture breast cancer tissue in porous 3D scaffolds by using a perfusion-based bioreactor system that allows the maintenance and expansion of tumour microenvironment.

METHODS: Freshly excised breast cancer specimens were fragmented and cultured in a 3D "sandwich-like format" between two layers of porous collagen scaffold under perfusion flow (U-CUP). DMEM/F12, supplemented with 10% autologous human serum, was used as a culture medium. We assessed the ability of tumour and non-malignant cells to survive and expand into the scaffold in perfusion culture, as well as their capacity to recapitulate features of the original breast cancer tissue. The maintenance of immune-infiltrating cells allowed testing of immune blockade therapy in vitro using anti-PD-L1 and anti-CTLA4 antibodies alone or in combination.

RESULTS: The U-CUP culture system preserved tissue viability better compared to a static culture and promoted the expansion of breast cancer cells from surgical specimens together with accompanying stromal and immune cells into the porous scaffold. Tumour tissues were viable after 21 days and mostly recapitulating the initial histology with formation of glands. Administration of anti-PDL1 antibody, alone or in combination with anti-CTLA4, to the culture medium was associated with increased expression of markers of immune-activation (i.e. IFNg) and decreased expression of immunosuppressive cytokine IL10.

DISCUSSION & CONCLUSIONS: Our results show that culture of breast cancer tissue in a 3D perfusion-based bioreactor might represent a promising system for the pre-clinical evaluation of immune-mediated therapies. Preserving malignant, interstitial and immunocompetent cells comprised in surgically excised breast cancer samples might allow a direct evaluation of the effects of various treatments on the complex tumour microenvironment. This engineered in vitro model could be extended as a platform allowing the testing of innovative approaches for the treatment of human malignancies, possibly in the direction of personalized medicine.

Non-destructive 3D optical toxicity assays for 3D liver models

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INTRODUCTION: Conventional 2D hepatocyte cell monolayers are increasingly found less physiologically-relevant [1] when compared with 3D liver models to perform pre-clinical drug toxicity testing. However, standard toxicity assays have been optimised to perform for 2D cell monolayers and are therefore poorly compatible with larger liver-like 3D tissues. In order to address this issue we investigated a novel approach to assess cell viability and metabolism in 3D tissue culture based on optical coherence phase microscopy (OCPM) quantitative imaging

METHODS: Human hepatic C3A cells were encapsulated in modular HyStem® Hydrogels (Thermo Fisher), and then cultured for 7 additional days in 200µl MEME/10%FBS in 96-well plates until spheroid formation/ viability was assessed. 3D liver constructs were then exposed to paracetamol (acetaminophen) on culture day 8. We developed an inverted OCPM in common path configuration based on a Callisto OCT engine (Thorlabs), centred at 930nm and a custom scanning head coupled to a Microscope stage. In-depth microstructural imaging was performed by processing the real part of the OCT signal, while phase fluctuations, originating from the imaginary part, were measured by collecting several successive B scans at the same location. Statistics on the first time derivative of the phase, i.e. time fluctuations, were analysed over the acquisition time interval to retrieve overall cell viability [2,3]. Power spectral density of the fluctuations were further analysed as a readout correlated to metabolic activity [4]; whereas spheroid size and phase fluctuation activity, were directly compared with biochemical assays (ATP; Prestoblue).

RESULTS: Cell viability and overall microstructural information were retrieved successfully from optical coherence phase tomography measurements. They correlated well with standard biochemical assays (ATP, Presto blue) when investigating paracetamol cytotoxicity in 3D liver culture. In addition, we introduced a new analysis based on power spectral density to quantify phase fluctuation imaging, and to derive a quantitative parameter of cell activity/metabolism (Fig. 1).

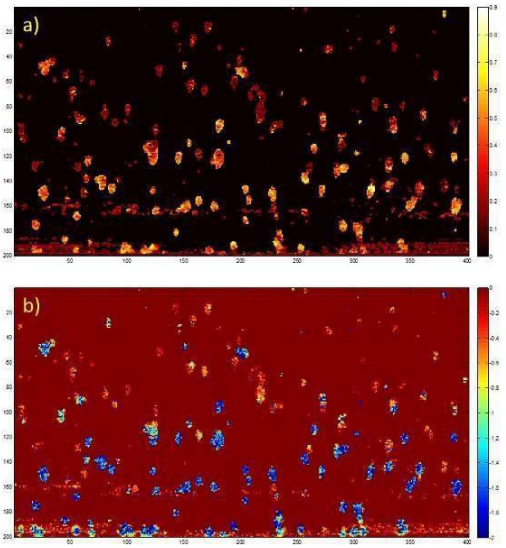

Fig. 1: Images represents a single cross-section through an entire volume of gel containing liver cell spheroids. a) Heat map of phase fluctuations' standard deviation showing cell viability b) Heatmap of the power spectral density slope is related to cell metabolic activity. Pictures are 200 pixels in depth (1mm) by 400 pixels in X (2mm).

DISCUSSION & CONCLUSIONS: We demonstrate the potential of optical coherence phase tomography to perform non-destructive optical toxicity assays. This was exemplified by monitoring change in cell viability of 3D liver tissue models following the addition of a well-known toxic compound. Together with this new set of algorithms, OCPM has the potential to assess noninvasively and label-free drug toxicity in 3D tissue models.

Osteoarthritis-on-a-chip: A novel 3D hydrogel chondrocyte disease model

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INTRODUCTION: Osteoarthritis (OA) can be induced by severe trauma, repetitive microtrauma, or strenuous exercise. A European study showed that 5-11% of the population aged 25-79 suffer from OA[1]. OA is a considerable socioeconomic burden in terms of lost time at work and early retirement. The prognosis for OA patients remains poor as there are no effective therapies that alter the course of the disease[2].
Advances in microfluidics have led to the development of disease-on-a-chip. The goals of this study are: to establish an on-chip injury model to elucidate onset and progression of OA; to study effects of new therapies in an OA-like injury model; and to facilitate high-throughput technology not reliant on animal testing.

METHODS: Articular cartilage was harvested from horses euthanized for reasons unrelated to OA. Cartilage samples digested in collagenase; isolated chondrocytes were routinely cultured until passage 1-2 prior to cryopreservation for further use.

Microfluidic devices were manufactured as previously described[4]. Surfaces were plasma activated and bonded at 70°C overnight.

Chondrocytes were stained with CellTracker™ Dye prior to suspension in fibrin matrix. 3D fibrin matrix was prepared to [20%] with 700 cells/mm3. Cells in 2D culture were maintained in culture flasks. Cells were cultivated for 21 days using routine medium, exchanged every 48hr. Cell morphology was considered elongated when the length was twice the width and spherical when cells showed round physiologic morphology[3]. Diffusion in the hydrogel was determined via fluorescently labelled bovine serum albumin[4]. Fluorescence intensity and biomolecule distribution of determined via image analysis.

RESULTS: Cells were viable for 21 days; morphologic evaluation showed 99 % of cells in 3D maintained physiologic morphology compared to cells in 2D with less than 5% physiologic morphology.

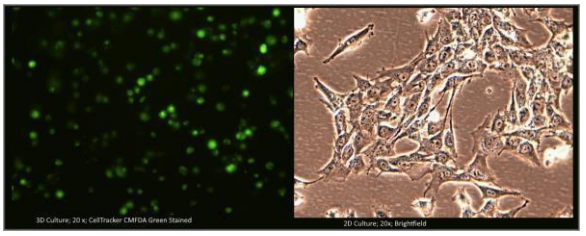

Fig. 1: Chondrocytes in 3D vs 2D culture. 20 x Magnification.

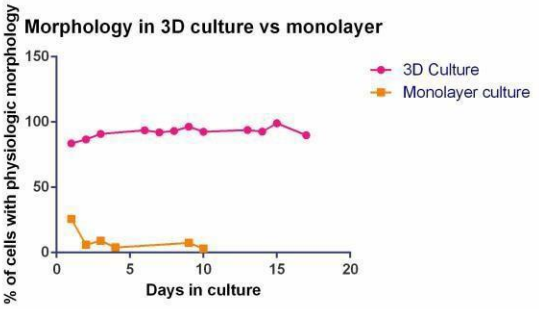

Figure 2. Cell count of Morphology

DISCUSSION & CONCLUSIONS: Microfluidic 3D chondrocyte culture has a high potential for establishment of a novel disease model. The gradient formation is similar to physiologic cartilage and cells remained viable for an extended time.

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

A 3D *in vitro* bone model for the investigation of the role of triggering receptor expressed on myeloid cells 2 (TREM-2) in bone homeostasis

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INTRODUCTION: TREM-2 is a transmembrane receptor expressed on myeloid cells including macrophages, microglia and osteoclasts. In humans, missense mutations in TREM-2 gene significantly increase susceptibility for late-onset of Alzheimer Disease (AD) [1]. Recent studies showed that TREM-2 may play a protective role in AD by suppressing inflammatory responses as well as promoting the removal of extracellular neurotic plaques containing ®-amyloid peptides [2]. In bone, TREM-2 has been shown to be essential for normal osteoclastogenesis with a role in bone homeostasis still unknown. While targeting TREM-2 may provide new opportunities for AD diagnosis and treatment, it is relevant to assess the effect of over-activation in bone homeostasis. Towards this aim, here we established a perfusion based 3D model using human progenitors differentiated into osteoblasts and osteoclasts. Cells were cultured in perfusion on collagen type I scaffold in order to allow a long-term cell culture and to achieve bone- like matrix deposition.

METHODS: Human Bone Marrow Cells (hBMCs) were seeded on a collagen type I scaffold and cultured, with osteogenic medium, in a perfusion bioreactor system (U-CUP, Cellec Biotek) for 3 weeks. Monocytes from peripheral blood were added to the system and further cultured with osteoclastogenic medium for up to 3 weeks. Extracellular matrix (ECM) and calcium deposition were analysed histologically, by Masson's Trichrome and Alizarin Red staining. Qualitative evaluation of cell differentiation was performed by tartrate-resistant acid phosphatase (TRAP) staining for osteoclasts and Bone Sialoprotein (BSP) immunohistochemistry for osteoblasts. Osteoclast activity and bone metabolism were also assessed by ELISA for TRAP5b, N-terminal Telopeptide (NTx-I) and C-terminal pro-peptide of collagen type I (CICP). TREM-2 receptor and shed TREM-2 (shTREM-2) were detected by FACS analysis and ⟨-LISA, respectively.

RESULTS: In the co-culture, a reproducible and homogeneous ECM deposition was achieved (Fig. 1A) confirmed by quantification of CICP. The presence of mineralized matrix and differentiated osteoblasts were observed (Fig.1B and D). Analysis for TRAP5b and NTx-I together with TRAP staining (Fig. 1C) demonstrated the presence of active osteoclasts. TREM-2 was detected both on pre-osteoclasts and in the culture supernatants. A correlation between TRAP5b and shTREM-2 levels in ELISA analysis was observed, suggesting a possible role of shTREM-2 in osteoclastogenesis.

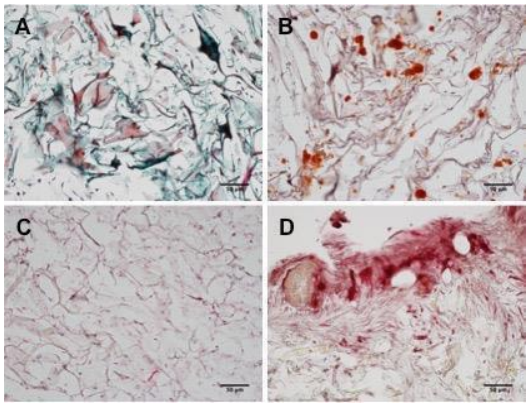

Fig. 1: Co-culture histology. Staining for Masson's Trichrome (A), Alizarin Red (B), TRAP (C) and BSP (D).

DISCUSSION & CONCLUSIONS: Before TREM-2 can be considered as a potential target for AD treatment, its role in bone homeostasis has to be elucidated. Here we present a 3D *in vitro* bone model based on the co-culture of osteoblasts and osteoclasts in an engineered bone-like environment. Our model recapitulates the dynamic interplay of bone components, providing a useful tool to study the effect of the TREM-2 pathway on bone homeostasis. Current studies are ongoing to evaluate the system response to over-activation of TREM-2, using an agonist based approach.

ACKNOWLEDGEMENTS: This study was supported by Roche Pharmaceuticals.

Cryogel based high throughput spheroid culture platform for drug toxicity screening

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INTRODUCTION: Despite advances in 3D cell culture systems, drug toxicity screening studies still rely on 2D systems due to lack of versatility and reproducibility of 3D systems. Further, the spheroids formed in 3D culture should possess robust cell-cell interaction. In this study, the ability of poly N-isopropylacrylamide (pNIPAAm), a temperature-responsive polymer, to facilitate cell spheroid formation on novel 3D poly(NIPAAm-*co*-Gelatin) (pNG) cryogel scaffolds was explored. The spheroids thus formed were employed for drug toxicity screening.

METHODS: The spheroid culture was carried out in pNG cryogels inserted in open-ended 96-well plates. Hep G2 cells were seeded on these cryogel scaffolds in presence of different concentrations of pNIPAAm and their viability, functionality and morphology were analyzed. The concentration of pNIPAAm at which the cell spheroids showed best functionality was used to assess the toxicity of anticancer drugs. Compatibility of the platform with other cells was also assessed.

RESULTS: The physicochemical characterization of novel cryogel showed its potential as cell culture scaffolds. The open-ended 96-well platform was drainage & leakage protected and allowed for easy collection of flowthrough (Figure 1). The LDH release profile of Hep G2 showed that pNIPAAm at concentration higher than 0.05% is toxic to cells. The maximum viability and functionality was observed on Day 5 of culture when cells were seeded in presence of 0.03% pNIPAAm (Table 1). Microscopic analysis also showed formation of larger and organised spheroids (Figure 1) and bile canaliculi in presence of pNIPAAm. The cells responded in dose dependent manner in terms of viability and functionality when treated with anticancer drugs with significant increase in IC_{50} values in case of spheroid culture. The platform was also found to be compatible to MCF7 and A549 cells.

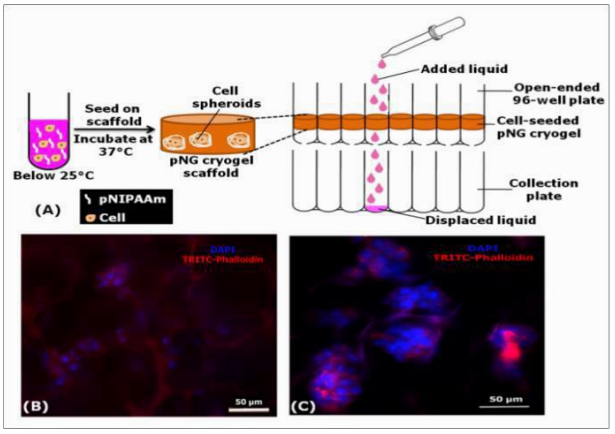

Fig.1: (A) Schematic of pNIPAAm assisted spheroid formation and cryogel-based high throughput platform. Fluorescent staining of Hep G2 cells on day 5 of culture seeded in (B) absence and (C) presence of 0.03% pNIPAAm showing formation of sturdy spheroids.

Table 1. Relative change upon usage of 0.03% pNIPAAm on day 5 of culture.

		Increase (%)
Glucose consumption		44.22
Albumin production		15.75
Phase I metabolic activity	CYP1A1	36.44
	CYP2A6	32.05
Phase II metabolic activity		27.02
IC_{50} Tamoxifen	Hep G2	11.23
IC_{50} Tamoxifen	MCF7	37.03
IC_{50} Paclitaxel	Hep G2	15.03
IC_{50} Paclitaxel	A549	66.98

DISCUSSION & CONCLUSIONS: The transition of pNIPAAm from soluble to insoluble state above 32 °C, tends to bring cells in close proximity and facililate spheroid formation with improved functionality and resistance to drugs. The developed cryogel based platform is widely applicable, versatile and is suitable for drug toxicity screening.

ACKNOWLEDGEMENTS: The authors thank CSIR, India; TATA Innovation fellowship; DBT, India and IIT Kanpur.

Biocompatibility of SiC samples prepared in the form of mono- and polycrystal with a graphene modification

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INTRODUCTION: Silicon carbide (SiC) is considered promising material for biomedical applications [1]. It is also expected that using the graphene modification could further improve SiCs properties in contact with biological environment [2]. This would, however, require a strong connection of the graphene layer with the ceramic. The aim of this study was to determine and compare the biocompatibility of the SiC samples prepared in the form of mono- and polycrystals with a graphene layer on their surface.

METHODS: Studies were performed on the two types of SiC materials: mono- and polycrystal. The SiC monocrystals (6H polytype) were obtained by the physical vapour transport method (PVT). Polycrystals ceramic samples were prepared using a granular SiC (SIKA Tech, FCP 15 RTP), by uniaxial pressing. The sintering process ran at 2150°C for 3 hours, at 500 mbar in the argon atmosphere. The density of the sintered samples were measured according to the Archimedes principle. The microstructure of the samples was characterized by using scanning electron microscopy (SEM, Zeiss). X-ray diffraction measurements were performed on the Siemens D500 powder diffractometer. Micro-Raman scattering analysis was performed by the Renishaw in Via Raman Microscope at room temperature, using the 532 nm line from Nd-YAG as a source of continuous wave excitation. For cytocompatibility studies MG-63 cells were seeded on the surface of all SiC ceramic samples – with and without graphene layer on their surface. The adhesion, morphology and viability of cells cultured on the ceramic samples were analysed at two different time points. Live/Dead fluorescent staining was performed for qualitative assessment of cell viability (Fluorescein diacetate – viable cells; propidium iodide – dead cells). Additionally, cell viability was measured by means of a quantitative Alamar Blue fluorescent test and compared between all the materials. Cells seeded on the standard polystyrene culture plates were treated as a control.

RESULTS: The SEM analysis showed the homogeneity of the microstructure and a high relative density (more than 95%) of the ceramic samples. X-ray analysis confirmed the crystallographic phase of the mono- or polycrystal. Optimization of the sintering process resulted in obtaining the graphene structure on the surface of the SiC ceramic materials. It was confirmed in Raman Microscope – spectrum presented three basic bands: D, G and 2D with displacement 1350 cm^{-1}, 1580 cm^{-1} and 2680 cm^{-1}, respectively. As revealed by Live/Dead assay, most of the cells seeded and cultured on the surface of the investigated materials were spread and viable during the whole observation, performed up to day 7. As further confirmed by Alamar Blue assay, cell number increased with time.

DISCUSSION & CONCLUSIONS: Presented results confirmed the possibility of obtaining a high quality graphene layers strongly connected with the surface of both mono- and poly-crystalline SiC samples, by means of controlled sublimation processing. The results demonstrate in vitro cytocompatibility of all types of SiC materials – prepared in the form of mono- and polycrystals, both with and without graphene layer. This study is a preliminary, encouraging step towards the possible use of the graphene-modified SiC samples for biomedical application.

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ACKNOWLEDGEMENTS: This work was supported by the Firs Faculty of MUW, research project no 1M15/PM12/15.

Understanding why breast implants fail: Physiochemical and fibrotic encapsulation analysis of failed implants

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INTRODUCTION: Breast implants are used for both reconstructive (e.g following mastectomy) and augmentation purposes. These implants, however, invariably fail and it has been reported that up to 28.3% require revisional surgery at 8 years[1.] Failure can occur due to capsular contracture, leakage, extrusion and rupture. Relatively little is, however, understood about the mechanisms of aseptic failure. Further understanding of the failure mechanisms could led to the development of new breast implants with longer life spans. Here we investigated if retrieved implants had altered physicochemical properties (which may causes rupture or leakage) compared to fresh implants. We also investigated if the physiochemical properties of the retrieved implants were related to biological responses in particular fibrotic encapsulation.

METHODS: Ethical approval for retrieval and analysis of breast implants and the surrounding capsular tissue was obtained from Royal Free Hospital Ethical Committee. Demographic and clinical data was recorded (including: age, duration of implant, adjuvant therapy and previous revisional surgery/complications). Each retrieved breast implant was cleaned with iso-propanol and the inner silicone gel removed. Stress-strain curves, tensile, elongation and tear strength and Young's modulus of samples from the anterior implant shells were performed using the Instron 5565 UK. FTIR and contact angle measurements were performed to determine wettability and chemical composition. Tissue samples from surrounding capsule (if present) were fixed with formalin, paraffin embedded and H&E stained. Currently 11 implants have been retrieved and 8 samples of tissue obtained for analysis. There mean patient age was 43.2 years (29-53) and mean age of retrieved implants was 191.3 months (5 – 300). The most common indications for revisional surgery were capsular contracture (n=4), implant rupture (n= 3), scheduled replacement (n=3).

RESULTS: Preliminary results revealed that the mechanical properties of the implants changed with the duration of implantation, with a positive correlation between implant duration and maximal tensile stress (R= -0.6573 p=0.018, strain at break (R= -0.7430, p =0.005) and tear strength (R= -0.7443 p 0.004 significantly reduced with length of implantation. The changes in the mechanical properties suggest chemical changes have occurred during implantation, but further analysis is required to determine what these changes are and the causes of these changes. Fibrotic membranes were found around all implants, the thickness of this membrane and fibrotic organization require further analysis but may be related to both the material properties and duration of implantation.

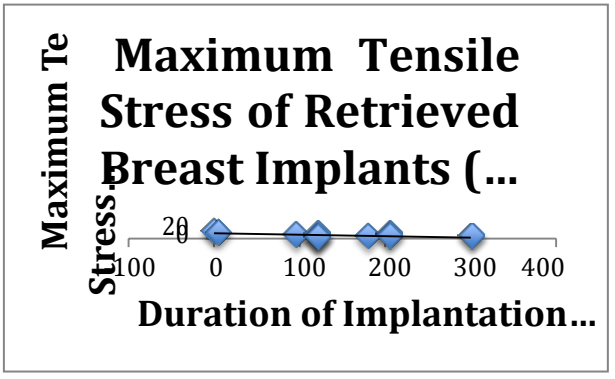

Figure 1: Maximun tensile stress of retrieved breast implants with increasing implantationtime

DISCUSSION & CONCLUSIONS: This analysis of retrieved silicone breast implants demonstrates that the shells mechanical properties significantly weaken with increasing implantation time. We have also demonstrated the cellular response over time to the implants and correlated this with their mechanical properties. As such further research into the mechanism of implant failure as well as development of newer more robust materials is required to prolong the life span of breast implants reducing complications and the need for revisional surgery in this cohort of patients.

Effect of direct current electrical stimulation on cell morphology, proliferation, and osteogenic differentiation of human mesenchymal stem cells

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INTRODUCTION: Direct current electrical stimulation (DC ES) has been utilized as clinical solution to promote healing in bone nonunions as well as delayed unions. Piezoelectric nature of bone suggests plausible role of electrical field in bone remodelling signalling pathways. While the mechanism is still poorly understood, *in vitro* studies showed that DC ES alters several MSCs' behaviours [1, 2]. In this study we investigate the effect of DC ES on morphology, proliferation and osteogenic differentiation of human bone marrow derived mesenchyemal stem cells (hMSCs), during 3 weeks in different types of culture conditions.

METHODS: We compared electrically stimulated (ES) versus not stimulated (Control) cell groups, cultured both in osteogenic induced medium and growth medium, at different time points (week 1, 2, and 3). A laboratory-made device used for applying electrical stimulation; which basically is the L-shaped pure platinum electrodes secured to the top lid of 6-well cell culture plates and connected to a DC electrical power generator [3]. 100mV/mm of DC ES was applied to the cells of ES group for 1hr per day. We modelled the electrical field distribution in the culture wells by Finite Element Method (FEM) using COMSOL-Multiphysics software. To observe cell-shape changes and actin filament distribution, Phalloidin and DAPI staining were applied to both groups. Proliferation was assessed by Alamar Blue assay and DNA concentration measurement. Calcium accumulation, as an indicator for osteogenic differentiation, were evaluated by AlizarinRed staining. AlizarinRed stain was quantified by reading absorbance with spectrophotometer. Calcium amount in cell lysate was measured by colorimetric calcium assay. Total RNA was isolated in each time point from cell lysate and expressions of osteogenic genes (Runx2, Osteopontin, Col1A2, BMP2, ALP) were assessed by RT-qPCR technique.

RESULTS: FE model of the lab-made device showed that electrical field in the culture area between the electrodes is almost homogeneous (80%). Based on the software calculations the average electrical field, which is sensed by the cells, is 88.4 mV/mm. Actual temperature test revealed that there is no significant change in the temperature of cell culture area while it is stimulated. Actin filament staining shows cytoskeleton changes in electrical stimulation groups versus controls. ES significantly accelerates proliferation of hMSCs. ES change osteogenic gene expression patterns and caused a meaningful increase in mRNA levels of BMP2, Osteopontin and ALP at first and second week.

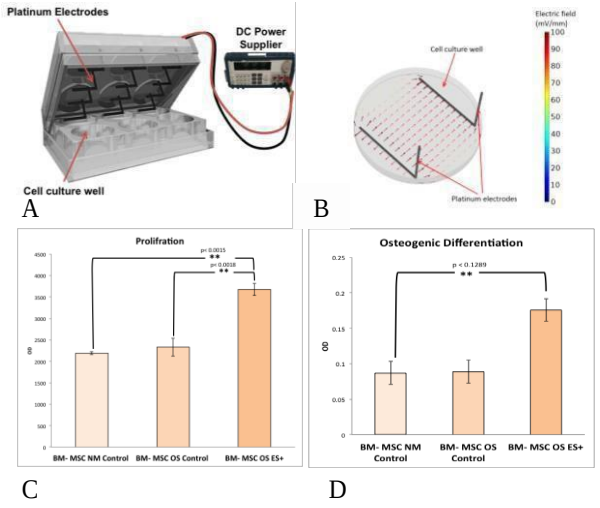

Fig. 1: A) lab-made device for applying electric field to the cultured cells. B) FE modelling of electrical field by COMSOL software. C) Effect of ES on proliferation and D) Differentiation.

DISCUSSION & CONCLUSIONS: This study shows that hMSCs evidently react to electrical stimuli and this reaction is dissimilar in different culture condition. Proliferation of the cells rises by means of ES. Osteogenic induced medium and ES have synergic effect on acceleration and augmentation of osteogenic differentiation of hMSCs.

ACKNOWLEDGEMENTS: This work is financially supported by BBSRC BB/M013545/grant.

Exosomes derived from pluripotent stem cells increase the reprogramming efficiency of MBD3 silenced fibroblast cells

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INTRODUCTION: Methyl-CpG binding domain3 protein (MBD3) ubiquitously expressed in all somatic cells, and promote transcriptional repression through being an essential subunit of Nucleosome Remodeling and Deacetylase (NuRD) complex which possess nucleosome remodeling and histone deacetylation activities [1]. Its suppression has been shown to remove epigenetic barriers of somatic cells [2]. Exosomes are specific type of secreted cellular products carrying mRNAs, miRNAs and several other transcription factors such as pluripotency factors in case of embryonic stem cells [3, 4]. In the present study, we aimed to analyze the impact of the exosomes derived from pluripotent stem cells on the capacity for reprogramming of fibroblasts into pluripotent state.

METHODS: Exosomes were isolated from mouse embryonic stem cells by density centrifugation after the culture of 2 days. To eliminate the effect of feeder layer cells and fetal bovine serum during the derivation of exosomes, feeder-free system was used by culturing ESCs on Nunclon Vita Surface culture plates. Human fibroblasts (foreskin) were modified by shRNA mediated MBD3 knockdown under the control of TET-ON system. GFP expression was observed as a reporter of silencing by flow cytometer and fluorescence microscopy. Exosomes and fibroblast cells were co-cultured for 10 days, and exosome transfer were characterized by PKH26 staining of exosomes. The effect of exosomes on fibroblasts was determined by gene expression analyses for pluripotency genes and telomerase activity.

RESULTS: Following the MBD3 silencing, somatic cells were reprogrammed with exosomes. MBD3 silenced cells showed enhanced cell proliferation and increased stemness character. The integration of exosomes and cells were observed to complete within 16 hrs. The repeated application of exosomes (5-times during 10 days of culture) increased the effect. The considerable change in the gene expression profile was observed in the MBD3-silenced cell line after the exosome treatment. The expression of Nanog and Oct4 genes were observed 2300 and 1600 times compared to the unmodified fibroblast cells, respectively, while c-Myc expression down-regulated related with exosome treatment.

DISCUSSION & CONCLUSIONS: The complete differentiation into ESC-like cells was not observed even in the modified and exosome transferred cell line after 12 days of culture. However, pluripotency factors were increased significantly after the co-culture with exosomes, including Oct4, Nanog, UTF1, Sox2, and Lin28. As future investigation, complete reprogramming might be achieved by long term culturing and culture medium-based changes with additional inhibitors.

Primary human skeletal stem cell bio-physical characterisation for high-purity microfluidic cell sorting and enrichment

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INTRODUCTION: Skeletal stem cells (SSCs) are found in human bone marrow with adipogenic, chondrogenic and osteogenic differentiation capacity. SSCs offer significant potential for skeletal tissue regeneration, however, in the absence of a specific cell surface marker their current isolation methods (FACS and MACS) lack the purity required for clinical translation.[1] Microfluidics provides novel sorting techniques that can isolate cell populations based on the bio-physical properties of single cells.

Here we report the size, deformability and dielectric characterisation of enriched populations of SSCs with the aim to find a bio-physical fingerprint that will allow their label-free isolation with significant clinical impact.

METHODS: Human bone marrow stromal cells (hBMSCs), obtained from patients undergoing total hip replacement surgeries, were enriched by magnetic selection using Stro-1 and Stro-1$^+$ cells were analysed immediately or after adherence to tissue culture plastic. When not selected by plastic adherence, SSCs were identified as fluorescently-labelled CD146$^+$ cells. Cell deformability was measured by real-time deformability cytometry (RT-DC) by image analysis of single cells captured when flowed at high-speed through a narrow constriction (30 µm x 30 µm) in a PDMS micro-channel.[2] Cell size and dielectric properties were measured using microfluidic impedance cytometry coupled to fluorescence optical detection.[3] AC voltages were applied at fixed frequencies to electrodes on the top of a micro-channel and the differential current flowing to bottom electrodes was measured.

RESULTS: RT-DC measurements of culture expanded Stro-1$^+$ hBMSCs (Fig. 1a) show that enriched populations of SSCs are stiffer and larger than the three main human leukocyte populations (lymphocytes, granulocytes and monocytes), all found in the bone marrow. Impedance measurements of these cells (Fig. 1b) reveal similar differences in size with opacity values identical to leukocytes. The size of SSCs (Stro-1$^+$/CD146$^+$) in freshly isolated hBMSC was smaller than that of expanded cells, averaging 9 ± 1.2 µm, but still present within the larger fraction of all hBMSCs (Fig. 1c).

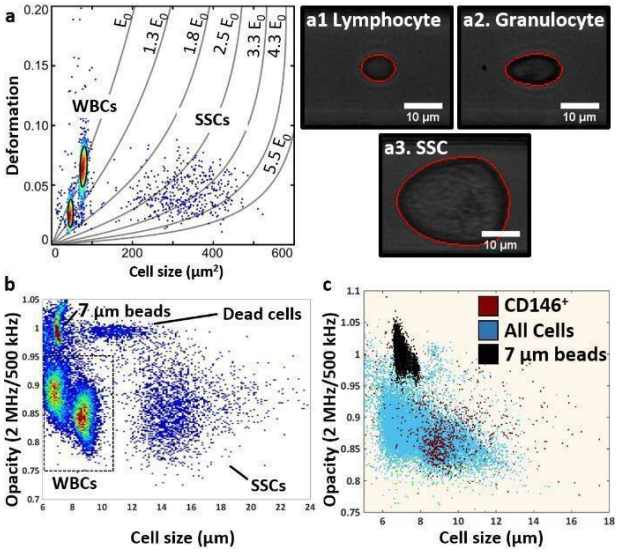

***Fig. 1**: (a) Deformability and (b) opacity vs cell size of Stro-1$^+$ SSCs at passage 0 mixed with white blood cells (WBCs) from the peripheral blood of healthy volunteers. (a1-a3) show captured cells when flowing through the RT-DC micro-channel. (c) shows opacity vs cell size of Stro-1$^+$/CD146$^+$ SSCs in a heterogeneous hBMSC sample.*

DISCUSSION & CONCLUSIONS: While the dielectric properties of SSCs closely resembles that of other cells in the bone marrow (rendering dielectric-based sorting unlikely), SSCs show clear differences in deformability from WBCs. With our findings we aim to design a size and deformability-based cell sorting mechanism to enrich SSC populations from human bone marrow, offering significant therapeutic implications.

ACKNOWLEDGEMENTS: This work was supported by the European Union's Seventh Framework Programme FP7/2007-2013 under REA grant agreement no. 607350.

Human skin decellularization protocol and local tissue reaction in nude mice graft model

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INTRODUCTION: Decellularization of human skin can be helpful in life-threatening condition of skin wounds and injuries. Various methods such as cell transplantation, materials science, and bioengineering are employed to reach the opportunities for restoring normal function of damaged or abnormal tissues and organs [1]. We aim to develop an efficient method for decellularization of human skin based on optimal protocol. Histopathological evaluation biomechanical assessments were done prior to implantation into the nude mice (sub-dermally) in order to evaluate local tissue reaction, cell cytotoxicity and cell seeding into the decellular matrix.

METHODS: Abdominoplasty excess skin of adult patients who underwent abdominal cosmetic surgery was used. First the skin was decellularized with detergent based methods then washed several times with phosphate buffered saline (PBS) to remove residual toxic materials. All skin scaffolds were evaluated by hematoxylin and eosin (H&E), Movat's Pentachrome, Sirius red, trichrome staining and SEM to confirm decellularization and preservation of extracellular matrix. A biomechanical evaluation was performed to evaluate tensile properties after decellularization. Finally the specimens were implanted into the nude mice for in vivo cytotoxicity and evaluation of local tissue reaction.

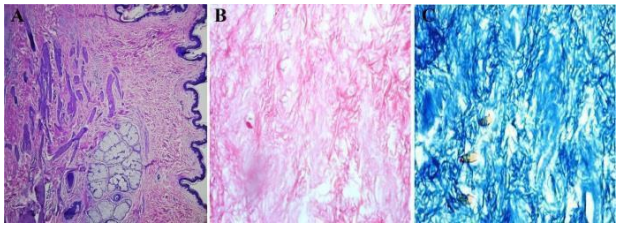

Fig .1: HE and trichrome staining of control and acellular skin

RESULTS: Histological examination confirmed the skin was completely decellularized. The extracellular matrix (ECM) evaluation showed that the collagen fibers of decellularized tissue were preserved compared to control group. Biomechanical data demonstrated no significant change and tissue ultrastructure has remained intact.

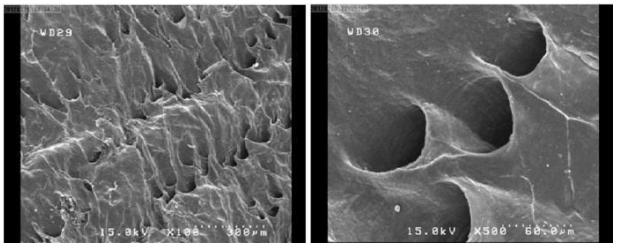

Figure 2. Scanning electron microscopy

DISCUSSION & CONCLUSIONS: The present optimal method of skin decellurization with intact ECM without any ultra-structural distortion is simple, safe, and minimally toxic to the cell growth with in-vivo cell growth in nude in nude mice. Data suggests that finding optimal decellularization protocol could pave the road for safer clinical application in skin tissue engineering with minimal in vivocytotoxicity.

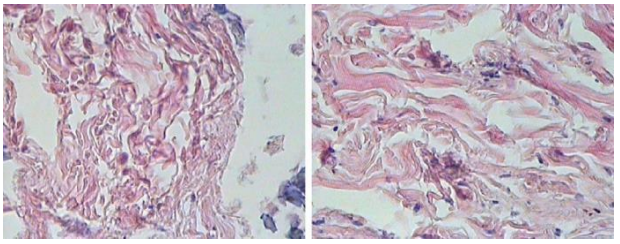

Fig.3: Histology of grafted acellular skin in nude mice subdermally.

1

DISCLOSURES: Authors declare no conflict of interest

Comparison of two different decellularization and recellularization protocols of kidney orgn in efficiency of cell removal, biocompatibility and further recellularizaion potential

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INTRODUCTION: Extracellular matrix (ECM) has been considered as a platform for kidney engineering [1-2]. We evaluated the efficacy of decellularization and further recellularization of three- and two-dimensional rat kidney scaffolds. Static- and perfusion-based techniques were compared both in decellularization and recellularization processes.

METHODS: Whole kidneys were obtained from 12 healthy Sprague Dawley rats and divided into three groups. In GI (N=4, control), specimens were kept in phosphate buffer saline. Kidneys of GII (N=4) were cannulated via the renal artery under sterile condition and decellularized with a detergent-based method. To mimic cardiac cell composition, we reseeded these constructs with Green Fluorescent Protein (GFP) renal cells with a peristaltic pump. Thin slices of the other 4 kidneys (GIII, N=4) were decellularized and recellularized with static method. Histological staining, scanning electron microscopy (SEM), and DNA quantification, were performed in all natural, decellularized and recellularized kidneys.

Fig 1: decellularization of whole organ with perfusion-based method

RESULTS: According to the results of histopathological evaluations and DNA quantification, all cellular and nuclear components were completely removed from the scaffolds of both groups. However, SEM analysis revealed that ECM was better preserved in scaffolds of GII. Similarly, cells were effectively seeded in both scaffolds with better arrangement and a higher density in perfusion-based technique.

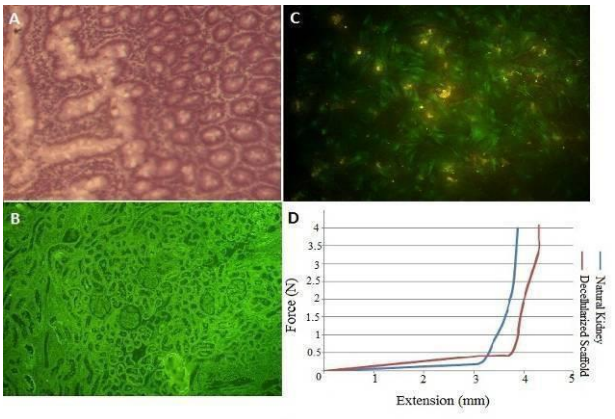

Fig 2: (A-B) H&E and immunofluorescent evaluation of recellularized scaffolds (C) cultured renal cells (D) tensile test

DISCUSSION & CONCLUSIONS: Scaffolds obtained from perfusion-based method seems to have satisfactory geometrical properties in ECM for further cell seeding of renal cells. The results of the current study suggests that dynamic technique may have more promising results in organs with distributed micro-vasculature system as compared to organs with empty chambers and poor vasculature system.

DISCLOSURE: Authors have nothing to disclose.

Optimization and critical evaluation of decellularization and recellularization strategies to develop bioengineered heart: comparison of two different protocols

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INTRODUCTION: The extracellular matrix (ECM) provides structural support for heart engineering [1-2]. We determined the superiority of perfusion-based or static-based decellularization method of heart tissue for cell removal and ECM preservation. Histological characteristics of these techniques in recellularization of decellularized heart matrices were also evaluated.

METHODS: Heart tissues were obtained from 12 healthy Sprague Dawley rats and separated into 3 groups. GI: Natural tissues. GII: cannulated via superior vena cava and one of the pulmonary veins and decellularized with perfusion-based method. Inferior vena cava and other pulmonary veins were cautiously ligated to conduct the solutions to the heart cavities. Dynamic cell seeding with green fluorescent protein (GFP)-positive heart cells was performed with the same method. In GIII, thin slices of heart tissue were decellularized and recellularized with static-based method. Histological examination, scanning electron microscopy (SEM), DNA quantification and immunohistochemical (IHC) staining were performed.

Fig1: whole organ decellularization with perfusion-based technique

RESULTS: DNA quantification and histological evaluations confirmed the removal of all cellular and nuclear components in GII and GIII. However, SEM analysis showed that ECM was better preserved by applying perfusion-based method. After recellularization process, heart cells were more dominant on the inner surface of the scaffold as compared to the outer layer in GII. While recellularization process was promising both in whole organ and slices of heart tissue, cells were better seeded on slices.

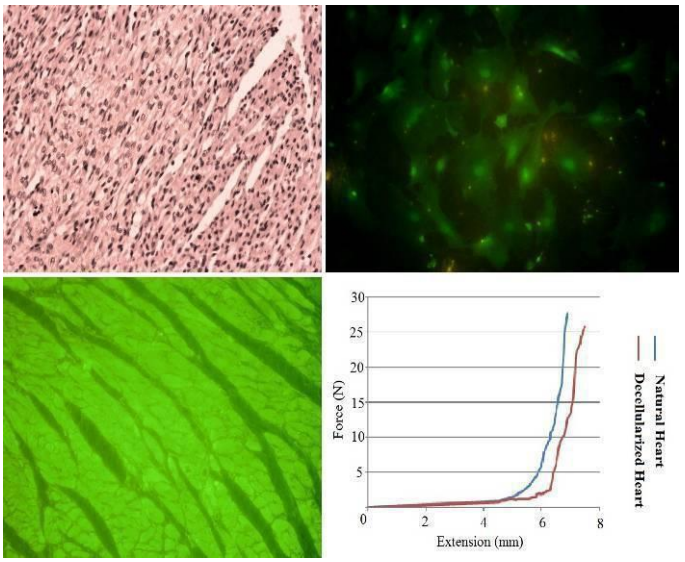

Fig 2: (A-B) H&E and immunofluorescent evaluation of recellularized scaffolds (C) cultured heart cells (D) tensile test

DISCUSSION & CONCLUSIONS: Although, perfusion-based method may be a promising technique for tissue-engineering of organs with a defused vasculature system within the tissue, its application for organs with empty cavities and thick muscular walls may not be aseffective.

2

DISCLOSURE: Authors have nothing to disclose.

Different xenon conditions for preservation of viable tissue in order to use in regenerative medicine

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INTRODUCTION: Nowadays skin graft preservation is a highly important procedure due to several reasons. On the one hand it is an obligatory tool to treat different skin burns. On the other hand it is an endless source of different cells using in regenerative medicine that can be sown on scaffolds to make bioengineered skin or other constructs. Therefore it is so important to preserve this tissue appropriately to maintain sufficient viability. Basic preservation methods do not provide sufficient viability to stored skin grafts or have time limitation. Meanwhile the technique based on xenon (Xe) clathrate formation probably seems rather useful for it because it was shown the possibility to save viability of some bioobjects after storage in these conditions. Thus, this study dedicated to comparison different xenon preservation approaches either clathrate or hyperbaric actions as well as usual DMSO cryopreservation technique.

METHODS: The clathrates were obtained in custom-made gas chamber with temperature and gas pressure regulation. Skin grafts were obtained from the 28 albino rats tails and separated into four groups. The experimental groups were the following: 10% DMSO with deep-freezing (195K), Xe hyperbaric (30–60 psi, 277K) and Xe chlathrate (90–105 psi, 277K). Skin samples were auto-transplanted after 7 days preservation on the new freshly-made tail places. As a control measure, the unpreserved fresh skin samples were auto-transplanted immediately. We assessed the viability of the graft's sections after one week preservation by 5 microscopic criteria immediately and then the engraftment was assessed 14 days after transplantation by 3 macroscopic criteria in accordance to described techniques [1]. Statistical data was assessed on GraphPad Prizm 6.0h.

RESULTS: It was shown lack of significant differences between Xe clathrate and intact (unpreserved) skin groups by micro- and macroscopic criterions. In the Xe clathrate preservation group, we observed better engraftment and microstructure of dermal as well as epithelial layers that were comparable with unpreserved fresh skin. On the other hand, the lowest engraftment and more destruction in all layers were shown using the usual method with DMSO as a main preservation agent and Xe hyperbaric protocol.

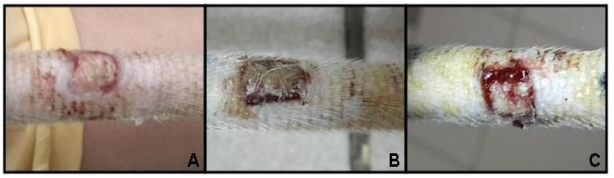

Fig. 1: Engraftment of preserved skin grafts on 14 day of transplantation. A- Xe clathrate skin. B- DMSO skin. C- Xe hyperbaric skin

Table 1. Integral viability indicators of different preserved skin grafts

	Intact skin	DMSO	Xe clathrates	Xe hyperbary
Macro points	4,0±2,2	2,7±2,2 p≤ 0,05	3,5±2,0	3,3±2,3
Micro points	8,0±0	6,0±1,7 p≤ 0,05	7,7±0,7	5,3±2,2 p≤ 0,05

* - p≤ 0,05 compare to intact skin by Kruskall-Wallis test

DISCUSSION & CONCLUSIONS: Significant Xe clathrate preservation effect that was observed in this study might be linked to clathrate anabiosis hypothesis. Accordingly its there is no possibility for biochemical reactions in clathrate immobilized cellular water. Thus, this state can reversibly preserve viable tissue that was shown in our results. Contrary it is so important to provide exact clathrate conditions because the usage of simple Xe hyperbaric action does not guarantee the proper result due to keep water in liquid phase. Performed study might be the basis of further Xe research probably linked to ovarian tissuepreservation.

3D scaffold-free cartilage transplants (SFCTs) as a new way to the cartilage tissue engineering

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INTRODUCTION: The limited self-repair capability of damaged hyaline cartilage is the main cause of joint arthrosis. Therefore, different tissue-engineering methods have been developed to address this particular problem. We focused on the development of a new technology to produce a 3D scaffold-free cartilage transplant (SFCT) by intermittent mechanical stimulation of the chondrocyte pellet cultures.

METHODS: For the production of SFCTs the equine chondrocytes of the stifle joint were used. After cultivation and proliferation in monolayer cultures, cells were transferred into a three-dimensional structure without any artificial matrix, growth or differentiation factors. In order to produce scaffold-free transplants and extracellular matrix, the samples were exposed to undifferentiated mechanical pressure (manually) [1].

The SFCTs were analysed after 4-6 weeks of culturing. Chondrogenesis in SFCTs was documented by macroscopic, biochemical, immunohistological and biomechanical analyses. For biomechanical investigations, the E-modulus of samples was determined using a material testing machine INSTRON® 4466 (100 N). The uniaxial compression of the specimens was effected with a testing velocity of v = 1 mm/s, with an initial load of F = 0.1 N and with a strain of 30%. Native equine joint cartilage was used as a control.

RESULTS: The biochemical results clearly demonstrate that SFCTs have a high amount of proteoglycan and collagen (20% and 45% of the native tissue, respectively). The biomechanical results show that the E-modulus of SFCTs was about 30% of the native tissue E-modulus (see Tab.1).

Also, histochemical examination revealed clear staining for proteoglycan and collagen. Even though immunohistological analyses indicate the presence of collagen type I, collagen type II was the main component of extracellular matrix in SFCTs. All SFCTs exhibit a remarkable size (see Fig.1).

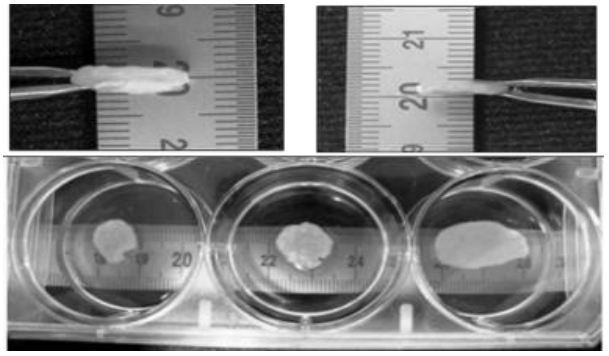

Fig. 1: Macroscopic view of 3D scaffold-free cartilage transplants (SFCTs).

Table 1. Biochemical and biomechanical characteristics of native cartilage and SFCTs.

Specimens	Collagen	E-modulus
SFCT	30.1±1.9 µg	0.26±0.12 MPa
native	64.9±5.0 µg	0.89±0.11 MPa

DISCUSSION & CONCLUSIONS: The presented results lead to the conclusion that tissue engineered constructs created by scaffold-free technology can be applied to the rehabilitation of damaged articular cartilage in veterinary and human medicine. Mechanical stimulation can be used as a stimulatory factor for cartilage development *ex vivo.*

ACKNOWLEDGEMENTS: This study is a part of research project supported by the German Ministry of Economy and Technology: INNO-WATT Reg. Nr.: IW091034.

Evaluation of different biomaterials and cell types for use in skeletal muscle engineering

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INTRODUCTION: Skeletal muscle can repair/regenerate efficiently due to resident muscle stem cells called satellite cells. However, this process is ineffective for restoring significant muscle loss following extensive trauma, such as after road traffic accidents or tumour removal. Intramuscular injection of myogenic cells can improve muscle regeneration, but this approach is inefficient for large scale trauma. One strategy to treat volumetric tissue loss is grafting myogenic cells together with a biocompatible scaffold to provide a supportive environment for both donor and host cells.

METHODS: Immortalised murine (C2C12) or human myoblasts (C25Cl48[1]), primary murine satellite cells or muscle fibres were encapsulated in different 3D scaffolds. These were composed of polyethylene glycol-fibrinogen hydrogel (PEG-FN, 8-10mg/ml), collagen I (1.6mg/ml) or bovine fibrin (5mg/ml). Scaffolds were immobilised between two silicon posts to generate tension across the biomaterial[2], and myoblast proliferation and differentiation assessed.

RESULTS: Immortalised murine (C2C12) and human myoblasts (C25Cl48) were capable of proliferation in all three materials, as shown by Ki67 immunostaining after two days of culture. They also readily differentiated into multinucleated myotubes, with the generated tension facilitating myotube alignment parallel to the direction of force. Spontaneous contraction was observed for C2C12 encapsulated in PEG-FN after over 20 days in culture. Primary satellite cells embedded in 3D scaffolds generally survived but did not differentiate well, with no evidence of spontaneous contraction. However, freshly isolated myofibres with their associated satellite cells were able to produce many myoblasts that proliferated and differentiated into large multinucleated contractile myotubes. This effect was observed with fibres encapsulated in all three different scaffolds. The number of myoblast progeny generated and the strength of contraction appeared strongest if the myofibres were embedded in bovine fibrin. Tension generated across scaffolds facilitated orientation of myotubes derived from all myoblast sources in all biomaterials, and so favours myotube maturation independently of the biomaterial.

DISCUSSION & CONCLUSIONS: All three biomaterials were used at concentrations that have been shown to be feasible for skeletal muscle tissue engineering. There was no obvious difference in myoblast behaviour in the varying biomaterials. However, the study shows that satellite cells delivered in an intact niche proliferate and differentiate more efficiently than expanded satellite cells. Importantly, while tension across the 3D scaffold facilitates aligned myotube formation, it does not seem to increase myotube contraction. It can be speculated that beneficial effects of the alignment would be more noticeable after a longer culture period.

The enhanced myotube contractility observed when satellite cells are used with their niche could be influenced by trophic factors and/or associated fibroblasts.

ACKNOWLEDGEMENTS: This work was funded by the European Commission FP7-NMP- 2010-LARGE-4 (Grant number: 262948-2) Rational Bioactive Materials Design for Tissue Regeneration (BIODESIGN).

Tissue photo-bonding using biopolymer crosslinked with rose bengal

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INTRODUCTION: Visible light-activated photo-bonding of tissues presents an attractive alternative to surgical suturing for closing of dermal wounds. The advantages of photo-bonding include reduced inflammation and less scarring. However, the materials currently used for light-activated wound closure allow for only a limited, if any, ability to modulate the biological and mechanical properties of the resulting bonded tissue. Particularly, the micrometric void within dermal wounds presents another obstacle for the photo-bonding materials, since collagen structures are required for the photobonding to occur. Therefore, developing a light-activated biomaterial glue that can overcome these limitations would represent an important advance in translational potential. To this end, we have developed a new generation of photo-bonding materials based on vinyl-modified collagen and rose Bengal that are crosslinkable using visible light.

METHODS: Different formulations of hydrogels were prepared from vinyl-modified collagen mixed with positively charged amino acids, and rose Bengal, which is used as a photoinitiator, so that the gel can be cross-linked by green light illumination. Different conditions of illumination were used to produce scaffolds with varying physical and biological properties.

RESULTS: The optimal illumination parameters were determined by measuring the bleaching of the photoinitiators with increasing illumination time and subsequent differential scanning calorimetry experiments to determine denaturation temperature. Gels containing rose Bengal bleach most significantly within 5 min, after which the denaturation temperature of the gels was ≤ 40°C, while 10 min of irradiation produced materials with denaturation temperatures of about 48°C. The swelling properties of the gels were examined in saline solution at 37°C. The gels exhibited weight loss associated with water displacement from the matrix, which was partially rescued by amine containing amino acids. Finally, we demonstrated successful photo-bonding properties of the photo-crosslinkable hydrogel; it was able to bond a biopolymer as well as samples of mouse skin *ex vivo*. Different compositions of the hydrogel resulted in different mechanical properties of the photo-bonded system (Table 1).

Table 1. Mechanical properties of a biopolymer and mouse skin photo-bonded using different variations of the light-activated hydrogel. MC – modified collagen, RB – rose Bengal.

Hydrogel composition	Modulus (MPa)	Breaking strain (%)
Biopolymer (control)	**2.4**	**6.1**
MC+RB	1.6	5.8
MC+RB+Arg	1.4	5.4
MC+RB+0.025% Lys	0.3	8.9
MC+RB+0.05% Lys	0.2	4.4
Skin (control)	**0.6**	**35.2**
MC+RB	0.1	17.2

DISCUSSION & CONCLUSIONS: Using the visible light-excited photoinitiator rose Bengal, we produced vinyl-modified collagen scaffolds with tunable properties. Scaffolds illuminated for 10 min are stably crosslinked within the physiological temperature range. Gels exhibit weight loss associated with water expulsion, which was partially prevented by adding positively charged amino acids. The hydrogel was capable of bonding a biopolymer and mouse skin tissue *ex vivo*, but further *in vivo* experiments are needed to characterize its suitability for use in dermal wound closure. In summary, this new photo-crosslinkable hydrogel presents attractive properties for future clinical translation in tissuephoto-bonding.

ACKNOWLEDGEMENTS: This work was funded by an operating grant from Vetenskapsradet to MG, a Burroughs Wellcome Fund Travel Grant Program to EIA and IK, and by Discovery Grants #342107 to EJS and RGPIN-2015-06325 to EIA from the Natural Sciences and Engineering Research Council.

Bone tissue regeneration – functionalization of biomimetic scaffolds with a central, growth factor-loaded depot for cell attraction and stimulation of angiogenesis

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INTRODUCTION:

The classical tissue engineering approach is still limited by the lack of vascularization leading to an insufficient oxygen- and nutrient supply of cells in inner scaffold regions. In this study, porous biomimetic scaffolds out of mineralized collagen [1] were functionalized with a central, growth factor-loaded hydrogel-depot in order to promote angiogenesis and to attract cells with regenerative potential. Hypoxia conditioned medium (HCM) from human mesenchymal stroma cells (hMSC) served as autologous growth factor mix, which was integrated into the hydrogel depot. Chemoattractive and angiogenic factors released from the depot generate a gradient within the scaffold expected to induce angiogenesis and cell migration towards the central depot (*in situ* tissue engineering).

METHODS:

For scaffold preparation a mineralized collagen suspension was freeze-dried and stabilized by EDC-crosslinking leading to an interconnected porous structure [1]. HCM was generated by hMSC (hTERT-MSC, Prof. Schieker; LMU Munich), which were exposed to hypoxia for 72 h (1 % O_2) [2]. HCM composition was analysed by an angiogenesis array (R&D Systems) and its cell-attractive potential was investigated by transwell assays. For depot integration, HCM was concentrated by dialysis and freeze drying and resuspended in either 1 % alginate, 1 % alginate with 1 mg/mL heparin or 1 % hyaluronic acid. The growth factor-loaded hydrogels were injected in the scaffolds centre. To characterize the angiogenic and chemoattractive potential of the depot, hMSC and human umbilical vein endothelial cells (HUVEC) were seeded as coculture on top of the scaffolds. The formed prevascular network was fluorescence stained for CD31 and analysed regarding length, branching and penetration depth of the tube structures into the scaffold.

RESULTS:

hMSC cultivated under hypoxic conditions secreted various angiogenic growth factors like VEGF, angiogenin, pentraxin-3, thrombospondin and fibroblast growth factor-7 (VEGF: 25 - 35 ng/ mL). HCM highly stimulates cell migration and angiogenesis (Fig. 1). Compared to VEGF-loaded depots (100 ng), HCM-depots led to a clearly more dense prevascular network on the scaffolds surface and tube structures, sprouting deeper throughout the interconnected pore system towards the central depot.

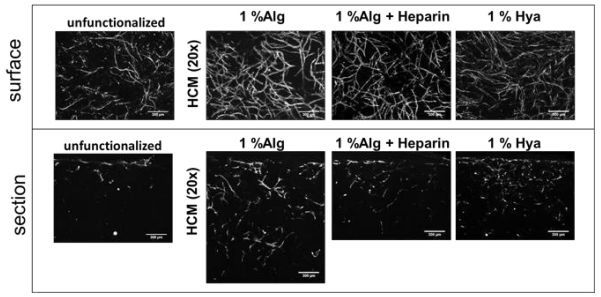

Fig. 1: Prevascular networks on/in scaffolds functionalized with HCM-loaded hydrogel depots after 10 d of incubation, fluorescence stained for CD31

DISCUSSION & CONCLUSIONS:

Our findings show that HCM is a potent alternative to recombinant VEGF to stimulate angiogenesis and cell migration. A HCM-loaded hydrogel depot is a suitable strategy to promote angiogenesis and cell ingrowth in scaffolds and expect to accelerate bone defect healing.

ACKNOWLEDGEMENTS:

This study is founded by the German Research Society (DFG) (Collaborative Research Centre/Transregio 79 (SFB/TRR 79, subproject M4).

Species specific staining of extracellular matrix proteins reveals remodelling of decellularized pulmonary heart valves in a pig-to-sheep transplantation model

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INTRODUCTION: Fresh decellularized allogeneic heart valves are currently tested in two multicentre clinical trials (ESPOIR, ARISE). Preclinical results using large animal models and first clinical experiences reveal the superiority of these valves compared to currently available heart valve substitutes in terms of durability and performance [1,2]. Especially the growth adaptation shown in paediatric patients is a unique feature of these valves, which has the potential to make reoperations in children obsolete. However, the processes leading to the clinically observed increase in the orifice area of the valve during somatic growth of the patients are still unresolved. Therefore, we investigated repopulation and matrix turnover in decellularized porcine heart valve matrices using an orthotopic xenotransplantation model, which allowed the discrimination of donor and host extracellular matrix proteins by applying species-specific antibody staining.

METHODS: Three detergent (SDS/Triton X-100) decellularized porcine pulmonary heart valves have been implanted orthotopically in black-headed sheep for 9 months. Performing echocardiography at the time of implantation and explantation revealed an absolute physiological functionality of the valves. Explanted valves have been embedded and frozen in O.C.T. in order to investigate matrix repopulation and remodelling by immunofluorescence. With the aim of finding species specific antibodies for Collagen I, different anti-Col1A1 antibodies were tested using decellularized porcine and native ovine heart valves. Finally, a double staining using a porcine specific Collagen I-antibody combined with an antibody that binds to porcine as well as to ovine Collagen I was applied. In order to identify cells responsible for matrix production, described double staining was combined with an anti-proCollagen I staining.

RESULTS: DAPI staining of cryosections revealed a higher repopulation density of the proximal valve part in direct comparison to the distal part. In all three cases valve leaflets were only repopulated at the surface. The species specific Collagen I staining showed that most of the Collagen I detected in the valve leaflets was of porcine origin. In the vessel part of the valves, porcine Collagen I was also found. However, at sites of repopulation porcine specific Collagen I stain was less intense or absence, indicating a replacement of the porcine by ovine Collagen I. ProCollagen I positive cells were mainly found in the neo-tissue at areas of proximal as well of distal anastomoses and in the repopulated valve vessels at the border between ovine and porcine Collagen I.

DISCUSSION & CONCLUSIONS: The applied xenogeneic implantation model (porcine valves into sheep) provided results comparable to prior conducted allogeneic orthotopic valve implantation studies in terms of repopulation and inflammation [3]. Specific staining of porcine Collagen I identifies the valve leaflets as a critical area, were most of the porcine Collagen I was still present and not exchanged by ovine Collagen I after 9 months of implantation. On the other hand we found considerable evidence that in repopulated areas of decellularized porcine valve matrices porcine Collagen I was replaced by its ovine counterpart. Furthermore, we assume, that first repopulating cells are also responsible for collagen remodelling as procollagen I producing cells were mainly found in boarder zones between unpopulated and repopulated areas of the vessel wall. Taken together, xenogeneic implantation of porcine decellularized heart valves into sheep in combination with the application of species-specific collagen staining enables new insights concerning remodelling characteristics of decellularized tissues in vivo.

Remote activation and spatial modulation of Wnt signalling for regulating hMSC osteogenesis and bone formation

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INTRODUCTION: Wnt signalling regulates many biological processes, including stem cell maintenance and osteogenesis. Enhancing tissue repair by regulated activation of Wnt pathways in adult stem cells holds promise for a variety of disorders. Therefore, the development of techniques for localized targeting and spatial Wnt gradients may provide a suitable therapeutic option. Previous work has demonstrated the feasibility of remote activation of Wnt pathways[1]. Here, we demonstrate nanomagnetic activation of the Wnt signalling cascade with peptide-functionalized Magnetic Nanoparticles (MNP) *in vitro* and show how this strategy can be employed to set up spatial cues in an *ex vivo* chick femur model. In addition, we report how gradients of localised Wnt binding can regulate the differentiation of mesenchymal stem cells (MSC) in hydrogels and regulate bone *ex vivo* bone formation.

METHODS: MNP (Micromod) were covalently coated with Wnt receptor targeting peptides. hMSC (Lonza) were labelled with MNP (25µg/mL) for 1.5h before stimulation in an oscillating magnetic force bioreactor (MICA Biosystems). Cultures were stimulated in 1h/3h sessions. Immunocytochemistry was performed to monitor mobilisation of the intracellular messenger β-catenin. MSC were also transiently transfected with a Wnt TCF/LEF luciferase reporter. Luciferase activity was monitored over 24h after treatment with MNP. For *ex vivo* experiments, femurs from day 11 Dekalb chicks were extracted and organotypically cultured for 24h. Femur's were then injected with MNP labelled hMSC and magnetically stimulated for 10h over 14 days. Femurs were analysed by micro computerised tomography (µCT) and histological analysis. Statistical significance was determined using ANOVA. Additionally, the effect of Wnt gradients on MSC was assessed using Wnt immobilised substrates. MSC were seeded over immobilised Wnt protein then overlaid with collagen1 hydrogel. Immunocytochemistry was used to assess cell migration and expression of stem cell and osteogenic markers.

RESULTS: Treatment with peptide-MNP initiated mobilisation of β-catenin and increased TCF/LEF controlled luciferase expression over 24h, indicating active Wnt signaling. An added effect was observed when magnetic fields were applied to MNP. Injection of MSC labelled with MNP resulted in increased mineralised bone volume. Histological analyses confirmed new bone was formed around MSC injected epiphyseal areas of the femur. Presentation of active Wnt gradients using immobilised Wnt platforms led to increased cell migration through collagen gels. Cells distal to the Wnt signal showed enhanced osteocalcin expression whilst cells at the basal layer, proximal to the Wnt signal maintained Stro1 expression.

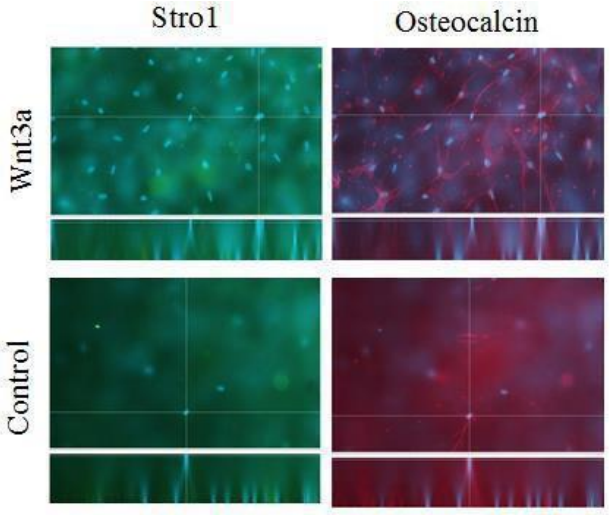

Fig. 1. Wnt induces cell migration with migrated cells showing increased OCN expression.

DISCUSSION & CONCLUSIONS: These studies demonstrate the potential for remote nano-activation and localised regulation of Wnt pathways for stem cell conditioning. These techniques have clear applications for optimising stem cell therapies in bone tissue engineering.

ACKNOWLEDGEMENTS: The authors acknowledge Dr S. Habib (Kings College London) for providing the immobilised Wnt platforms.

Spatial characterization of different types of human nasal cartilages: Basis for successful nasal cartilage engineering

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INTRODUCTION:

For tissue engineering of the nose, a detailed knowledge of biochemical and biomechanical characteristics of different types and regions of human nasal cartilage is required. Correlations of biochemical and biomechanical findings as well as spatial and morphological analyses will enable a new understanding of structural and functional properties of human nasal cartilage. This understanding will be the key for successful tissue engineering of nasal cartilage.

METHODS:

Ten entire fresh-frozen human noses (average age: 62.6 ± 8.6 years) were obtained from Science Care (Phoenix, Arizona). All tissue donors gave informed consent to Science Care. Furthermore, the study was approved by the University of Ulm Ethical Committee (44/15). Septal mucosa and perichondrium were removed carefully. Specimens of septal and alar cartilages respectively were evaluated by confined compression testing. Furthermore, hydroxyproline, glycosaminoglycan- and DNA-quantification was performed. In order to analyse structural characteristics in detail histological (H&E and alcian blue) and immunohistochemical analyses (Collagen I and II, COPM and matrilin-3) were performed.

RESULTS:

All anatomical sites showed a significant increase of equilibrium modulus with increasing compression loads. There was a significant difference of mean equilibrium modulus between septal and alar cartilage, with septal cartilage possessing a significantly higher equilibrium modulus. Hydroxyproline content of septal and alar cartilage was comparable. GAG content, DNA content as well as cell number was significantly higher in alar than in septal cartilage. Alcian blue staining revealed a three-zonal structure of septal cartilage with differences in cell shape, alignment and number. In the superficial layer numerous flat, elongated chondrocytes were found. In the intermediate zone there were less cells with a more oval morphology, aligned palisade-like. In the central zone, cells with heterogeneous morphology were distributed sparsely and were surrounded by large intercellular areas. The structure of the alar cartilage resembled those of nasoseptal cartilage but exhibited a less obvious and less prominent three-layered structure.

Fig. 1: Immunohistochemical staining for COMP (cartilage oligomeric matrix protein) with typical 3- layer structure in the different types of nasal cartilage.

DISCUSSION & CONCLUSIONS:

Hyaline cartilage from closely adjacent nasal anatomical subunits differs significantly in functional, biochemical and morphological features. Alar and septal human nasal cartilages exhibit a layer-based architecture with distinct distribution of cartilage specific matrix proteins such as COMP. Successful tissue engineering efforts will need to take care of these obvious differences in order to produce tissue, which mimics the origin most closely.

Antibiotic-free scaffold for the treatment of infection and regeneration of bone

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INTRODUCTION: The bone infection osteomyelitis (OM) is most commonly caused by S. *aureus* and *S. epidermidis.* Following surgical debridement, long-term systemic high dose antibiotics and bone grafting are typically required. Antibiotic resistance is an increasingly concerning issue; 70% of bacteria are resistant to some/all antibiotics [1]. An alternative approach is to utilise non-antibiotic antimicrobials – however, these materials must be delivered locally and a fine balance is needed between mammalian and bacterial cell toxicity. The overall aim of this project is to develop a biomaterial scaffold that can 1) deliver non-antibiotic antimicrobials at the site of infection, and 2) regenerate bone.

METHODS: Metal salts – copper chloride, silver nitrate, and zinc chloride – were dissolved in deionised water and chitosan was dissolved in 1% (v/v) acetic acid. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the metal salts and chitosan against *S. aureus*, *S. epidermidis*, and *E. coli* was determined. Their effect on the cellular viability of a mouse pre-osteoblast cell line (MC3T3) was determined by PicoGreen® DNA assay. For the controlled, prolonged release of ions, chitosan/metal salt microparticles were produced by a spray-drying process. The antimicrobial materials were then incorporated into freeze-dried collagen scaffolds.

RESULTS: Metal ions and chitosan are toxic towards bacterial and mammalian cells, necessitating a controlled release system:

A

	S. Aureus		*S. Epidermidis*		*E. Coli*	
	MIC	MBC	MIC	MBC	MIC	MBC
Chitosan (mg/ml)	0.94-1.88	-	0.938	-	1.875	-
Copper chloride (mM)	8	8	8-16	8-32	8	8-16
Silver nitrate (mM)	0.5-1	1-2	<0.25-1	2-4	<0.25-0.5	0.5-2
Zinc chloride (mM)	1-8	16	1-2	8-32	2-8	16-32

B

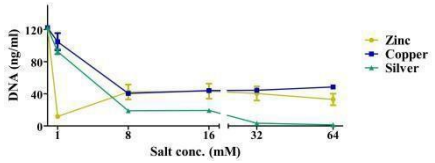

Fig.1: Chitosan and silver, copper, and zinc ions are effective against S. aureus, S. epidermidis, and E. coli (A) but toxic towards mammalian MC3T3 cells with increasing metal ion concentration (B).

To control the release of ions, spray-dried chitosan/metal ion microparticles were produced and incorporated into collagen scaffolds:

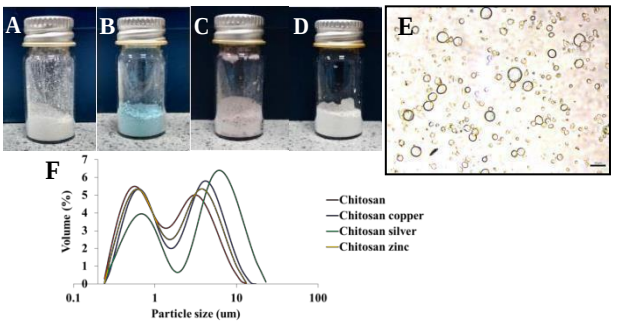

Fig. 2: (A) Chitosan, (B) chitosan/copper, (C) chitosan/silver,& (D) chitosan/zinc microparticles. Morphology (E), & size (F) of microparticles.

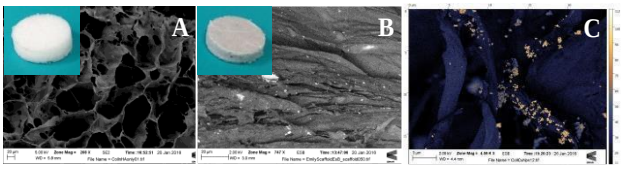

Fig. 3: SEM image of collagen scaffold (A). SEM images with backscatter electron detector showing (B) silver & (C) copper nanoparticles. Note: bright nanoparticles against dark collagen background.

DISCUSSION & CONCLUSIONS: The materials examined were effective against both gram-positive and gram-negative bacteria, demonstrating their potential for use in wide range of infection applications. Although a reduction in viability of MC3T3 cells with increasing metal ion concentrations was observed, chitosan/metal ion particles were successfully produced and characterised with the aim to control the release of ions, minimise toxicity, and prolong bioactivity. These were incorporated into collagen scaffolds previously developed in the TERG with immense capacity for bone repair [2] and assessment of their bioactivity is ongoing.

ACKNOWLEDGEMENTS: This research was funded by the Irish Research Council.

Participation of epithelial-to-mesenchymal transition of mesothelial cells in the pathogenesis of peritoneal adhesions

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INTRODUCTION: Adhesion formation is a frequent complication following abdominal surgery and occurs through the development of fibrotic bands between bowel loops, solid organs and the parietal peritoneum [1]. Peritoneal adhesions (PAs) represent a serious health problem with no effective treatment, partly because its pathogenesis is not well understood. Under pathological conditions, mesothelial cells (MCs) that line the peritoneal cavity undergo a mesothelial-to-mesenchymal transition (MMT), transforming into myofibroblasts, which are abundant in the peritoneal fibrotic tissue [2, 3]. In the induction of MMT, TGF-β1 is considered the master molecule.

METHODS: PA biopsies from patients subjected to abdominal surgery were analyzed by immunohistochemistry, immunofluorescence and qRT-PCR. C57BL/6 mice were used for peritoneal adhesion formation using ischemic buttons. TGF-β1 pathway was blocked using peptide P144.

RESULTS: We show that MC-derived myofibroblasts can be found in submesothelial areas of PAs from patients. Additionally, MMT-related markers are modulated in fibrotic adhesion zones when compared to distant normal peritoneal tissues of the same patients. Moreover, in a PA mouse model, MCs were also observed to give rise to myofibroblasts. In this context, the role of TGF-β1 was studied in MMT and adhesion development. We observed that blockage of TGF-β1 with P144 resulted in molecular reprogramming of markers related to MMT—interfering with it—and in a significant decrease in the severity of the adhesions in a dose-dependent manner (Fig. 1). Finally, by immunofluorescence we observed that the peritoneal structure was preserved upon P144 treatment.

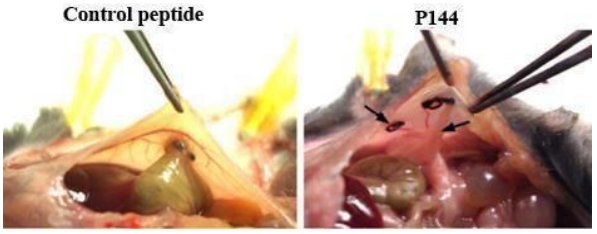

Fig. 1: TGF-β1 blocking peptide ameliorates adhesions in mice. Severe adhesion between cecum and parietal peritoneum in control group (left) and adhesion severity amelioration in mice treated with P144 (right).

DISCUSSION & CONCLUSIONS: Our results demonstrate that intraperitoneal blockage of TGF-β1 prevents the mesenchymal conversion of MCs, decreases adhesion severity and preserves the peritoneal membrane structure. In conclusion, this study shows for the first time that MC-derived myofibroblasts through a MMT contribute to PA development. We suggest that the MMT could constitute an alternative target in the treatment and/or prevention of peritoneal adhesions through strategic modulation of this process.

ACKNOWLEDGEMENTS: This work was supported by grants SAF2013-47611R and S2010/BMD-2321 (FIBROTEAM Consortium) from the Spanish "Ministerio de Economia y Competitividad" and "Comunidad Autónoma de Madrid", respectively.

Autophagy is needed during the differentiation of adipose derived stem cells to functional smooth muscle cells for use in tissue engineering

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INTRODUCTION: Tissue engineering using smooth muscle cells may provide a treatment option for diseases with smooth muscle pathology such as bladder dysfunction, urinary incontinence, and erectile dysfunction. As autologous smooth muscle cells (SMC) cannot be harvested from organs with end-stage disease there is a need for other cell sources. Adipose derived stem cells (ADSC) can be easily harvested and differentiated into SM tissue [1]. We have shown that autophagy, a conserved lysosomal degradation pathway, is required for cell survival and differentiation of stem cells. ADSC undergoing differentiation to SMC efficiently remodels their cytoskeleton and shape in an energy-consuming process [2]. We investigated functional role of autophagy during differentiation and remodeling of ADSCs to SMC in vitro.

METHODS: Rat and human ADSCs were characterized and induced towards SMC using induction medium for 1 to 6 weeks. The changes in gene and protein expression level for SMC markers: calponin, smoothelin, α-SMA, MyH11; and autophagy: LC3, Atg5, Beclin1, were investigated by ICC and WB.

RESULTS: Upon induction, up-regulation of Atg5-Atg12 and free Atg5 was observed during 4-6 weeks. This was supported by an increase in conversion of cytosolic LC3I to membrane-bound LC3II protein. At the same time the contractile proteins calponin, MyH11 and smoothelin were up regulated during 1-3 weeks and decreased after 5-6 weeks of differentiation detected by WB and ICC. Pharmacological blocking of autophagy by 3-methyladenine (3-MA) during the differentiation abolished the differentiation capacity of stem cells.

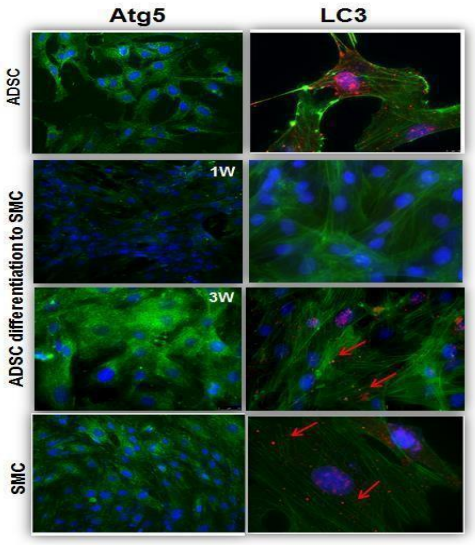

Fig. 1: ADSC and bladder derived SMC were stained with anti- atg5 and LC3. Atg5 in green, punctuated staining in red indicates LC3 and autophagosome formation. Samples were detected using Cy3 conjugated secondary antibody (red), or FITC (green) and DAPI (blue).

DISCUSSION & CONCLUSIONS: Our study demonstrates that autophagy plays an important role in ADSC differentiation to SMC. Influencing autophagy by pharmacological agents might be used to further optimize the induction to SMCs.

ACKNOWLEDGEMENTS: The authors recognize the research efforts of those who have contributed to this field of study and declare no conflict of interest. The authors' work is supported by Helmut Horten Foundation.

Influence of 2D and 3D microenvironments on human bone marrow stem cells under different oxygen tensions

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INTRODUCTION: The extracellular micro environment of differentiated and stem cells plays a significant role on the success of tissue engineered constructs. *In vitro* systems mostly use 2D test media and *in vivo* conditions can best be mimicked if a 3D test environment is created [1]. In addition to the spatial signals, physiological cues such as arterial oxygen pressure (12%) play a vital role in maintaining osteoblast function [2]. In this study 2D and 3D systems were investigated for bone tissue engineering under low oxygen environment native to stem cells [3], arterial pO_2 and typical *in vitro* test environment.

METHODS: Films of micropatterned collagen, fibroin and a collagen-fibroin blend were produced by solvent casting on a silicone elastomer mold and crosslinked [4]. Swelling degrees of the films were determined gravimetrically and glass transition temperatures (T_g) were determined with differential scanning calorimetry (DSC). Human bone marrow stem cells (hBMSCs) were seeded on the films (2D) and on their 3D versions which were produced by rolling longer films prepared in a similar fashion. TCPS was used as control. Cells were cultured in an osteogenic medium and Alamar Blue assay was performed on Days 1 and 3 to determine the cell numbers. Each group was analyzed statistically with ANOVA followed by Tukey's test ($p \leq 0.05$).

RESULTS: The films were characterized and swelling degrees of collagen films (163%) was found to be much higher than that of fibroin films (47%), also by adding collagen to fibroin in a ratio of twice significantly increased the swelling capacity for blend films (139%) when compared with fibroin film. Glass transition temperature (T_g) of collagen (55.5^{O}C) was lower than that of fibroin (81.5^{O}C) and T_g of the blend (58.8^{O}C) was in between. 12% O_2 level significantly contributed to cell adhesion when 2D constructs were tested on Day 1 and in the meantime no significant difference was observed for 3D scaffolds. While cell adhesion on 2D scaffold was the lowest in 21% O_2 tension, for 3D it was the contrary. After 2 days cell number was the lowest on 2D scaffold which was kept in 21% O_2 tension. Alignment of hBMSCs on microchannels was confirmed visually and 21% O_2 tension led to cell proliferation at the inner part of the 3D scaffold *(Figure 1)*.

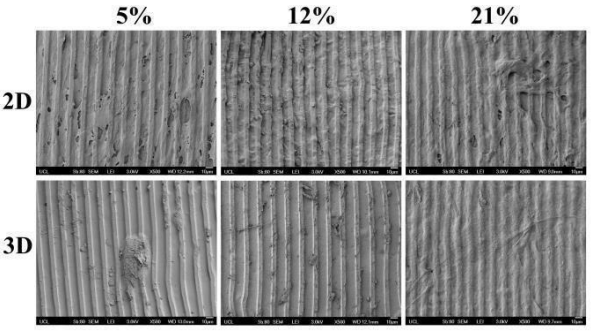

Fig. 1: SEM micrographs of hBMSC seeded 2D and 3D scaffolds (innermost layer) on Day 3. Cells were subjected to oxygen tensions of 5, 12 and 21%.

DISCUSSION & CONCLUSIONS: In this study 2D and 3D microenvironments were formed successfully and correlations between oxygen levels and hBMSC adhesion and proliferation were shown to depend on oxygen levels in media for TCPS and 2D films and within the 3D scaffolds.

ACKNOWLEDGEMENTS: The authors would like to acknowledge METU (BAP-07.02.2013. 101), BIOMATEN, METU Ctr. Excellence in Biomaterials and Tissue Engineering established by the Ministry of Development (Turkey) for their financial support. E.S. gratefully acknowledges the scholarship by the Scientific and Technical Research Council of Turkey (BIDEB 2214/A).

What's for dinner? The impact of cell culture media on annulus fibrosus and nucleus pulposus cells from human cervical intervertebral discs.

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INTRODUCTION: Intervertebral disc (IVD) degeneration is under consecutive investigation using mainly *in vitro* studies. It's well known, that cultured IVD cells undergo morphological and biochemical changes during primary monolayer culture. The cells are influenced by serum, glucose and oxygen concentration as well as growth factor supplementation [1, 2]. However, the cell culture media itself and its possible impact on the behaviour of human intervertebral disc cells have not been investigated, yet. Current studies utilise a broad variety of disposed cell culture media and there is no common cell culture protocol for IVD cells available. In this study, we evaluated the most common media used for monolayer culture of annulus fibrosus (AF) and nucleus pulposus (NP) cells.

METHODS: AF and NP tissue was obtained from human cervical discs of three donors (≤ 55 years) with mild to moderate degenerative changes (Modic≤2, Pfirrmann≤3). Cells were isolated and cultured using seven different cell culture media all containing 10% human serum and 1% antibiotics. The media used were: DMEM+FGF (2ng/ml), DMEM, Ham's F-12 (F-12), alpha-MEM (alpha), DMEM/F-12, alpha/F-12 and DMEM/alpha (Biochrom. Germany). The expression of glycosaminoglycans (GAGs), which is specific for IVD or cartilage cells, was analysed using dimethylmethylene blue assay (DMMB) and alcian blue staining. Statistical analysis was performed with GraphPad PRISM 6.0 (2way ANOVA, Bonferroni compensation).

RESULTS: Isolated AF and NP cells were expandable in all tested cell culture media. The cells showed a fibroblast-like morphology, independently from the used media. In passage P2, the cells reached a population doubling level (PDL) of 9.7±0.9 and 9.0±1.1 for AF and NP cells, respectively. In all cell culture media, both cell types showed the highest PDL when cultivated with DMEM including FGF. The effect wasn't statistically relevant, except compared to Ham's F-12 (Figure 1). However, the supplementation of FGF did not enhance proliferation or GAG expression. GAGs were present in all cells and supernatants of monolayer passage P0, but not P1 or P2 (Figure 2), independent of the used media.

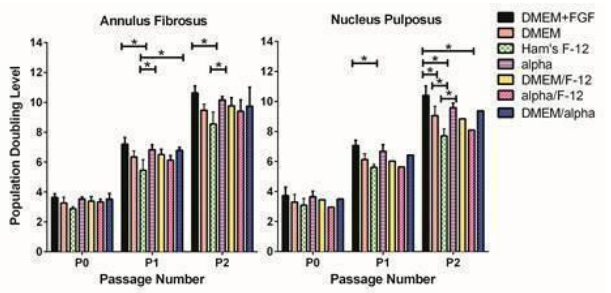

*Fig. 1: Cell growth of AF and NP cells cultured with different media, *p≤0.05.*

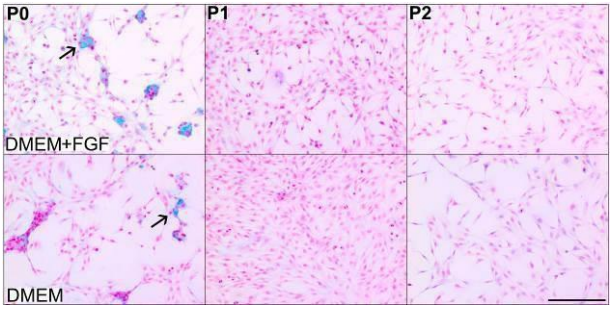

Fig. 2: GAG expression as shown by the alcian blue staining (arrows) of AF cells cultured in DMEM w/wo FGF, scale bar 200 µm.

DISCUSSION & CONCLUSIONS: All tested cell culture media were suitable for monolayer expansion of AF and NP cells. However, our results show that the choice of cell culture media affects proliferation capacity. The usage of single cell culture media had a larger variation in PDL than a media mixture, especially for NP cells. As the genetic phenotype of IVD cells is influenced by *in vitro* expansion [3], the usage of a media mixture is recommended. This might be useful for the standardisation of future *in vitro* studies.

Controlled release of hepatocyte growth factor from MPEG-b-(PCL-ran-PLLA) diblock copolymer for scarless vocal fold regeneration

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INTRODUCTION: In situ-forming gel system comprised of MPEG-b-PCL and MPEG-b-(PCL-ran-PLLA) diblock copolymer could be used in accurately controlled drug delivery and tissue remodeling. Degradation of MPEG-b-(PCL-ran-PLLA) diblock copolymers were easily manipulated by adjusting PLLA contents, enabling them to act as protein drug depots. The purpose of this study is to demonstrate favorable vocal folds (VF) regeneration by using an in situ-forming gel composed of MPEG-b-(PCL-ran-PLLA) diblock copolymers (C97L3; CL/LA ratio 97:3) and hepatocyte growth factor (HGF).

METHODS: A scar was made with microscissors on both VFs in 32 rabbits, followed by injection of either HGF only, C97L3 only, or HGF-C97L3 composite gel in the left side VF, while the right side VF was left untreated. VF scars were evaluated with in vivo fluorescence live imaging system (IFLIS), endoscopic examination, histology, and videokymography (VKG) twelve weeks after the injection.

RESULTS: Gradual and sustained release of HGF from C97L3 diblock copolymer was detected and biochemical properties of released HGF were maintained. IFLIS demonstrated that C97L3 enabled the sustained release of injected HGF in the scarred VF. The histological analysis showed increased hyaluronic acid accumulation and controlled collagen deposition by HGF-C97L3 composite gel. VKG analysis showed more favorable vibrations of HGF-C97L3 injected VF mucosa, compared to the other treatment group. In conclusion, the sustained HGF release helped to regulate ECM synthesis, and the eventual functional improvement of the scarred VF.

DISCUSSION & CONCLUSIONS: The results of the present investigation suggest that C97L3 in situ-forming gel has the potential for use as an HGF delivery carrier for enhancement of wound healing and improvement of functional remodeling following VF injury.

REFERENCES: [1]Kang YM. et al (2010) A biodegradable, injectable, gel system based on MPEG-b-(PCL-ran-PLLA) diblock copolymers with an adjustable therapeutic window, Biomaterials **31**:2453-60. [2]Choi JW. et al (2014) Small intestine submucosa and mesenchymal stem cells composite gel for scarless vocal fold regeneration, Biomaterials **35**:4911-8.

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

This research was supported by a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI14C2108).

Human subchondral bone pieces can be effectively rescued after cold ischaemia with addition of a new blood-derived supplement (SPRF) to culture medium

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[1] Institute of Clinical Experimental Research, Semmelweis University, Budapest, [2]University of Physical Education, Budapest, [3]Polyclinic of the Hospitaller Brothers of St. John of God, Orthopaedic Department, Budapest

INTRODUCTION: The most important aim during a bone transplantation process is that the graft should be kept alive until it gets implanted to the recipient. Storing bone grafts creates stress factors through temporal oxygen and glucose deprivation (OGD), which negatively affects cell viability. It is widely known, that fetal calf serum (FCS) is an essential ingredient in stem cell culture media and it may be of value in transfer solutions as well. Moreover, novel human blood derivatives such as serum from platelet rich fibrin (SPRF) may replace animal-derived material. We compared the effects of FCS and SPRF supplemented DMEM on bone explants subjected to OGD.

METHODS: Human subchondral bone pieces (~1mm^3) harvested during hip replacement surgery from the femoral head were kept under normal cell culture conditions (37 °C, 5% CO_2) over 48 hours. Thereinafter, OGD was carried out in order to model ischaemic conditions: the cell culture medium was changed to glucose-free, while oxygen was purged by nitrogen from the incubation chambers. The seven hours long OGD happened either at 4 °C or at 37 °C. For this purpose, a custom-built device was designed with two independent parallel chambers. After OGD, explants were separated into 3 groups (only DMEM, DMEM with FBS, DMEM with SPRF, n=6 per group) and incubated for 5 days. XTT assay was applied to determine the viability of the explants. Finally, alkaline-phosphatase (ALP) activity was measured from the culture media in order to monitor osteoblast-like activity.

RESULTS: Figure 1. shows the viability difference between bones subjected to cold or warm OGD, and then incubated for 5 days in the three special media types. It was observed that the viability of cells is consistently higher after cold OGD in all groups, especially in SPRF-medium. Significant ALP activity was only detected in SPRF-treated explants (Figure 2.).

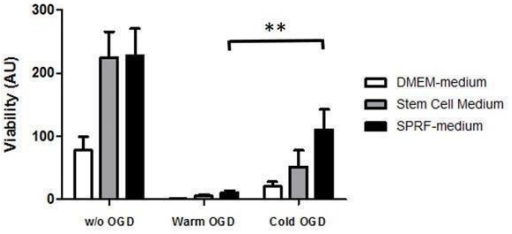

Fig. 1: Rescue effect of three different media after cold (4 °C) and warm (37 °C) OGD on human subchondral bone pieces.

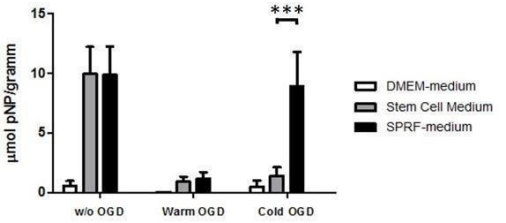

Fig. 2: ALP-activity measured from the cell culture medium showed high values after OGD followed only by SPRF treatment.

DISCUSSION: We demonstrated that after cold OGD circumstances the inactivated cells' viability can be completely restored with SPRF-medium. No medium or treatment was able to restore the inactive cells' function if cells were incubated at body temperature. Furthermore, the SPRF-medium treated bone chips showed more relevant ALP secretion compared to FBS supplemented ones. Higher ALP activity is presumed to be due to either increased cell number or more active cell metabolism of osteoblasts.

CONCLUSIONS: Live human bone explants are significantly damaged by the lack of glucose and oxygen at body temperature, so it advisable to transfer them as early as possible into a cold solution. Supplementing the transfer fluid with SPRF rather than FCS can effectively restore the viability of the explanted grafts to pre-ischemia levels.

ACKNOWLEDGEMENTS: Supported by OrthoSera GmbH

The influence of sterilization on tubular hybrid construct remodeling

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INTRODUCTION: Clinical implementation of novel biomaterials requires a validated sterilization method. However, sterilization may affect the material characteristics, resulting in altered material remodelling. Previously, a γ-irradiated tubular collagen construct was subcutanrously pre-implanted to create functional artificial urostomies. Although successful, contraction was observed. One month after pre-implantation, the material was completely remodelled and might have resulted in the observed contraction. We hypothesize that ethylene oxide (ETO) degassing may improve outcome, by providing prolonged strength. In this study, we compare the effect of sterilization on *in vitro* and *in vivo* performance of tubular constructs, sterilized by either γ-irradiation or ETOdegassing.

METHODS: Tubular constructs were prepared from 0.7 % (w/v) collagen type I and a reinforcing Vicryl polymer, and subsequently sterilized by 25 kGray of γ-irradiation or ETO degassing. Constructs were evaluated by Scanning Electron Microscopy (SEM), histology and tensile strength analysis (TSA) and compared to native goat ureter (target tissue for anastomosis). Degradation assays were performed for both constructs in goat urine and Simulated Body Fluid (SBF) for 28 days and evaluated by histology and tensile strength. Subsequently, γ- and ETO-sterilized constructs were subcutaneously pre-implanted in 6 goats. After one month, constructs were evaluated by histology and TSA. In 4 goats, an artificial urostomy was created with γ-sterilized (n=2) or ETO-sterilized (n=2) constructs and evaluated after maximally 3 months.

RESULTS: Macroscopic and microscopic evaluation of γ- and ETO-sterilized constructs show a porous structure with integration of the Vicryl polymer. TSA of the construct indicated that the ETO-sterilized construct approximated the native tissue more closely than the γ-sterilized constructs (Fig. 1). Evaluation of both construct types in urine resulted in fast degradation of the polymer mesh (<7 days), and therefore reduced strength (<3 days). Constructs incubated in SBF still contained polymer remnants at 28 days and maintained strength up until 14 days. Subcutaneous pre-implantation of both constructs resulted in a well-vascularized tube. TSA of the pre-implants demonstrated that after pre-implantation the γ-sterilized construct resembles the native ureter more closely than the ETO-sterilized construct (Fig. 2). The γ-sterilized construct showed more tissue remodelling and degradation of the polymer, indicated by histology. Preliminary results of functional application of the pre-implants as artificial urostomies are in favour of the γ-sterilized construct where both animals successfully completed the experiment. In one animal with an ETO-pre-implant the ureter and the implant was disconnected after three weeks.

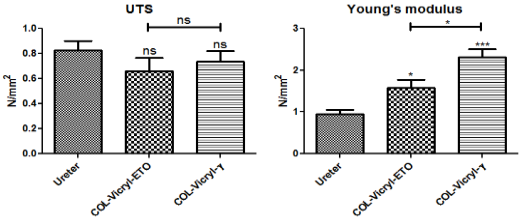

Fig. 1: TSA analysis of ETO- and γ-sterilized constructs.

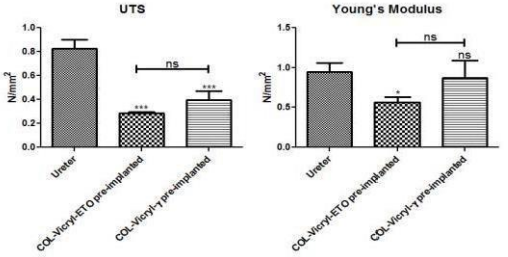

Fig. 2: TSA analysis of pre-implanted ETO- and γ-sterilized constructs.

DISCUSSION & CONCLUSIONS: This study shows that sterilization alters material characteristics in a way that cannot be predicted by *in vitro* analyses alone. Here, in vitro analyses indicated a preference for ETO-sterilized construct, while γ-sterilized construct resulted in a better tissue remodelling and functional outcome *in vivo*.

Engineering skin and cornea living substitutes *in vitro* with macromolecular crowding and oxygen tension

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INTRODUCTION: Advancements in tissue engineering have enabled the development of scaffold-free substitutes; a technology termed tissue engineering by self-assembly [1, 2]. Despite efficacious *in vitro* and *in vivo* results, very few products have been commercialised, primarily due to prolonged time required to develop an implantable device. Herein, we assessed the potential of combining macromolecular crowding and low oxygen tension to produce a rich in extracellular matrix tissue substitute.

METHODS: We assessed the influence of serum [foetal bovine serum (FBS) and new born calf serum (NBCS)] concentration (0.5 % and 10 %), oxygen tension (0.5 %, 2 % and 20 %) and macromolecular crowding (75 μg/ml carrageenan) in extracellular matrix deposition of human corneal and dermal fibroblasts after 3, 7 and 14 days in culture, using SDS-PAGE and complementary densitometric analysis and immunocytochemistry for various extracellular matrix molecules.

RESULTS: Macromolecular crowding consistently enhanced extracellular matrix deposition in corneal fibroblast culture, while the % serum did not affect extracellular matrix deposition. Among the various oxygen tensions, the 2 % oxygen tension was the most effective, with respect to highest extracellular matrix synthesis and deposition (Figure 1).

Dermal fibroblasts cultured at 2 % oxygen tension, in the presence of carrageenan and at 0.5 % serum concentration deposited within 3 days in culture more extracellular matrix than cells grown for 14 days, at 20 % oxygen tension, 10 % serum concentration and in the absence of carrageenan (Figure 2).

DISCUSSION & CONCLUSIONS: Herein we demonstrated that low oxygen tension, coupled with macromolecular crowding, accelerated extracellular matrix deposition in human corneal fibroblast and dermal fibroblast culture, opening new avenues in the *in vitro* development of tissue-like modules.

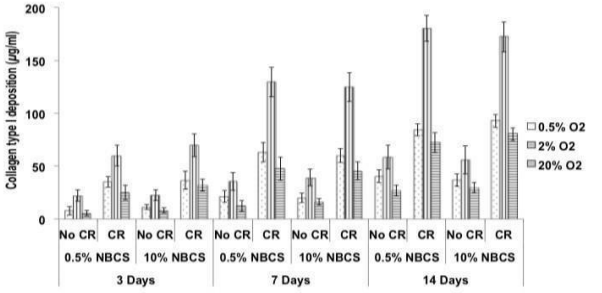

Figure 1: Complementary densitometric analysis of SDS-PAGE demonstrates that 2% oxygen tension and CR supplementation (75 µg/ml) significantly increased collagen type I deposition at all time points and FBS concentrations in human corneal fibroblast culture.

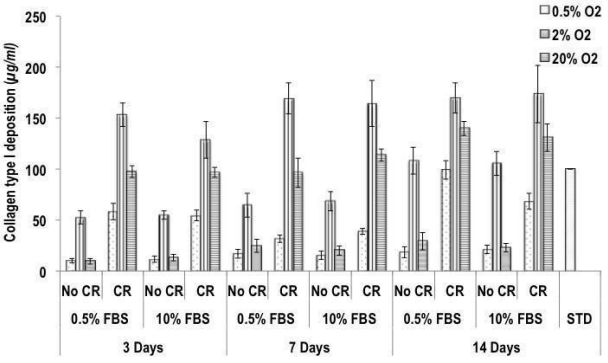

Figure 2: Complementary densitometric analysis of SDS-PAGE *demonstrates that human dermal fibroblasts, cultured at 2 % oxygen tension, synthesised and deposited significantly more collagen type I, than cells cultured at 0.5 % and 20 % oxygen tensions.*

ACKNOWLEDGEMENTS: The authors would like to acknowledge the Irish Research Council, the Health Research Board, the Science Foundation Ireland, the EU FP7/2007-2013, Marie Curie, IAPP award and the EU FP7/2007-2013, NMP award for financial support.

Synthetic mRNA for therapeutic applications: a versatile tool to engineer cells

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INTRODUCTION: In recent years, the synthetic messenger RNA (mRNA)-based therapy for *de novo* protein expression in cells is gaining in importance[1]. Due to the lack of genomic integration of synthetic mRNA into the host genome, the risk of mutations and tumor development are minimized. Cells can be engineered by mRNA for reprogramming, vaccination, tumor treatment, expression of surface receptors on cells, production of industrial grade human growth factors, and treatment of several diseases that are caused by missing or defective synthesis of proteins in cells due to genetic or acquired disorders (Figure 1).

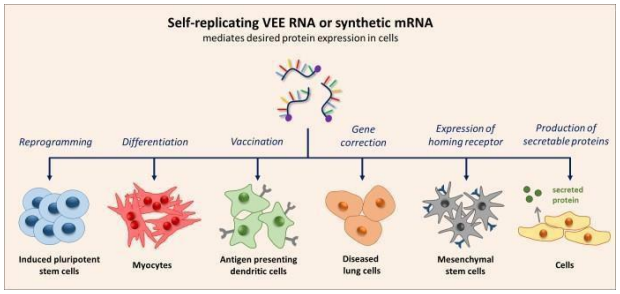

Fig. 1: Perspectives of self-replicating VEE RNA and synthetic mRNA.

Nevertheless, for the reprogramming of cells the mRNA-based method is work intensive and time consuming. Due to the fast degradation of mRNA in the cytosol daily transfections over several days are required. To overcome this hurdles, a self-replicating RNA replicon can be applied which is derived from a non-infectious, self-replicating Venezuelan Equine Encephalitis (VEE) virus able to induce continuous protein synthesis[2] in cells.

METHODS: The VEE RNA replicon is a positive-sense, single stranded RNA with a 5'-cap (ARCA) and 3'-poly(A) tail that mimics cellular mRNA. VEE replicons encode structural proteins for self-replication in one open reading frame (ORF) at the 5'-end and the target ORF(s) and a puromycin resistance (for positive selection) at the 3'-end. Replicons were synthesized by *in vitro* transcription with an ORF encoding for GFP (VEE-GFP). VEE-GFP RNA was transfected into HEK293 cells and human foreskin fibroblasts (HFFs) using a cationic lipid-based transport vehicle. Analysis of fluorescence signals was performed by fluorescence microscopy and flow cytometry (Figure 2).

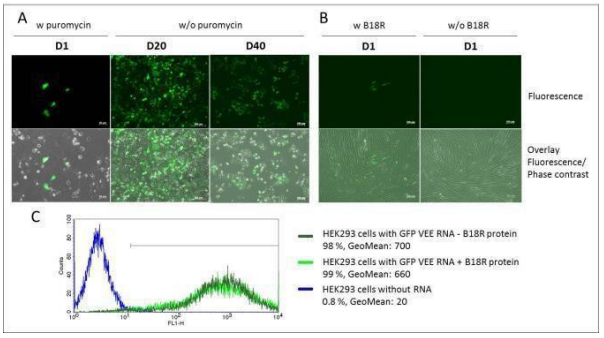

Fig. 2: VEE RNA-mediated GFP expression in cells. A: HEK293 cells at day 1 (D1) and day 20 (D20) after transfection (+/- puromycin) in absence of B18R protein. B: HFFs at day 1 (D1) +/- B18R. C: Flow cytometry analysis of HEK293 cells at day 20 (D20) w/o puromycin, +/- B18R.

RESULTS: After a single transfection of VEE-GFP RNA replicon (1-1.5µg) in HEK293 cells and HFFs, the fluorescent signal was detected for a time period of over 6 weeks and multiple passages. Surprisingly, in HEK293 cells the presence of the interferon inhibitor B18R protein was not necessary, compared to fibroblasts. The positive selection with puromycin resulted in about 98% of VEE-GFP positive cells.

DISCUSSION & CONCLUSIONS: The results show that daily transfections with relatively fast degrading synthetic mRNAs can be circumvented by using a self-replicative RNA replicon which leads to stable expression of desired protein over a long period of time. Furthermore, the optimization of RNA-based methods to mediate desired protein expression in cells is an ongoing process and holds significant potential for biomedical research and regenerative medicine, which certainly will be better accessible to more research labs in the future.

Stability of electrospinning produced vascular grafts depending on the presence of blood cell impermeable inner layer

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INTRODUCTION: Stability of linear dimensions, structure, tensile strength during long term functioning as well as biocompatibility are among the main requirements for the vascular grafts (VG). To increase the biocompatibility of VG we offer to introduce blood cell impermeable inner layer (BCIL) into the graft wall[1]. In the present study we have investigated the stability of VG produced by electrospinning from polycaprolactone (PCL)/gelatin (GL) blends in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) with or without BCIL and implanted in the abdominal aorta of rats for a different time intervals.

METHODS: VG (1,7 mm diameter, 150 ± 30 um wall thickness) made from 5% PCL (w/v %) blended with 10% of fluorescein labeled GL (w/w % to PCL) in HFIP with or without BCIP, were prepared as described[2]. VG were implanted into infrarenal position of abdominal aorta of 8 Wistar rats for 2 h, 2, 4 and 12 weeks intervals. After explantation VG were rinsed with PBS, fixed with formalin, sliced into 1 mm rings and investigated by scanning electron microscopy (SEM) on JSM-6460 LV (Jeol) and fluorescent microscopy on Axiovert 200M (Zeiss). Pairwise comparison of control and test was performed using fluorescent microscopy (fluorescence intensity per square was compared) to normalize the data obtained. Tensile strength was measured using Zwick/Roell Z100 machine 2 mm rings.

RESULTS: Fluorescence data demonstrated that VG without BCIL lost fluorescence faster than those with BCIL and are prone to the destruction of wall structure. In contrast fluorescence in VG with BCIL decreased in the first 2 weeks and then remained stable. SEM demonstrated that fiber diameter was 0,25±0,073 um and did not change during the first month but increased up to 0,5 ±0,15 um after 3 months. No more that 20% decrease of tensile strength was found in 3 month VG (with BCIL) but elongation at break in the same VG was found to be 2-3 times lower.

DISCUSSION & CONCLUSIONS: Addition of GL to PCL was shown to increase the strength and biocompatibility of electrospun scaffolds[3]. Only a minor amount of GL leaves the fibers and the remaining GL is stably bound to the surface or located inside the fibers (theses of the same authors).

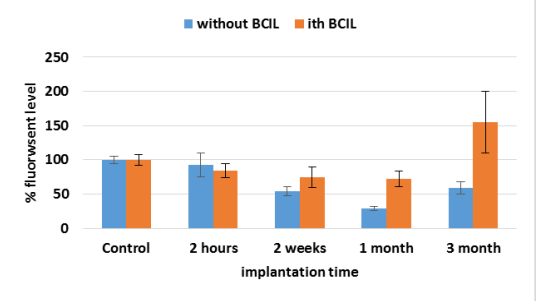

Fig. 1: Fluorescence of the matrices after 2 hours, 2 weeks, 1 month and 3 months of implantation, as compared to control.

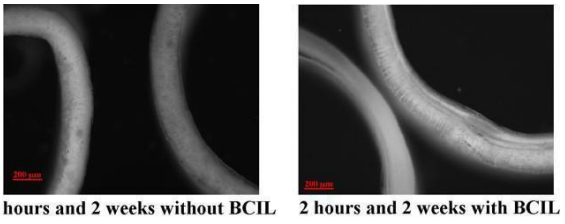

Fig. 2: Fluorescent image of VG after explantation

Here we demonstrate that clotting in the walls of VG decreases fluorescence of the fibers and disrupts the structure of the scaffold obviously due to clot retraction and subsequent cellular clot disintegration. The increase of the fluorescence of VG after 3 months implantation may be concerned with the increase of the efficacy of the GL exposure and fluorescence in swollen fibers or autofluorescence of the newly formed tissue. This newly formed tissue apparently compensates for the loss of strength but not elasticity of the scaffold.

ACKNOWLEDGEMENTS: This work was supported by a Russian Science Foundation grant (№ 14-15-00493).

Factor-screening for the regeneration of anulus fibrosus defects after disc herniation

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INTRODUCTION: Intervertebral disc (IVD) degeneration is a major source of back pain. For the IVD regeneration after disc herniation and for a possible restoration of the nucleus pulposus (NP) using Tissue Engineering approaches, a fast closure of defects in the anulus fibrosus (AF) is crucial. Here, the use of different factors to induce and enhance AF-matrix formation will be tested in an *in vitro* micro mass pellet model.

METHODS: Human AF-cells were isolated enzymatically from native tissue biopsies using collagenase II and P, and hyaluronidase [1,2]. Native human tissue samples from 3 volunteers were obtained during discectomy. The study was approved by the ethics committee of the Charité – Universitätsmedizin Berlin (EA2/102/15). Passage 3 cells were transferred to 3D pellet micro mass cultures and differentiated (serum-free) with supplementation of growth and differentiation factors in concentrations of 10, 50 and 100 ng/ml. Among them were factors of the transforming growth factor beta (TGFβ) superfamily (TGFβ3, bone morphogenetic proteins, and growth differentiation factors), and platelet rich plasma (PRP). After 28 days of induction the production of proteoglycans was examined using alcian blue and safranin O staining. Collagen type I and II was detected immunochemically.

RESULTS: All used factors induced proteoglycan production demonstrated by alcian blue and safranin O staining in at least one of the tested concentrations. A scoring revealed that TGFβ3 delivered the strongest staining and was able to induce proteoglycans in all three concentrations (Fig.1 A-D). Furthermore, it was the only factor able to facilitate collagen type II production (Fig.1 E-F). According to the scoring, the presence of collagens and proteoglycans was even higher in TGFβ3 induced samples compared to native tissue. The scoring grade of proteoglycan formation of all other factors was between the native tissue controls and the non-induced samples.

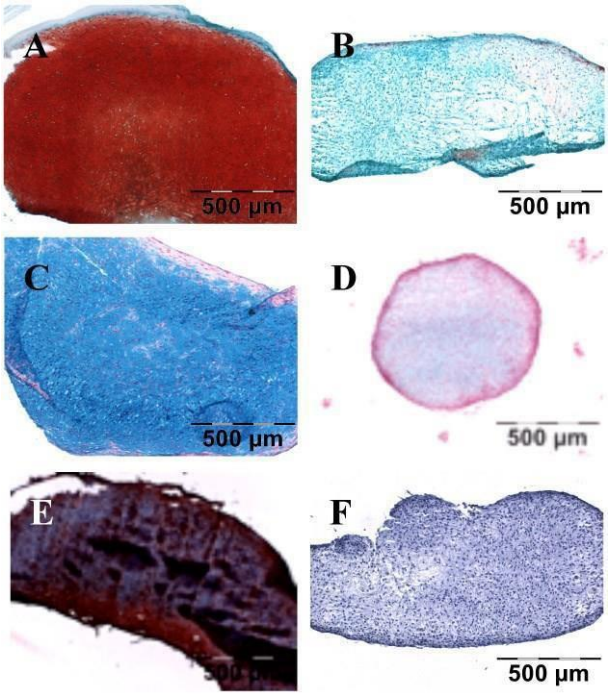

Fig. 1: Safranin O (A,B), alcian blue (C,D) and immunochemical collagen type II staining (E,F) of cryo sections of TGFβ3 induced (A,C,E) and non-induced (B,D,F) AF cell pellets after 28 days.

DISCUSSION & CONCLUSIONS: The study revealed that different factors from the TGFβ superfamily, and other factors were able to induce the formation of proteoglycans and partially of collagens *in vitro* in AF-cell. Therefore, the tested factors are promising candidates for matrix formation in AF-defects after disc herniation.

ACKNOWLEDGEMENTS: This study was funded by the Bundesministerium für Bildung und Forschung (BMBF-Nr.: 13N13437).

DISCLOSURE: The authors see no possible conflict of interest, financially or otherwise.

Recellularization of tissue engineered vascular grafts

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INTRODUCTION: Due to the physical barrier of dense concentric tunica media layers within blood vessels, cells tend to migrate longitudinally, compromising effective radial recellularization; therefore, posing difficulty for complete repopulation of decellularized constructs which potentially may reduce efficacy [1-3]. To overcome this barrier, we modified decellularized carotid artery scaffolds with radial microchannels. Our first hypothesis was that modifying decellularized arteries with a microneedle device would facilitate cell migration and repopulation of arteries in the radial direction. To achieve this, we seeded cells using an injection technique [1-3]. Furthermore, to confirm that modified scaffolds would facilitate the recellularisation of tissue engineered scaffolds independently of the seeding system, we used a dual cell seeding bioreactor combining vacuum and perfusion system.

METHODS: Scaffolds were produced by decellularization of arteries according to [1]. Subsequently, a microneedle device was developed to create radial microchannels using 200 µm diameter acupuncture needles with 600 µm space between each needle. Scaffolds were characterised by image analysis and mechanical tests. A dual vacuum-perfusion cell seeding bioreactor was developed. Scaffolds were seeded with rat MSCs and evaluated using histochemistry to analyse (i) seeding by the injection method [1]; and (ii) seeding by a novel bioreactor for control and microchannel specimens.

RESULTS: A minimal quantity of extracellular matrix was removed by the microneedle device. Creating radial microchannels did not affect the mechanical properties, and aided cells to disseminate through the tunica media radially. We confirmed the repopulation of decellularised scaffolds in the radial direction using both seeding methods (*Figure 1).* At 24 hours, we also observed 0 cells/mm^2 in samples with microchannels absence, and 60 cells/mm^2 using the vacuum-perfusion technique, confirming robust radial recellularization.

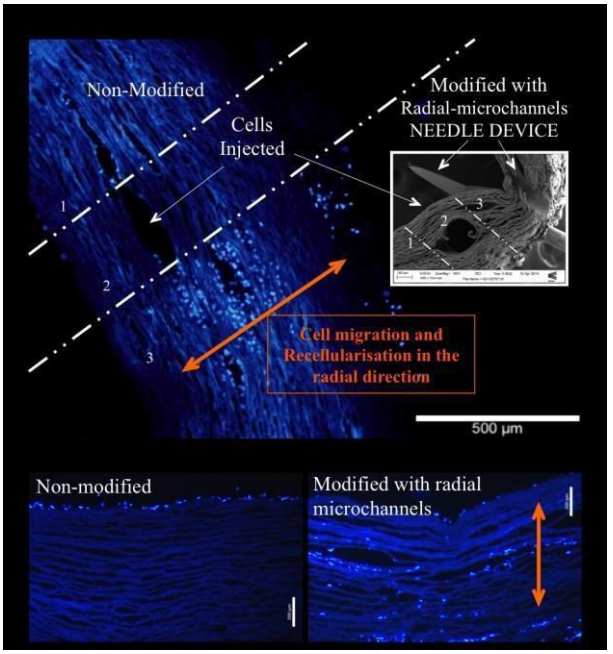

Fig. 1: Histochemistry images of bioscaffolds seeded by A) injection seeding technique; B) dual vacuum-perfusion cell seeding bioreactor system.

DISCUSSION & CONCLUSIONS: The recellularization in the radial direction of decellularized arteries was successfully achieved by modification with a radial microneedle device, as verified by two different methods. Thus this confirmed that independent of the seeding system, modified scaffolds facilitate the repopulation of tissue engineered vascular graft, therefore confirming our hypothesis. Furthermore, the combination of vacuum and perfusion as a method to seed cells promotes an improvement in radial cell migration and better cell distribution. Hence these are steps towards a complete recellularisation of decellularized arteries without compromising the mechanical integrity.

ACKNOWLEDGEMENTS: Prof. F. O'Brien, Dr. D. Kelly and funding SFI/12/RC/2278.

Synergistic effects of chemical, biological and electromechanical stimulations on cardiomyogenic differentiation of BMSCs

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INTRODUCTION: Heart diseases are still the leading cause of deaths worldwide and there is an urgent need for a fully functional cardiac patch to be used in myocardial infarction or atrial and ventricular septal defects (ASD, VSD) [1]. Some of the fundamentals that need to be met in cardiac regeneration are; the selection of a human cell source, establishment of cardiac tissue matrix, electromechanical cell coupling, robust and stable contractile function, and functional vascularization which we have tried to address in this study [2].

METHODS: Differentiation of bone marrow mesenchymal stem cells (BMSCs) was studied in two steps for cardiac patches. 1) Differentiation was induced by chemical and biological stimulators in 2D culture condition. The effect of chemical stimulators (5-azacytidine, dimethyl sulfoxide, gold nanoparticles, 5-azacytidine/gold nanoparticles) and biological stimulators (TGF-β1, FGF-2, BMP-2) were studied. 2) Cells were seeded in the acellular bovine pericardium scaffolds and exposed to electromechanical stimulations inside the bioreactor. Patches were cultured for 21 days under increasing uniaxial strain and expression of cardiac markers (GATA4, MEF2C, NKx2.5, CACNA1C) and protein expressions (Sarcomeric alpha actinin, N-CAD, Cx43, Cardiac heavy chain myosin, troponin T) were evaluated. Statistical significance was tested by Two-way ANOVA ($p<0.001$).

RESULTS: Acellular scaffold with the pore size of 54.5µm was obtained. BMSCs were characterized as 99.3% for CD29 and 99.2% for CD90. RT-qPCR results of 2D culture showed that synergistic chemical and biological stimulations give better results in cardiomyogenic differentiation of BMSCs. Cell infiltration results showed that at least 7 days is needed to achieve complete infiltration of the cells in acellular scaffolds (Fig. 1A). Live&dead assay showed that, electrical stimulation does not have any adverse effect on viability (Fig. 1B). Electromechanical stimulation had explicit results on cardiomyocyte like cell morphology and enhanced the differentiation (Fig. 1C, D).

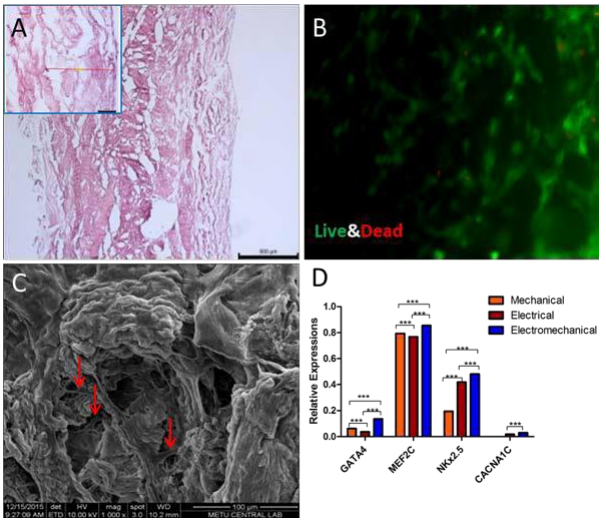

Fig. 1: Engineered cardiac patches. (A) Cell infiltration in acellular scaffolds. (B) Cell viability. (C) Cell morphology. (D) Gene expressions.

DISCUSSION & CONCLUSIONS: In the context of this study it was found synergistic chemical, biological and electromechanical stimulations are of paramount importance in order to achieve cardiac patches with better functional properties.

ACKNOWLEDGEMENTS: Study is supported by The Scientific and Technological Research Council of Turkey, grant number 213M685 and Hacettepe University, Scientific Research Projects Coordination Unit, grant number 7924.

Disclosures: Authors have nothing to disclose.

Induction of endothelial cell sprouting by poly (ε-lysine) dendron tethered with an angiopoietin-1-mimicking peptide

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INTRODUCTION: Coronary heart disease and stroke are leading causes of mortality in Europe, resulting in a loss of function of the affected tissue[1]. The challenge is to restore the tissue functionality using growth-factor mimicking peptides to stimulate the development of pre-existing blood vessels and enhance tissue regeneration. In this research, poly (ε-lysine) dendrons of different branching generations (Gx) will be used as a molecular scaffold to present, at their uppermost branching generation, the Angiopoietin-1 (Ang-1) mimicking peptide, QHREDGS, which has previously been shown to promote blood vessel formation and stabilisation[2].

METHODS: G0 and G1 dendrons were synthesised by a manual solid-phase peptide synthesis (SPPS) method. G0 and G1 were designed starting from a molecular root of two phenylalanine residues (FF) and branching of the molecule by addition of lysine monomers to expose either two (G0) or four (G1) active amino groups. Finally, G0 and G1 dendrons were functionalised, using the same SPPS method, with the bioactive peptide sequence, QHREDGS, mimicking Ang-1. Non-functionalised and functionalised dendrons were analysed by both analytical HPLC and micro time-of-flight mass spectrometry (TOF-MS). The *in vitro* cytotoxicity of G0 and G1 dendrons functionalised with the Ang-1-mimicking peptide was evaluated in terms of cell viability of human umbilical vein endothelial cells (HUVEC) in a 2D culture model. A linear QHREDGS sequence was used as control. After 72 hours in culture, cells were stained for their cytoskeleton organisation by Rhodamine-Phalloidin staining and for immunostaining of CD31 (membrane protein that mediates endothelial cell-cell interaction and angiogenesis). HUVEC were analysed by confocal microscope.

RESULTS: Poly (ε-lysine) G0 and G1 dendrons were synthesised successfully, as confirmed by micro TOF-MS and HPLC. Fragmentation peaks (G0 MH^+=440.25 *m/z*; G1 MH^+=724.46 *m/z*) correspond to the expected dendron fragment sizes and HPLC chromatograms showed that the increased molecular weight led to a decrease in peak retention time from 9 to 8 minutes. Laser confocal images with Phalloidin-Rhodamine staining show the effect of branched Ang-1-mimicking peptides. Indeed, while in the control samples cells grew as a monolayer, the Ang-1 peptide-functionalised G1 dendrons clearly induced the formation of a network of endothelial sprouting where cells appeared to establish connections also in 3D (data not shown). Immunofluoresce results (Fig.1) shown the expression of CD31 protein only when HUVEC are treated with the Ang-1 peptide-functionalised G1 dendrons probably demonstraiting the synergistic action imparted by the dendrons branched structure and by the Ang-1 peptide (QHREDGS) sequence, in guiding the 3D organization of the endothelial sprouting.

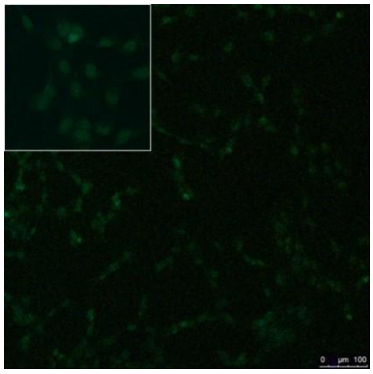

Fig.1 Immunofluorescence anti-CD31 antibody on HUVEC treated with the Ang-1 peptide-functionalised G1 dendron.

DISCUSSION & CONCLUSIONS: The present study for the first time unveils a novel biomaterial approach to stimulate angiogenesis through nano-structured biomaterials and emphasises the need for a temporospatial presentation of the relevant peptide sequence to obtain endothelial sprouting.

ACKNOWLEDGEMENTS: This work was funded by the EC FP7 Marie Curie ITN, AngioMat, grant agreement n. 317304.

Human adipose stem cells cultured on braided polylactide scaffolds is a potential approach for tendon tissue engineering

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INTRODUCTION: Growing number of musculoskeletal defects increases the demand for a functional engineered tendon which has not been available.

METHODS: In this study we optimized tenogenic differentiation medium (TM) for human adipose stem cells (hASCs). We differentiated hASCs on different 3D structures in the optimized TM, and compared braided poly(L/D)lactide (PLA) 96L/4D and foamed poly(L-lactic-co-ε-caprolactone) (PLCL) 70L/30CL scaffolds as potential structures for tendon tissue engineering. Mesenchymal origin of the hASCs was verified with flow cytometric analysis [1]. Cell viability was analyzed with Live/Dead fluorescence staining. Cell number was analyzed based on the total amount of DNA in the sample by CyQUANT assay. qRT-PCR was used to detect relative changes in mRNA expression of tendon marker genes (scleraxis, tenomodulin, collagen type I, and III). Total collagen content assay was used to evaluate tenogenic differentiation. Immunocytochemical staining of tendon markers (tenomodulin, collagen type I) with specific antibodies was performed to verify tenogenic differentiation. Quantitative cell number, qRT-PCR, and total collagen content results were analyzed to determine statistical significance.

RESULTS: Cell viability, proliferation, and tenogenic differentiation of hASCs were supported on braided PLA 96/4 scaffolds in TM condition compared to foamed PLCL 70/30 scaffolds. Strong immunocytochemical staining result for tenogenic proteins collagen type I and tenomodulin was seen in the braided PLA 96/4 scaffolds compared to poor result in the foamed PLCL 70/30 scaffolds in TM condition at 14 days.

The elastic modulus of PLA 96/4 scaffold at 280±20 MPa resembled more the Young's modulus of 870±200 MPa reported for native Achilles tendon.

PLA 96/4 scaffolds supported the formation of a uniform cell layer when hASCs were cultured in TM compared to maintenance medium (MM) condition after 14 days of culture. Cell number, gene expression of tenogenic marker genes, and total collagen content were significantly higher with TM compared to MM condition after 2 weeks in braided PLA 96/4 scaffolds.

DISCUSSION & CONCLUSIONS: Our study showed that the optimized TM is needed for the efficient and rapid *in vitro* tendon-like matrix production of hASCs. Braided PLA 96/4 scaffolds combined with the optimized TM significantly enhanced tenogenic differentiation of hASCs. This tissue engineering strategy of stimulating hASCs with TM in braided PLA 96/4 scaffolds prior to *in vivo* transplantation might be potential for functional musculoskeletal tissue engineering applications.

ACKNOWLEDGEMENTS: This study was financially supported by the Finnish Funding Agency for Technology and Innovation (TEKES) and Academy of Finland.

Altered microRNA expression profile in exosomes derived from mesenchymal stem cells during osteogenic differentiation

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INTRODUCTION: Exosomes are membrane nanovesicles that have been shown to be secreted by human mesenchymal stem cells (hMSCs) and able to contribute to injury repair of various tissues including heart, kidney and liver [l-3]. However, how hMSCs-derived exosomes mediate the cell-to-cell communication in the injury site to exert their biological functions is still not clear. The aim of this study was to profile the microRNAs (miRNAs) expression in exosomes derived from hMSCs during osteogenic differentiation and further predict the possible regulatory functions of these exosomal miRNAs.

METHODS: Human MSCs were cultured with osteogenic additives in exosome-free media for 21 days. Media were collected in the early (Day 3) and late (Day 21) differentiation stages. Medium from MSC expansion culture was used as control. Exosomes were isolated from conditioned media by ultracentrifugation. RNA from exosomes was extracted and RNA size distribution was examined using Agilent 2100 Bioanalyzer. Exosomal miRNAs were polyadenylated and reverse transcribed into cDNA and labelled by SYBR green. MiRNA expression profile was detected on Exiqon miRCURY LNA™ Universal RT miRNA PCR Human panel I containing 372 miRNAs. DIANA mirPath v.3 was applied to predict the genes and pathways targets of the interested exosomal miRNA candidates.

RESULTS: Exosomes derived from hMSCs during expansion (Exo-P6), early (Exo-D3) and late (Exo-D21) differentiation stages showed altered miRNA expression profiles (Fig.1). There were 9 and 16 miRNAs having significant change when comparing Exo-D21 with Exo-P6 and Exo-D3, respectively. Interestingly, expression of specific miRNAs related to osteogenic differentiation such as miR-21, miR-31 and miR-10b, had significantly changed. Forty-one and sixty-three KEGG biological pathways were significantly enriched ($p<0.05$, FDR corrected) among these differentially expressed exosomal miRNAs. Among them, ECM-receptor interaction, PI3K-Akt signalling pathway, signalling pathways regulating pluripotency of stem cells, FoxO signalling pathway, MAPK signalling pathway, Wnt signalling pathway, mTOR signalling pathway, and Hippo signalling pathway were the most prominent pathways related to osteogenic differentiation.

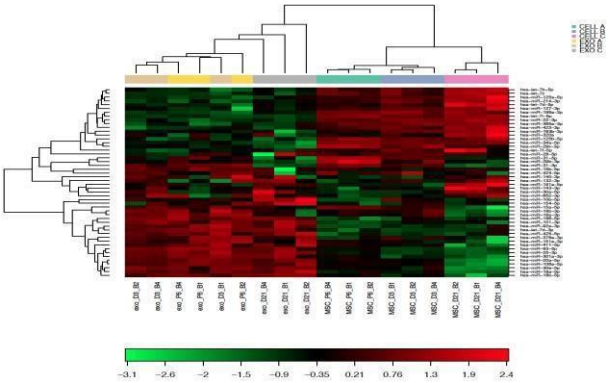

Fig. 1: Altered miRNA profiles of exosomes and hMSCs during expansion (P6) and osteogenic differentiation (D3 and D21) of hMSCs.

DISCUSSION & CONCLUSIONS: The results revealed that miRNAs were differentially expressed in exosomes derived from MSCs during osteogenic differentiation. Moreover, the miRNA target prediction revealed that several biological pathways related to osteogenic differentiation were regulated by these differentially expressed miRNAs.

ACKNOWLEDGEMENTS: This study is supported by the Swedish Research Council (grant K2015-52X-09495-28-4, LUA/ALF grant, the Area of Advance Materials, Chalmers and GU Biomaterials, the BIOMATCELL VINN Excellence Center of Biomaterials and Cell Therapy, the Region Västra Götaland, the Hjalmar Svensson Fund, the Inga Britt and Arne Lundberg Research Foundation and the Adlerbertska forskningsstiftelsen.

The impact of fucoidan from fucus vesiculosus on angiogenesis in OEC and MSC co-culture system

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INTRODUCTION: The main purpose of this study is to investigate the influence of fucoidan, a highly sulfated polysaccharide extracted from fucus vesiculosus revealing heparin-like properties and the ability to bind to vascular endothelial growth factor (VEGF), on angiogenesis and vascularization of bone tissues. Fucoidan might be used to control VEGF levels and in consequence the angiogenic process while it can also be used for the site specific delivery of VEGF. To study the effect of free fucoidan we treated co-cultures of outgrowth endothelial cells (OECs) and mesenchymal stem cells (MSCs) with fucoidan. Previously we have shown that OECs derived from the human peripheral blood possess a high potential of angiogenesis [1]. On the other hand MSCs derived from human bone marrow secrete high levels of VEGF and platelet-derived growth factor to the supernatant of co-cultures which promote the formation of angiogenic structures by OECs [2].

METHODS: Human MSCs and OECs were isolated and cultured as previously described [1,2]. MSCs differentiated in osteogenic medium were seeded in 24-well-plates to establish MSC mono-cultures grown in the osteogenic differentiation medium (ODM). After one day, OECs were seeded to the MSC to setup co-culture systems in the endothelial growth medium (EGM-2). Fucoidan was applied 24h after establishing the MSC mono- or MSC/OEC co-cultures, respectively. The surface marker of endothelial cells VE-Cadherin, angiogenesis related marker CXCR-4 and nuclei were stained to monitor OECs in the co-cultures; DNA quantification, Elisa and RT-PCR were used to evaluate the effect of fucoidan on cell proliferation, growth factor and cytokines in the supernatants or gene expression.

RESULTS: In contrast to fucoidan treated groups in which most of OECs appeared as cell clusters forming a monolayers, control groups showed more angiogenic structures with elongated and orientated alignments of OECs. According to the results of DNA quantification, the cell numbers of fucoidan treated samples for both mono-culture and co-culture were slightly lower compared to untreated samples. The free VEGF levels in supernatant showed a significant decrease in the fucoidan treated groups compared to the untreated ones. The fucoidan groups showed a slight increase in the expression of endothelial markers in co-cultures and a decrease in the expression of VEGF in MSC mono-cultures.

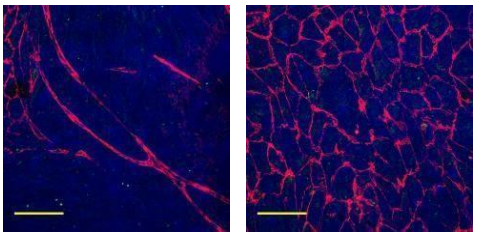

Fig. 1: Confocal laser scanning microscopy (CLSM) of co-cultures. Left: control group. Right: fucoidan treated group. Cells were stained with VE-Cadherin (red), CXCR4 (green) and Nuclei (blue). The scale bar: 100 µm.

DISCUSSION & CONCLUSIONS: The lower level of VEGF in the supernatant of fucoidan treated groups could explain the reduction of proangiogenic structures. As fucoidan can bind to free VEGF, it impaired the angiogenic process in the co-culture system in our study. On one hand Fucoidan was used as a vehicle for site-specific delivery of VEGF in bioartificial scaffolds and was shown to promote angiogenesis i*n vivo* [3]. On the other hand as the addition of fucoidan blocked the angiogenesis in the co-culture system it might be useful for the inhibition of angiogenesis in bone tumors for instance. In summary, the effect of fucoidan on angiogenesis depends critically on the experimental setup and way of application.

Utilization of adipose-derived cells to bio-engineer skin substitutes that include the epidermis, the dermis and the hypodermis

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INTRODUCTION: As an outer covering of the human body, skin serves multiples functions. Its structure and biology make it a perfect boundary, sensory and thermoregulatory organ. Our expertise in the field of regenerative medicine allows the generation of a personalised bio-engineered skin that brings back biological functions to damaged areas of the patient's body.

In some cases, however, there might be a shortage of skin cells for a production of a substitute, which led to a quest for other suitable sources of cells. It appeared that adipose tissue is a ubiquitous and accessible source of both committed mesenchymal cells and mesenchymal stem cells. The Stromal Vascular Fraction (SVF) is obtained after removal of mature adipocytes and blood cells from a fat biopsy and includes Adipose-derived Stem Cells (ASCs), endothelial cells, pericytes, and fibroblasts.

METHODS: SVF cells were isolated from adipose tissue of patients undergoing liposuction or excision surgery. Dermo-epidermal substitutes were prepared by seeding SVF cells into collagen hydrogels and cultured in conditions promoting formation of blood capillaries. Pre-vascularized grafts were transplanted onto immuno-compromised rats.[1]

In another approach ASCs were obtained from the SVF, embodied into a collagen hydrogel and differentiated into adipocytes. Finally, three layered constructs, composed of the epidermis, the dermis and the hypodermis, were created. These skin grafts were transplanted onto immuno-compromised rats.

RESULTS: The ratio of endothelial and stromal cells in the SVF was intrinsically optimal and constant, hence ideally supporting the development of capillary networks in hydrogels. Notably, after transplantation of pre-vascularized skin grafts the tissue engineered capillary plexus was rapidly connected to the rat's microvascular system.

We further present the successful creation of three-layered skin substitutes, both in vitro and in vivo. Sufficient numbers of adipocytes differentiated from ASCs were established. Moreover, these adipocytes survived in the hypodermal compartment after transplantation of three-layered skin substitutes.

DISCUSSION & CONCLUSIONS: On account of an uncomplicated isolation adipose tissue represents an attractive cell source for the regenerative medicine. Our focus on bio-engineered skin substitutes showed that adipose-derived cells can be utilized not only for the efficient pre-vascularization but also for enriching dermo-epidermal skin substitutes with the third layer: hypodermis.

ACKNOWLEDGEMENTS: This work was financially supported by the EU-FP7 project iTERM (grant agreement no. 607868), by the EU-FP7 project EuroSkinGraft (grant agreement no. 279024), by the EU-FP7 project MultiTERM (grant agreement no. 238551), and the Clinical Research Priority Program of the University of Zurich (CRPP: From basic research to the clinic: Novel tissue engineered skin grafts for Zurich). We are particularly grateful to the Foundation Gaydoul and the sponsors of „Dona Tissue" (Thérèse Meier, Robert Zingg, the Vontobel Foundation and the Werner Spross Foundation) for their generous financial support and interest in our work.

Bone tissue engineering for critical sized bone defects

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INTRODUCTION: Treatment of critical sized bone defects is an actual clinical challenge. The "gold standard" in this case is autologous bone grafting. The method disadvantage is associated with limited donor bone resources. Based on our preliminary clinical trial positive results on use of cultured bone marrow-derived multipotent mesenchymal stem/stromal cells (BM-MSCs) in traumatology, our aim was to develop 3D tissue-engineered bone equivalent (3D-TEBE) transplantation technology for restoration of critical sized bone defects.

METHODS: To fabricate 3D-TEBE we used devitalized allogeneic bone (blocks and chips) seeded with cultured autologous cells: BM-MSCs alone or in mix with periosteum progenitor cells (PPCs, 3:1), or with PPCs and endothelial progenitor cells (EPCs, 3:1:1) to promote the graft vascularization depending on bone defect size. BM-MSCs were obtained by bone marrow aspirate seeding and culturing in αMEM with 10% pooled human platelet lysate and 10 ng/ml bFGF under 5% O_2. PPCs were obtained via enzymatic digestion of *fibula* periosteum fragments and propagated in DMEM:F12 with 10% pooled human serum and 10 ng/ml bFGF under 5% O_2. EPCs were isolated by peripheral blood seeding and culturing in selective endothelium growth medium (EGM-2, Lonza) under 5% O_2. Quality of cell cultures was assured by flow cytometry (cell phenotype), cytogenetic analysis (GTG-banding), donor and cell cultures infection screening (IFA, PCR), functional analysis (cell kinetics, CFU analysis, multilineage differentiation assay, cell senescence assay via SA-β-gal activity detection). Quality assurance of cell seeded 3D-TEBE was done via combined staining with FDA/PI and MTT assay. The grafting was performed in 43 patients with 49 critical sized bone defects. The study was approved by Bioethics Committee of State Institute of Genetic and Regenerative Medicine. New bone formation was assessed by the radiographic examination and histological analysis where possible.

RESULTS: The patients were included in a program 4-10 months after injury, provided the ineffectiveness of conventional treatment methods. All cell cultures had a normal karyotype and phenotype, differentiation potential and functional properties, the CFU frequency of 30% and hadn't any signs of cell senescence. BM-MSCs and PPCs had a phenotype: $CD73^+CD90^+CD105^+CD34^-CD45^-HLA\text{-}DR^-$ and they were capable for direct osteogenic and adipogenic differentiation. $CD31^+$ EPCs were capable for capillary-like structures formation in 3D hydrogel. FDA/PI staining, MTT-assay and histological analysis of 3D-TEBE samples showed their regular seeding with viable cells. In all cases 3D-TEBE transplantation took place with any serious complications or adverse reactions. Histological analysis of 3D-TEBE biopsies 3 months after transplantation showed graft intensive remodeling and immature bone tissue formation (Fig. 1). Full restoration of a bone defect was observed after 5-6 months post-surgery by radiographic examination in all 43 patients (49 critical sized bone defects).

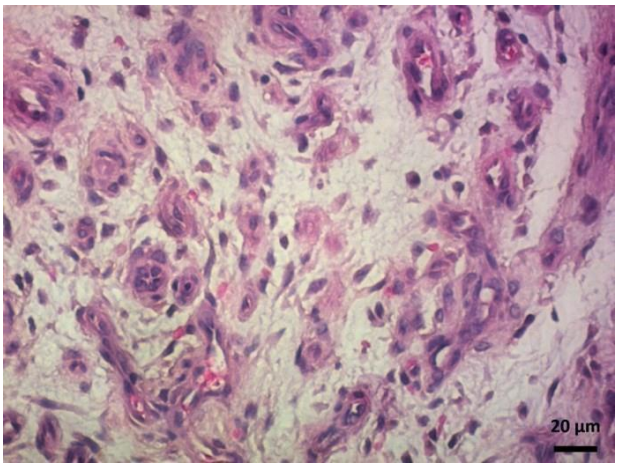

Fig. 1: Histological analysis of grafted 3D-TEBE. Remodeling and immature bone (osteoid) formation 3 months post-transplantation; biopsy taken during graft adaptation resection surgery; H&E stain.

DISCUSSION & CONCLUSIONS: The developed biotechnology of 3D-TEBE transplantation allows restoring the bone integrity, forming new bone tissue in a site of bone defect, and significantly reducing the rehabilitation period of a patient.

Repair of osteochondral defect in a mini-pig model by periosteum-derived osteoblast/BMP-2-embeded alginate/hyaluronic acid hydrogel

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INTRODUCTION: Large bone defects caused by fraction, tumor resection, and congenital deformity are a major challenge for reconstructive surgeons. There are many treatments for bone regeneration. Particularly, the use of biological bone grafts is considered as a common therapeutic treatment for the bone regeneration, however, limited availability of donor materials, additional painful procedure for the patients and risk of infection (autografts), in addition to the risk of immune responses and disease transmission (allografts) remain as significant drawbacks in the clinical field. In this study, we prepared injectable bioactive molecules [human periosteum-derived osteoblasts (PO) and/or bone morphogenetic protein-2 (BMP-2)]-embedded alginate/hyaluronic acid (HA) hydrogels for effective bone regeneration using a mini-pig model [1].

METHODS: Sodium alginate and HA with given ratios were dissolved in water including Na_2HPO_4 to be a polymer concentration of 2 wt%. Directly after $CaSO_4$ powders were homogeneously dispersed in water with different compositions (up to 1.0 wt%), the alginate/HA mixture solution was mixed with the $CaSO_4$ suspension having a same volume (final polymer concentration, 1 wt%) [2]. Alginate/HA solution was changed into gel state with time. The PO and/or BMP-2 were properly added during the hydrogel preparation procedures. The mechanical strength, BMP-2 release behavior, and *in vitro* osteogenesis of PO of the hydrogels were conducted. The animal study using a miniature pig defect model to investigate the effectiveness of the alginate/HA hydrogel for bone regeneration was also conducted.

RESULTS: It was observed that the BMP-2 was continuously released from the Alginate/HA hydrogels for more than 35 days (Fig. 1). The BMP-2-embedded hydrogels showed a positive effect for the osteogenesis of PO. Both PO and BMP-2-embedded hydrogels demonstrated the effective bone regeneration in a mini pig defect model (Fig. 2).

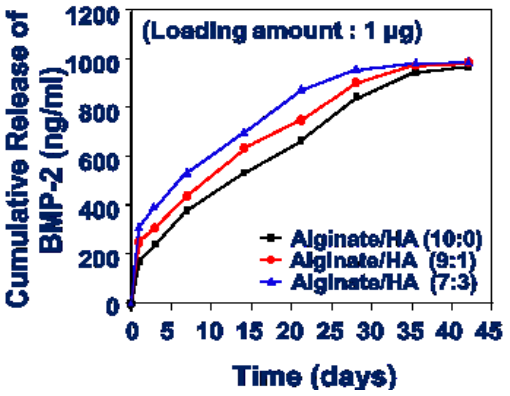

Fig. 1: Cumulative released amount of BMP-2 from the Alginate/HA hydrogels.

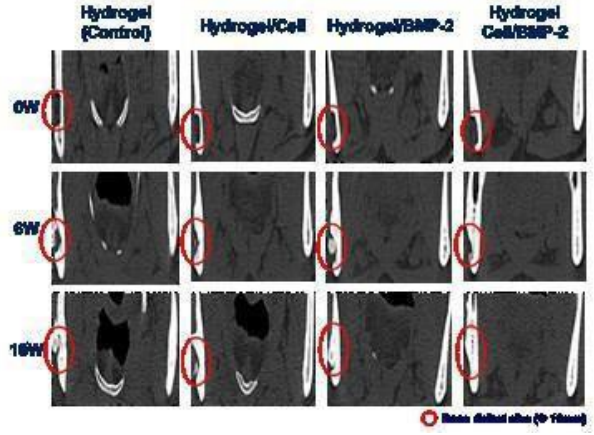

Fig. 2: Computed tomography images of mandibular bony showing the mineralized bone regeneration behavior of each group at different time periods.

DISCUSSION & CONCLUSIONS: Both PO and BMP-2-embedded alginate/HA hydrogel can be a good candidate as an injectable hydrogel system for the repair of osteochondral effect.

ACKNOWLEDGEMENT: This research was supported by the National Research Foundation of Korea (NRF- 2014R1A2A2A04003979).

Bioengineered silk vascular grafts for coronary artery bypass surgery

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INTRODUCTION: Cardiovascular malfunctioning is one of the leading causes of death globally. It is important to note that coronary artery occlusion incidences are more prominent [1]. Current surgical treatment methods require an adequate supply of native vascular constructs to replace the diseased vessels. Autologous vessels are most suitable target but nearly one-third of patients do not have their veins appropriate for grafting due to vascular disease and prior vein harvesting [2, 3]. In the current endeavor, we have developed a functional vascular conduit by combining the cell sheet engineering and patterned silk films.

METHODS: Both mulberry (*B. mori*) and non-mulberry (*A. Assama and P. ricini*) Indian endemic silk varieties were compared. Patterned silk films were characterized using FTIR, AFM, XRD, DSC and TGA. Vascular cells were co-cultured on to films and checked for maintenance of functional phenotype. Fabricated conduits were further characterized for blood compatability, burst strength and *in vivo* immunogenicity.

RESULTS: Non-mulberry silk films presented superior characteristics in terms of thermal stability, proteolytic degradation, cellular proliferation (~1.1-1.3 folds higher than mulberry silk) and alignment of vascular cells. Immunogenicity of films was assessed *in vitro* by looking into macrophage response in terms of TNF-α secretion. It was ~40.79-51.4% lesser as compared to positive control after 7 days. *In vivo* subcutaneous implantation of films in mice showed minimal fibrosis and inflammation after 28 days. Pattern induced alignment favored functional contractile phenotype of SMCs and they strongly expressed contractile markers calponin and α-SMA. Moreover, the expression of elastin in SMCs and punctuated pattern of vWF in ECs layer was observed. Burst strength of tubular construct ranged between 915-1260 mmHg, sufficiently higher than the physiological pressure.

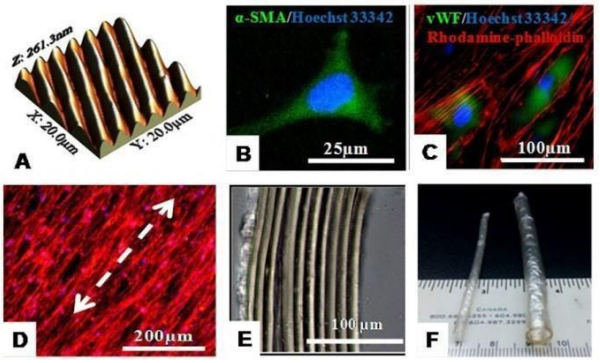

Fig. 1: A) AFM image of patterned silk film, B) Expression of α-SMA in SMCs, C) Expression of vWF in ECs, D) Alignment of vascular cells, E) Cross section of multilayered mature construct, F) Gross morphology of fabricated graft.

DISCUSSION & CONCLUSIONS: We demonstrated the formation of unidirectionally aligned monolayer of vascular cells on patterned silk films expressing their functional phenotype and were metabolically active. Constructs were mechanically suitable for bypass grafting since they withstood and did not fail even at significantly higher values than physiological arterial pressure. Non-mulberry silk varieties (*A.assama* and *P. ricini*) were superior to conventionally used *B. mori* silk in terms of stability, vascular cell compatibility and did not elicit any immunological response when implanted *in vivo* in mice. We envisage that future advancement in this direction would lead towards "off the shelf" clinical implementation of small diameter vascular graft.

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ACKNOWLEDGEMENTS: We are thankful to Department of Atomic Energy (DAE), Department of Biotechnology (DBT), Government of India for their funding support.

Proliferation and differentiation of osteoblasts exposed to pyrophosphate

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INTRODUCTION: Pyrophosphate is a calcium chelator used to control crystal size and enhance mechanical strength in a number of commercial bioceramics [3]. In vivo, pyrophosphate also participates in diverse biological functions, such as inhibiting mineralization, regulating extracellular and intracellular calcium concentration, enhancing proliferation and increased alkaline phosphatase activity [l,2]. Recent studies have suggested that pyrophosphate plays both a beneficial and inhibitory role in mineralization [2-4]. Osteoblast mineralization is inhibited by pyrophosphate delivered in the medium, and enhanced on ceramics containing pyrophosphate. The present study investigated the effect of exogenous pyrophosphate on osteoblast proliferation differentiation and gene expression.

METHODS: Materials characterization: Sodium pyrophosphate (50mM, Sigma) was dissolved in water and neutralized to pH 7.4. The precipitant was purified in alpha-MEM by addition of 1mM sodium pyrophosphate. After centrifugation at 3,000g, the precipitant was washed twice in distilled water, air-dried and characterized by x-ray diffraction (XRD).

In vitro analysis: Mouse calavarial osteoblast cell line, MC3T3, was seeded at 5,000/cm^2 for proliferation, or 10,000/cm^2 for differentiation and gene expression studies. Sodium pyrophosphate was added at a final concentration of 100uM every other day. Proliferation was determined by measurement of the fluorescent redox product of alamar blue. Alkaline phosphatase (ALP) activity was determined by measuring the conversion rate of p-nitrophenylphosphate into 4-nitrophenol. Total RNA was isolated 7 days after pyrophosphate treatment with Tri-reagent, 0.5ug was converted to cDNA (applied biosystems) and RTPCR was run using commercial primers (Invitrogen) for ALPL and collagen 1 (COL1). RTPCR data is expressed as fold over day-1 (FOD) values. A student's t-test was used to identify statistical significance. Each data point represents the average of 3 (RTPCR), or 4 (ALP) or 5 (alamar blue) samples.

RESULTS: Sodium pyrophosphate precipitated as amorphous calcium pyrophosphate in alpha-MEM media. XRD analysis confirmed the amorphous nature of the precipitant. After 7 days of exposure pyrophosphate precipitant affected ALP and COL1 gene expression, but not intracellular ALP activity. The proliferation rate was identical to controls after 24 hours of exposure, while ALP activity was slightly elevated (16%, p=0.35) above controls. ALPL gene expression increased approximately 2-fold higher in pyrophosphate, compared to controls (p=0.004). Collagen1 expression increased by 50% compared to untreated samples (p=0.01).

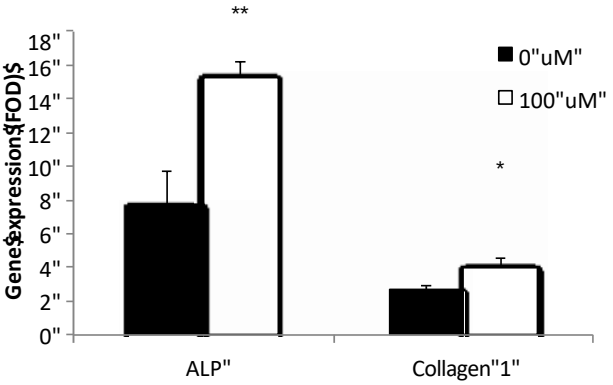

Fig. 1: Gene expression prolife of ALPL and COL1 after 7 days of exposure to 0uM or 100uM sodium pyrophosphate. Statistical differences of p< 0.05 (*), or 0.001 (**).

DISCUSSION & CONCLUSIONS: Pyrophosphate stimulates osteogenic gene expression in MC3T3, without affecting proliferation. Though increased ALP activity was not observed at 7 days, preliminary results suggest that ALP activity increases at later time points (10 days) following pyrophosphate exposure. This study confirms prior findings, and suggests that the reported positive long-term effects on mineralization may occur via changes in gene expression.

DISCLOSURES: Authors have nothing to disclose.

Biomimicking vascular grafts using natural polymers and dynamic stimulation

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INTRODUCTION: Cardiovascular disease is the leading cause of death worldwide, accounting for 29% of global deaths [1]. Arterial bypassing, for small diameter vessels (<6 mm), suffers from poor patency rates using synthetic or autologous grafts due to thrombosis, aneurysm formation, and compliance mismatch. Thus, this project's focus is the development of a bioengineered blood vessel for small diameter vasculature.

Elastin is a key component of native vasculature where it is responsible for the elastic recoil of vessels and modulates smooth muscle cell (SMC) behaviour [2]. Thus, we hypothesized that a bilayered tubular composite of collagen Type I & elastin would provide the optimal intrinsic physiochemical characteristics to support SMCs *in vitro* while subsequently having suitable viscoelastic properties for sustained mechanical stimulation in a custom pulsatile bioreactor.

METHODS: A novel biofabrication technique was developed to generate bilayered tubular scaffolds from a collagen Type I & elastin suspension. The dense film inner layer of the bilayered scaffold was formed via controlled dehydration of the collagen & elastin suspension while the porous outer layer of the tubular scaffold was fabricated via controlled freezedrying in a custom mold to form bilayered tubular scaffolds (Fig.1 A/B). Crosslinking was then utilised to tailor the scaffoldproperties.

Bilayered tubular scaffolds (L=30mm) were seeded with $4.5x10^6$ SMCs and cultured for 7 days statically followed by 14 days dynamic stimulation in a custom bioreactor (Fig.1C) at foetal pulse rates (120bpm) and strain rates (5%±1). Biomechanical properties were assessed with burst pressure, suture retention, and compliance. Biological assessment examined cell proliferation and collagen content, followed byhistology.

RESULTS: The biomimetic biomaterial partially emulates native architecture through its bilayered tubular structure with the outer porous layer having a pore size (~90µm) and porosity (~98%) in the ideal range to support cells while the dense inner layer was designed to support a confluent endothelium and increase the grafts mechanical properties. Carbodiimide crosslinking was determined to be the optimal crosslinking method due to the increased mechanical properties it imparted and so this was selected for further dynamic stimulation. Following 21 days culture (7 static + 14 dynamic) the SMC seeded scaffold was remodelled into a dense organised tissue (Fig. 1D) with a homogenous cell distribution. Dynamic bioreactor stimulation resulted in a 36% increase in burst pressure, improved compliance which closely matched native arteries, and enhanced cell proliferation ($p<0.05$). Furthermore, dynamic stimulation resulted in the circumferential organisation of collagen, improved tissue density, and the expression of the vascular proteins αSMA, calponin, and Coll III in the vessel wall.

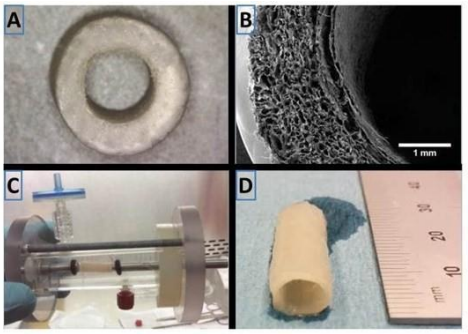

Fig. 1: Macroscopic bilayered collagen-elastin scaffold (A), under SEM (B), bioreactor loaded (C), and remodelled to a dense vessel (D).

DISCUSSION & CONCLUSIONS: Collectively, this study has led to the development of a composite bilayered tubular scaffold with optimised intrinsic physiochemical characteristics to support SMCs *in vitro* while subsequently displaying suitable viscoelastic properties for sustained dynamic conditioning in a custom designed pulsatile bioreactor. This bioengineered vessel represents an ideal *in vitro* platform for examining vascular cell interaction, disease progression, pharmacological toxicity, or cardiovascular medical device testing, in addition to its therapeutic potential.

ACKNOWLEDGEMENTS: European Research Council (grant agreement n° 239685) for funding.

Silica nanofibres –A novel biodegradable material for drug delivery

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INTRODUCTION: Over the last decade, electrospun nanofibres were approved to be an interesting material for application in regenerative medicine and drug delivery[1], exhibiting a number of unique properties including enormous specific surface area, high porosity and small pore size. Properties such as chemical and mechanical stability and degradation characteristics depend on certain electrospun material. While polymer nanofibres are variable in mechanical properties, functionality and degradation rates, they also exhibit several serious disadvantages such as water and protein adsorption and sterilization difficulties. In contrary, inorganic nanofibres represents a family of nanofibres unlimited by these disadvantages potent for medical applications. Silica nanofibres, being member of this family, combine traditional properties of nanofibres based on their structure and advantages of inorganic material. The aim of this study was to outline properties and performance of silica nanofibres as novel biodegradable, biodegradable, high performance matrix for regenerative medicine and drug delivery.

METHODS: Silica (SiO_2) nanofibres produced via modified sol-gel method and subsequent needleless electrospinning were evaluated on structural properties, degradation kinetics, biocompatibility, skin irritation and chromosomal aberration *in vitro*. Skin sensitization test *in vivo* (guinea pigs) was performed in addition. Based on very good biocompatibility results, the silica nanofibres intended as drug delivery system matrix for contaminated wounds treatment was evaluated on drug delivery performance on several antibiotics and antiseptics. Drug release kinetics, antimicrobial activity and biocompatibility were evaluated *in vitro*. This study provides a brief overview of results obtained.

RESULTS: The silica nanofibres were confirmed highly degradable in several simulated body fluids (SBF, PBS, SVF, SLF). The SEM study confirmed its ability to maintain structural characteristics during the degradation process. *In vitro* evaluation demonstrated no cytotoxicity (Vero, 3T3), irritant (EpiDerm™ model) or aberrant (human lymphocytes) effects. No sensitisation effect was observed *in vivo*. The drug release kinetics confirmed quick release in situ with good performance against *Escherichia coli K-12* and *Staphylococcus gallinarum* bacterial strains. The best results were obtained with Tetracycline (TET) antibiotic which also excellent biocompatibility and improving effect on cell viability.

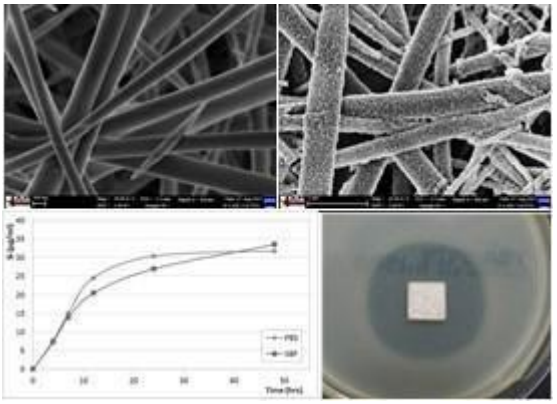

Fig. 1: SEM of silica nanofibres prior and after degradation (48 hrs, SBF), degradation kinetics of nanofibres in in PBS and SBF (150µg/ml, 48 hrs) and result of quantitative antimicrobial test on E. coli (TET).

DISCUSSION & CONCLUSIONS: Silica nanofibres were confirmed as biodegradable, biocompatible material able to maintain its structural characteristics during its degradation and having a huge potential as matrix for drug delivery and contaminated wounds and ulcers treatment.

ACKNOWLEDGEMENTS: The research reported in this paper was supported in part by the Project OP VaVpI CXI CZ.1.05/2.1.00/01.0005, in the framework of the targeted support of the NPU I programme LO1201 and project SGS 2015/16 (21066/115) at the Technical University of Liberec.

Ionic exchanges modulate the in vitro response of mesenchymal stem cells to biomimetic hydroxyapatite

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INTRODUCTION: *In vitro* assays are a potent tool for the evaluation of biomaterial performance. However, in the case of bioactive materials, like calcium phosphates (CaPs) the ionic exchange with the cell culture medium can make cell cultures very tricky. This is particularly relevant in the case of biomimetic hydroxyapatite, which has high specific surface area (SSA) and high surface reactivity. These features support its excellent *in vivo* performance, but can make the *in vitro* results difficult to interpret. The aim of this study was to analyse to what extent the outcome of cell culture studies on biomimetic calcium deficient hydroxyapatite depend on cell culture conditions, and more specifically on the ratio between cell culture and material volume, a parameter often overlooked.

METHODS: Calcium deficient hydroxyapatite (CDHA) discs were obtained from α–tricalcium phosphate (α-TCP) paste. To obtain β-tricalcium phosphate (β-TCP) the discs were sintered at 1100°C, and used as control. Two dimensions of discs were prepared: i) Large discs (~12 mm Ø x 2 mm height): CDHA -L and β-TCP-L; ii) Small discs (~5.5 mm Ø x 300 µm height): CDHA-S and β-TCP-S. SSA and porosity were determined by N_2 adsorption and mercury intrusion porosimetry respectively. Rat mesenchymal stem cells (rMSCs) were seeded (300 cells/mm^2) on the large and small discs, using always 2 mL of advDMEM complete medium, resulting in two cell culture conditions: i) low ratio between culture medium and biomaterial volume (V_{CM}/V_B ~ 10); and ii) high ratio (V_{CM}/V_B ~ 100). rMSCs proliferation was determined at 6h, 3, 7 and 14d by LDH quantification. Ca^{2+} and P_i concentration were monitored at the same time points. Quality of cell adhesion was assessed by visualization of phosphorylated focal adhesion kinase (pFAK) and actin stress fibres formation on CDHA-L and CDHA-S samples. For CDHA-S gene expression of RUNX2, ALP, COLL I, OC, ON, OP and BMP-2 was analysed by RT-qPCR..

RESULTS: Ca^{2+} and P_i concentrations in the culture medium were significantly altered only in CDHA-L (Fig. 1). Cells were able to proliferate on β-TCP (SSA=0.71 m^2/g), irrespective of the size of the discs. In contrast, cell behaviour on CDHA (SSA=19.13 m^2/g) was strongly dependent on disc size; even if cells adhered on both, they proliferated only on CDHA-S. pFAK was not detected and actin stress fibres did not form on CDHA-L. In contrast, both features were observed on CDHA-S. Noteworthy, upregulation of genes related to osteogenesis was observed onCDHA-S.

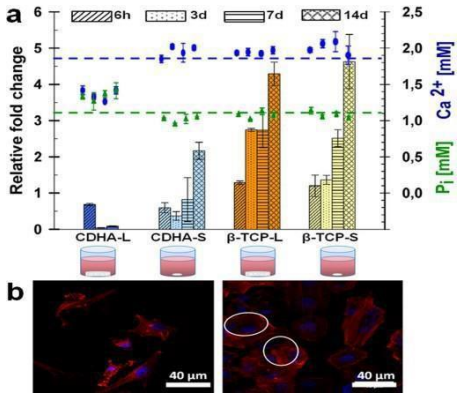

Fig. 1a) Proliferation of rMSCs and levels of Ca and Pi; b) pFAK and actin fibres on CDHA-L (left) and CDHA-S (right) at day 3.

DISCUSSION & CONCLUSIONS: Although rMSCs were able to attach both on small and large CDHA discs, the changes induced in the ionic extracellular environment by CDHA-L resulted in a decrease of cell number over time. This effect of V_{CM}/V_B was not significant for β-TCP, due to its smaller reactivity and SSA. The non-maturation of pFAK and actin stress fibres was an indication of a deficient cell adhesion on CDHA-L, which could lead to cell death via anoikis, a type of apoptosis that is known to be triggered by the deficiency of calcium [1]. In fact, when ionic fluctuations were reduced by increasing V_{CM}/V_B (CDHA-S), more mature focal adhesions and actin fibres were found. This demonstrates the need of adjusting cell culture parameters considering material/cell culture medium interactions.

ACKNOWLEDGEMENTS: Spanish government (MAT2015-65601-R).

Self-assembling peptide hydrogels for articular chondrocyte culture

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INTRODUCTION: Self-assembly has emerged as a powerful tool for the fabrication of novel functional biomaterials[1,2]. A variety of molecular building blocks can be used for this purpose, one of which has attracted considerable attention in the last 20 years: *de-novo* designed peptides[3]. Our group has recently developed a family of hydrogels that exploit the self-assembling properties of short peptides, typically 8 amino acids long[4,5]. The properties of these hydrogels can be tailored for use in a variety of biomedical applications ranging from tissue engineering[6] to drug delivery[7]. In this work we explore the use of our platform for the culture of articular chondrocytes.

METHODS: Cells were maintained in DMEM supplemented with 10 % FBS and cell-type specific supplements in a 5 % CO_2 humidified incubator at 37 °C. Peptide solutions were prepared at concentrations of 10-30 mg mL^{-1} and adjusted to pH 7.5 with 1M NaOH. Cells were suspended and cultured in 3D. Cell counts were performed in triplicate by dissolving the gel in excess media and then counting using trypan blue and a haemocytometer. Antibody staining was performed to observe Col I and Col II.

RESULTS: Peptide gels with different net charges and mechanical properties at pH 7.5 were produced. Bovine chondrocytes (BC) were cultured for 21 days. After an initial decline in cell numbers grown in both gels, due to environmental change, numbers increased on both gels at day 3 with F+ (overall positive charge) exhibiting a statistically significant increase in proliferation when compared to F= gel (no net charge). In both hydrogels cell numbers continued to increase until day 21. Antibody staining was used to visually observe Col I and Col II production (Fig. 1). Both systems exhibited Col I and some Col II at day 25, suggesting cell phenotype is maintained. Effect of charge on overall collagen production was also investigated (Fig. 2).

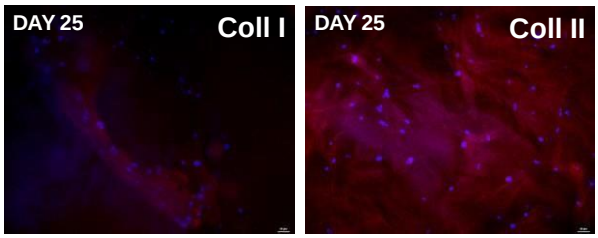

Fig. 1. Antibody staining of bovine chondrocytes cultured in F8 at day 25.

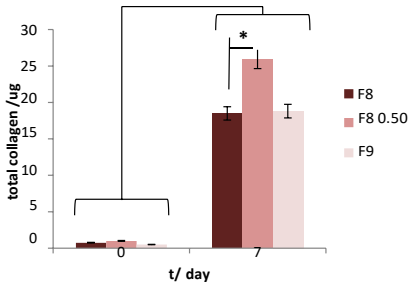

Fig. 2. Total amount of collagen measured when culturing bovine chondrocytes in F8, 50/50 F8/F9 and F9. All hydrogels had a G' of 10 kPa.

DISCUSSION & CONCLUSIONS: We have shown that our peptide hydrogel platform allows to tune the physical properties of the hydrogels in a controlled manner. The cell response was found to be dependent on the physical properties of the hydrogel in particular mechanical and charge.

ACKNOWLEDGEMENTS: This work is funded by an EPSRC fellowship (EP/K016210/1).

Preparation of β-glucan scaffolds by hydrogel foaming with supercritical CO_2

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INTRODUCTION: New materials and processing techniques are being constantly developed for the production of scaffolds for tissue engineering applications. Traditionally, foaming of polymers with supercritical fluids is one of the procedures used to create porous matrices. However, this technique cannot be applied to hydrophilic polymers which suffer degradation below their melting temperature. Foaming of hydrogels is an alternative to conventional gas foaming for the processing of hydrophilic polymers by dissolution of supercritical CO_2 in the water of the hydrogels [1]. Through a fast depressurization, a highly porous structure is obtained, which results in the foamed matrix of polymer after water removal.

In this work, β-glucan aerogels are produced by hydrogel foaming with supercritical CO_2. Among polysaccharides, β-glucans have not been widely explored yet for tissue engineering applications. Depending on their origin, they posses different structures and properties. In our study, hydrogels were created from barley and yeast β-glucans. The produced aerogels were characterized in terms of morphology, mechanical properties and degradation rate in physiological fluids.

METHODS: Hydrogels of 2.5% (w/w) yeast β-glucans (L-Naturae Nutraceutical, Naturae) and 4% (w/w) barley β-glucans (Glucagel, DKSH) were foamed in a high-pressure vessel at 37 ºC and different pressures in the range of 80-200 bar. The hydrogels were introduced in the pre-heated vessel and CO_2 was fed into it at the selected pressure. The system was kept at high pressure for 1.5 h and afterwards it was sudden decompressed in order to promote the foaming and at the same time freezing of the hydrogels, producing stable structures, which were further frozen at -80 ºC and subsequently freeze-dried.

The structure of the aerogels was analyzed by SEM (Leica Cambridge S360) and micro-CT (SkyScan 1272, Bruker). Compression tests were performed in a mechanical testing equipment (model 5543, Instron). Degradation tests were done by immersion of the aerogels on PBS at 37ºC and weight loss of the samples was followed for 3 weeks.

RESULTS: Porosity, pore size and interconnectivity of β-glucan aerogels were greatly increased due to the foaming (Fig. 1). A highly porous structure was obtained after foaming. Scaffolds with an average pore size in the order of 100 μm and 80-90% porosity were obtained and the porosity increased with the operating pressure.

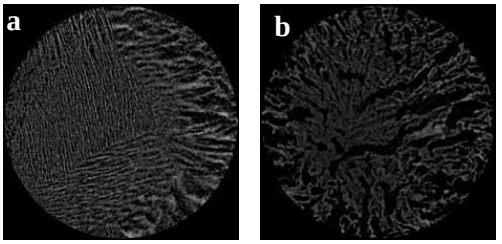

Fig. 1: Micro-CT images of non-foamed (a) and foamed at 80 bar (b) barley β-glucan aerogels.

On the contrary, their mechanical resistance diminished in comparison with non-foamed aerogels. Also, their degradation rate was higher when foamed, although they were not completely decomposed in any of the cases.

DISCUSSION & CONCLUSIONS: β-glucan hydrogels were successfully foamed with supercritical CO_2. The resulting scaffolds fulfill the morphological and mechanical requirements to be used as scaffolds in regenerative medicine.

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ACKNOWLEDGEMENTS: Authors thank MINECO (CTQ2013-44143-R) for financial support. M. Salgado thanks MECD for her FPU and mobility grants. S. Rodríguez-Rojo acknowledges MINECO and UVa for her Juan de la Cierva fellowship (JCI-2012-14992).

Study of soft-tissue regeneration on small intestinal submucosa scaffolds using a subcutaneous rat model: preliminary results

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INTRODUCTION: Biomaterials based on extracellular matrices (ECM) are a common choice as tissue scaffolds due to their composition and superior host response when compared with artificial materials. Porcine small intestinal submucosa (SIS) is an example of an ECM biomaterial composed mainly of a collagen structure suitable for supporting cell attachment, proliferation, differentiation and migration [1]. For this reason, it has been successfully used as scaffold for soft tissue healing. Although it has been seen that the therapeutic outcome of SIS as a vascular graft (VG) might be affected by the manufacturing process, SIS VGs have proven suitable in pre-clinical models [1-3]. In pursuit of the development of a SIS VG, further research is needed on the mechanism of neotissue formation. In this way, the current project's aim is to study the regenerative response of soft-tissues due to SIS with varying the material stiffness and membrane orientation, under passive conditions, using a subcutaneous animal model.

METHODS: SIS discs of 1-cm diameter were implanted subcutaneously in *Lewis* rats. On each animal, four different groups were sutured (n=5), varying the number of layers, the SIS side in contact with the muscle and the implantation period. Two groups had one layer, while the rest had four, and half of the samples were implanted with the luminal side of the intestine in contact with the muscle, whereas the other half with the abluminal side. Discs were explanted after 15 or 21 days. The harvested tissue was fixed in formaldehyde, paraffin embedded and sectioned for histological staining.

RESULTS: Gross analysis showed in all samples a dense vascularized fibrous capsule surrounding the SIS discs. Preliminary results of the histological analysis showed sparse cellular invasion inside the SIS membranes, regardless of the group and harvest times. Most cellular invasion occurred on the borders of the sample, especially on the multilayer samples, where vascularized tissue was found between the layers (*Figure 1)*.

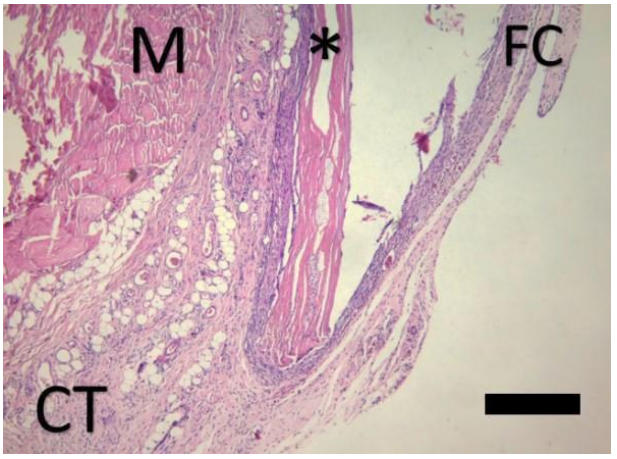

Fig. 1: H&E stain of a tissue interface with a SIS sample (*) of 4 layers after 21 days. It can be noticed a layer of connective tissue (CT) between the muscle (M) and the SIS, and a fibrous capsule (FC) with similar histological structure. Scale bar 200 um.

DISCUSSION & CONCLUSIONS: In this study we investigated the host reaction to the SIS scaffold. Although the results showed an active response to the material forming an early fibrous capsule, there was little invasion inside the membrane likely due to the low superficial porosity. Consequently, if the SIS membrane is used as a vascular graft, the porosity of the material should allow cellular invasion as it was seen in the borders. In that way, vascular remodelling processes, like trans-interstitial capillarization and surface endothelization with SIS scaffolds are induced when used as a vascular graft.

ACKNOWLEDGEMENTS: This work was financially supported by COLCIENCIAS Doctorate Scholarship Program 617-2 to Paolo Sánchez and National Science Foundation (IIS- 1125412).

Fabrication and characterisation of electrospun membranes containing strontium-substituted bioactive glass for bone regeneration

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INTRODUCTION: Guided bone regeneration is a dental procedure used to encourage the healing of bone tissue defects caused by periodontal disease. In this therapy, membranes are used to create a barrier that prevents the migration of cells originating from surrounding tissues into the defect, allowing bone cells to proliferate and produce new bone matrix without disturbance. Although effective, membranes used in the clinic generally lack the ability to directly stimulate the formation of new bone tissue. Our hypothesis is that barrier membranes containing an osteogenic component will be able to improve bone healing. Therefore, the aim of this study was to investigate the development of composite membranes with enhanced osteogenic properties made of bioresorbable polymers and bioactive glass, and manufactured using the solution-electrospinning technique.

METHODS: Electrospun composite membranes were fabricated using poly(caprolactone) (PCL) and particles of strontium-substituted bioactive glasses based on the 45S5 composition. For the substitution, calcium was partially (50%, Sr50) or fully (100%, Sr100) replaced with strontium on a molar basis. The effect of these compositional changes on glass density and solubility, as well as on the *in vitro* metabolic activity of rat bone marrow mesenchymal stromal cells (MSCs), was investigated. The expression of six genes associated with the process of osteoblast differentiation was studied on MSCs using RT-qPCR. Additional glass particles were added to the surface of the electrospun membranes using a method developed internally. The different membrane compositions were characterised using scanning electron microscopy and energy dispersive x-ray spectroscopy, and the dissolution of glass particles contained within the electrospun fibres was evaluated in water. *In vitro* membrane cytotoxicity was investigated by studying its effect on the metabolic activity of rat osteosarcoma cells cultured on the materials.

RESULTS: The substitution of calcium by strontium resulted in increased glass density and solubility. The metabolic activity of MSCs was inhibited in the presence of increasing amounts of glass powders, with more severe reductions observed in the case of Sr100. However, it was enhanced in the presence of <20 mg of Sr50. The bioactive glasses also stimulated the expression of osteogenic genes on MSCs, with a greater effect of strontium-substituted bioactive glasses on alkaline phosphatase and osteocalcin [1]. The electrospun composite membranes were made of fibres exhibiting regions of increased diameter where the glass particles accumulated. The embedded glass dissolved after immersion in water, increasing the local pH. Further evidence suggested accelerated polymer degradation due to interactions between PCL and the glass. All materials exhibited good *in vitro* biocompatibility, except those with >0.5 mg of additional surface glass particles.

DISCUSSION & CONCLUSIONS: The addition of strontium to bioactive glass and its influence on material properties resulted in clear biological effects, such as a greater *in vitro* cytotoxicity due to the increased solubility. However, the enhanced metabolic activity observed in the presence of Sr50 suggested that a particular ratio in the content of strontium and calcium might be able to maximise the stimulatory effect of these compositions. The presence of the glasses also enhanced the expression of osteogenic genes of MSCs, showing that strontium-substituted bioactive glasses promoted osteogenesis in a differentiating cell culture model. These results suggested that these are promising compositions for bone tissue regeneration applications, including barrier membranes for guided bone regeneration or scaffolds for musculoskeletal tissue engineering. This is supported by the potential shown by electrospun PCL/strontium-substituted bioactive glass membranes regardingbiocompatibility.

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ACKNOWLEDGEMENTS: The authors would like to acknowledge the EPSRC for their support.

DISCLOSURES: The authors of this study have nothing to disclose.

In vitro analysis of mesenchymal stem cell interactions with collagen-based scaffolds cross-linked by EDC/NHS or genipin

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INTRODUCTION: The collagen-based scaffolds have potential to imitate an extracellular bone matrix and support human mesenchymal stem cells (hMSCs) adhesion, proliferation and osteogenic differentiation. However, the fast biodegradation rate and the low mechanical strength of the untreated collagen very often complicate *in vitro* and *in vivo* applications [l]. The stability of collagen scaffolds can be enhanced by cross-linking. Thus, the impact of various cross-linking agents (genipin, EDC/NHS/EtOH or EDC/NHS/PBS [2-4]) on scaffold properties were tested *in vitro* in this study.

METHODS: The cell metabolic activity (Cell Titer 96 AQueous One Solution Cell Proliferation Assay, MTS, Promega, USA) of the cells cultivated in scaffolds infusions (cytotoxicity determination), the metabolic activity, the number of cells adhered on scaffolds and fluorescence 2D/3D visualization of cell/scaffold interactions were determined. All procedures were performed after 48 h and 168 h of cell cultivation. Tissue culture treated polystyrene (PS) (TPP, CH) was used as a positive control. hMSCs were isolated from a bone marrow blood of three healthy donors.

RESULTS: Composite scaffolds (natural collagen, poly(DL-lactide) electrospun nanofibers, calcium phosphate and sodium hyaluronate) cross-linked by EDC/NHS/EtOH, EDC/NHS/PBS or genipin were tested *in vitro*. The scaffold infusions were checked for releasing of cytotoxic agents into cultivation medium. After 48 h, the metabolic activities of hMSCs cultivated in all three scaffolds infusions were comparable and higher than the PS control. After 168 h, the metabolic activities of the cells cultivated in genipin and EDC/NHS/EtOH cross-linked scaffolds infusions remained similar. However, the metabolic activity of hMSCs in EDC/NHS/PBS cross-linked scaffold infusion markedly decreased to 83 % of PS control. Metabolic activities of the cells seeded on the scaffolds was almost comparable among all three samples at 48 h and 168 h. Fluorescence visualization determined the cell ability to adhere on all the tested scaffolds. After 48 h, hMSCs on the genipin cross-linked scaffold revealed the best morphology and symmetric distribution. After 168 h, cells on all the scaffolds were similarly organized with comparable appearance. Cell penetration ability was comparable at both times. Genipin maintained constant mechanical properties in contrast to the rest of cross-linkedscaffolds.

DISCUSSION & CONCLUSIONS: All the scaffold infusions seemed to be non-cytotoxic; cytotoxicity is often determined by the 75% limit of the metabolic activity. However, the metabolic activities and penetrations of cells seeded on scaffold were comparable for all three scaffolds, the quality of cell morphology, adhesion and distribution was different. The results revealed that cross-linking agents can influence cell viability, condition and scaffold behaviour. In this study, the genipin assigned the best mechanical and biological features to created scaffolds for hMSC application. It will be used for subsequent *in vitro* 2D and 3D and *in vivo* experiments.

ACKNOWLEDGEMENTS: This study was supported by projects provided by: the Ministry of Health of the Czech Republic (AZV15-25813A); Charles University in Prague grants (SVV–2015 No.260170, SVV-2015–260209, GAUK 400215, PRVOUK-P24/LF1/3); the National Sustainability Program I (NPU I)Nr. LO1503 provided by the Ministry of Education Youth and Sports of the CR; and by project no.RVO: 67985891.

Biofunctionalization of patterned PLLA surfaces with RGD, REDV and YIGSR peptides to promote endothelial cells adhesion and migration

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INTRODUCTION: Angioplasty and stent implantation open the blocked artery and restore proper blood flow after atherosclerosis disease. Despite their utility, intracoronary stents induce two types of complications: in-stent restenosis and thrombosis. Recently, biodegradable poly-L-lactic acid (PLLA) stents have been inserted to restore the healed artery, reabsorb and consequently avoid late stent thrombosis [1]. Moreover, rapid stent endothelialisation is one of the main goals in order to heal the damaged tissue and decrease in-stent restenosis [2]. The present work combines topographical modification and peptide functionalization of PLLA surfaces to increase endothelial cells adhesion and migration, and thus enhance endothelialization.

METHODS: PLLA films were obtained by solvent casting with a solution of PLLA (PL65 from Purasorb) in chloroform (3.7%v/v). Linear topography pattern was done by hot stamping at 180ºC with PDMS stamps (periodicity: 10 µm, depth: 1 µm). RGD, REDV and YIGSR lineal peptides with and without a terminal carboxyfluorescein molecule (CF) were synthesized by solid-phase and covalently attached to the surfaces using EDC/NHS. Planar and/or non-functionalized surfaces were used as control. Crystallinity, thermal degradation and topography were evaluated by DSC, TGA and interferometry respectively. HUVEC adhesion was characterized by immunofluorescence. HUVEC migration was performed through a wound healing assay.

RESULTS: Homogeneous linear nanopatterned surface were successfully obtained with ~900 nm depth and ~10 µm periodicity. As expected, DSC indicated that hot stamping reduced crystallinity from 26,2% to 10,0%. TGA measured degradation temperature of PLLA films was 360ºC, higher than the temperature of the hot stamping process. Visualization of the immobilized CF-peptides by fluorescent microscopy shows a successful and similar adhesion of RGD, REDV and YIGSR peptides on PLLA films. Cell adhesion studies indicated higher number of adhered HUVECs onto RGD and YIGSR surfaces compared to REDV and non-functionalized films. The effect of linear topography was clear showing an elongation and alignment of the adhered cells following the pattern channels. HUVEC cells on the combined functionalized with YIGSR and patterned surfaces induced an accelerated HUVECs migration.

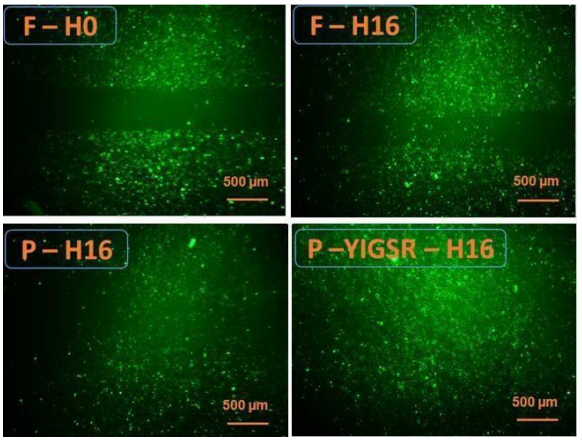

Fig. 1: HUVEC migration at initial point (H0) and after 16h (H16) of flat (F), patterned (P) and patterned and functionalized with YIGSR (P-YIGSR) PLLA films.

DISCUSSION & CONCLUSIONS: PLLA surfaces with linear patterning and functionalized with RGD, REDV and YIGSR peptides were successfully obtained. Topography induces cell orientation parallel to the linear pattern. Biofunctionalization with RGD and YIGSR peptides enhances cell adhesion compared to REDV and non-functionalized surfaces. Furthermore, functionalization and patterning accelerate HUVEC migration in the pattern direction. Overall, patterned PLLA functionalized with RGD or YIGSR may be excellent candidates to improve PLLA stent endothelialization.

ACKNOWLEDGEMENTS: R.S. would like to thank the Erasmus Mundus Doctoral Programme for funding through a DocMASE Scholars.

Decellularization of human articular cartilage for tissue engineering

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INTRODUCTION: Scaffolds derived from decellularized allogenic tissues provide a natural extracellular matrix (ECM) for tissue engineering strategies. Besides giving structural support, ECM is known to regulate cell behavior such as proliferation, migration and differentiation.

METHODS: Cartilage was harvested from human femoral heads. The protocols tested study can be grouped into three categories: SDS-based, SDS-free and enzyme-based. The protocols adapted from Kheir et al. [1] consist of freeze/thaw cycles, hypotonic treatment, decellularizing agent, washing and decontamination and optionally include enzyme treatment for targeted removal of certain ECM components. Ionic and non-ionic detergents, oxidizing agents as well as acids and bases were tested as decellularizing agents. The enzyme screening involved hyaluronidase, collagenase, pronase, pepsin, elastase and combinations thereof. Cell removal and changes in ECM components were evaluated via biochemical assays (DNA, DMMB), histological examination and SEM imaging. Furthermore, seeding tests with adipose derived stem/stromal cells were conducted to evaluate the cell's affinity to attach to the scaffolds.

RESULTS: By using SDS-based protocols satisfactory reduction of DNA content could be achieved, however scaffolds treated with SDS showed low cell attachment and cell viability. Among the other chemical agents, sodium hydroxide was established as most effective for glycosaminoglycan (GAG) reduction and hydrochloric acid proved to efficiently decellularize the scaffold, reducing the DNA content by 97% (Fig. 1). In a screening of various enzymes, pepsin emerged as best option for GAG depletion and was thus chosen to replace sodium hydroxide in the final protocol. Histological stainings indicated a strong reduction in GAGs while the collagen II staining was unchanged (Fig. 2). SEM images revealed the ECM to be less dense than in native tissue, possibly facilitating cell ingrowth. Seeding tests showed good adhesion of cells on top of the scaffold.

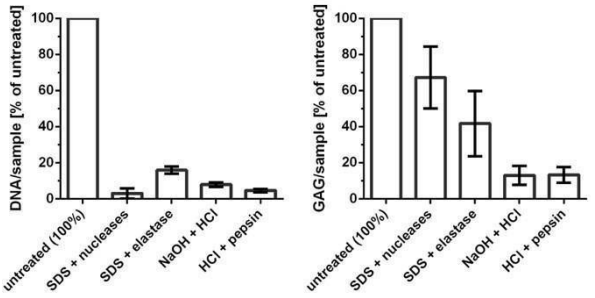

Fig. 1: DNA and GAG content of some of the tested protocols as percent of the content in untreated cartilage. Data presented as mean ±SD.

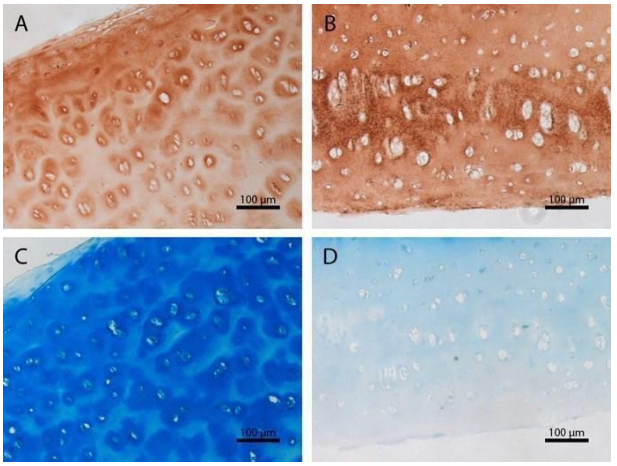

Fig. 2: Collagen II immunostaining (A, B) and alcian blue staining (C, D) of untreated (A, C) and decellularized and GAG-depleted (B, D) cartilage.

DISCUSSION & CONCLUSIONS: A protocol combining freeze/thaw cycles and treatment with hydrochloric acid followed by pepsin was established, achieving successful decellularization and depletion of GAGs within one week.

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ACKNOWLEDGEMENTS: We acknowledge the Austrian Research Promotion Agency FFG for funding the project 'CartiScaff' #842455.

In vitro evaluation of soy protein based hydrogels for wound-healing

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INTRODUCTION: To accomplish ideal wound dressings, hydrogels are highly interesting materials due to their intrinsic high water content. In contrast to animal protein, soy protein isolate possesses a lower immunogenicity and more controllable degradability [1]. Moreover, phytoestrogens from soy protein substantially accelerate wound repair [2]. In this study, a combination of soy protein isolate and alginate hydrogel was evaluated to developing novel matrices for wound dressing applications.

METHODS: Soy hydrogels were prepared by ionic gelation of 2% (w/v) soy protein and 2% (w/v) alginate solution in calcium chloride solution (0.1 M). Water uptake (A) and weight loss (B) kinetics was assessed by weighting hydrogels after incubation in DMEM and HBSS for indicated time-points. For fluorescence microscopic images, membranes were incubated for 10min. in 10 µg/ml hoechst 33342 solution and further analyzed with a Zeiss AxioObserver. Cell quantity was assessed via CellTiterGlo assay (Promega) according to the manufacturer´s instructions.

RESULTS: Water uptake is decreased in alginate/soy protein-composite membranes compared to pure alginate. Weight loss studies demonstrated an increased degradation of alginate /soyprotein-composite membranes. Human adult keratinocytes (HaCaT) were able to attach onto the membranes and were highly viable on alginate /soyprotein-composite hydrogel films in comparison to pure alginate.

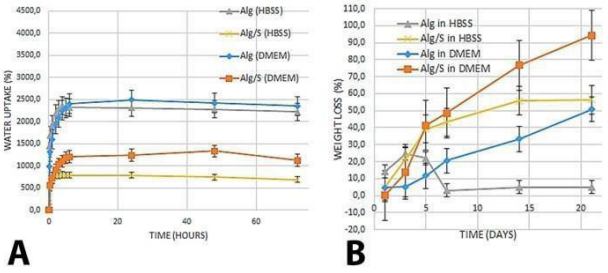

Fig. 1: Determination of water uptake (A) and weight loss (B) kinetics of alginate (alg) and alginate /soy protein hydrogel films.

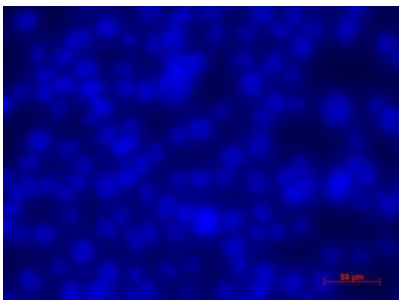

Fig. 2: Fluorescence microscopic image of HaCaT cells on alginate /soyprotein-composite hydrogel.

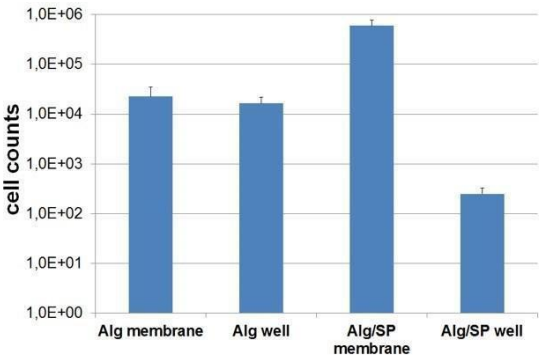

Fig. 3: Cell quantification on alginate (alg) and alginate /soy protein hydrogels.

DISCUSSION & CONCLUSIONS: In contrast to pure alginate hydrogels, the use of alginate-soy protein hydrogels offers a highly interesting approach for colonization of human keratinocytes on an easy to handle three-dimensional material. Further research will show the applicability of the new biomaterial in human wound-healing and wound care strategies.

Covalent immobilization of stem cell inducing/recruiting factor and heparin on cell-free small-diameter vascular graft for accelerated *in situ* tissue regeneration

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INTRODUCTION: The development of cell-free vascular grafts has tremendous potential for tissue engineering. However, thrombus formation, less-than-ideal cell infiltration, and a lack of growth potential limit the application of electrospun scaffolds for *in situ* tissue-engineered vasculature. To overcome these challenges, here we present development of an acellular tissue-engineered vessel based on electrospun poly(L-lactide-co-ε-caprolactone) (PLCL) scaffolds. Heparin was conjugated to suppress thrombogenic responses, neuropeptide substance P (SP) was immobilized to recruit host cells, and microfibers were fabricated to polarize macrophages and enhance cell infiltration.

METHODS: Linear and star-shaped PLCL copolymers were prepared by ring opening polymerization.[1-2] SP (Peptron, Daejeon, South Korea) and heparin (Sigma Aldrich) were conjugated with the terminal hydroxyl groups of star-shaped PLCL copolymers using carbonyldiimidazole (CDI) chemistry and esterification, respectively.[3] Bi-layered vascular grafts (Inner diameter = 2.0 mm, wall thickness = 200 μm, thin layer = 10–20 μm and thick layer = 180–190μm) were prepared using electrospinning. Vascular grafts were characterized for blood leakage, mechanical properties, burst strength, SP release, platelet adhesion, and *in vitro* cell migration. Biocompatibility and functional performance was assessed after subcutaneous implantation in rats (age = 7 weeks, n = 32) for up to 4 weeks. Explanted grafts were extensively characterized for histology, extracellular matrix deposition, inflammatory response, macrophage polarization, endogenous cell recruitment, smooth muscle cell regeneration, ECM deposition, and neovascularization. Data were expressed as the mean ± standard deviation. A two-tailed paired Student's *t*-test was used to compare the differences. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS: Bi-layered vascular grafts were successfully fabricated. SP was released in a sustained manner from scaffolds and recruited human bone marrow-derived mesenchymal stem cells. Histological analysis revealed a higher extent of accumulative host cell infiltration, neotissue formation, collagen deposition, and elastin deposition in scaffolds containing either SP or heparin/SP than in the control groups. We also observed the presence of a large number of laminin-positive blood vessels, von Willebrand factor (vWF^+) cells, and alpha smooth muscle actin-positive cells in the explants containing SP and heparin/SP. Additionally, SP and heparin/SP grafts showed the existence of $CD90^+$ and $CD105^+$ MSCs and induced a large number of $CD206^+$ macrophages to infiltrate the graft wall compared with that observed with the control group.

DISCUSSION & CONCLUSIONS: Cell-free, three-dimensional, bio-instructive small-diameter vascular grafts were prepared using electrospinning. The present study has demonstrated that vascular grafts containing SP and heparin/SP markedly enhanced cell infiltration, MSCs recruitment, blood vessel formation, and efficient regeneration of SMCs and ECMs components in comparison with that observed in regular PLCL vascular grafts. Our cell-free grafts could enhance vascular regeneration by endogenous cell recruitment and by mediating macrophage polarization into the M2 phenotype, suggesting that these constructs may be a promising cell-free graft candidate and are worthy of further *in vivo* evaluation.

ACKNOWLEDGEMENTS: This work was supported by the Ministry of Trade, Industry and Energy, Republic of Korea (Project Number 10052732).

Porous biomaterials: The methodology and characterisation of laser based additive and subtractive manufacturing techniques

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INTRODUCTION: Accurate control over biomaterials porosity is required in order to tailor the biomaterial towards tissue-specific applications. I will report on a hybrid of structuring techniques; using both an additive direct laser write, and a subtractive laser cutting fabrication technique, to tailor both the micro and macro porosity of emulsion templated foams for this application. This is an advanced manufacturing methodology approach to address the need to produce bespoke porous biomaterials at a large, reproducible scale. The laser based subtractive manufacturing of biomaterials at its current state is relatively unexplored in relation to the more recent additive manufacturing based techniques.

This work builds upon our previously developed additive manufacturing techniques; combining the tunability of emulsion templating with a direct laser write approach to produce porous polymeric structures from a photo curable material [1], with controllable mechanical properties [2]. This fabrication method mirrors the current laser cutting subtractive manufacturing technique I am presenting.

In all presented cases the pre-processing conditions control the micro-porosity of the biomaterial, and the post processing ones control the macro porosity. Laser cutting is a high throughput technique can be used to create a secondary layer of porosity which can be applied to a range of materials on a large scale, while still maintaining accurate control over the micro porosity of the scaffold.

METHODS: A photocurable emulsion was created using the monomers 2-Ethylhexy acrylate (EHA), Isobornyl acrylate (IBOA) and a triacrylate crosslinker with the photoinitiator (a 50:50 blend of diphenyl (2,4,6-trimethylbenzoyl)phosphine oxide and 2-hydroxy-2-methylpropiophenone) (Sigma). Stabilization of the emulsion was achieved using the surfactant Hypermer B246 (Croda). Constant agitation @ 350rpm was used to create the emulsion while adding the water dropwise. Porous structures were created using the emulsion by using either stereolithography (selectively scanning UV light (355nm) isolated from a ND:YAG microchip laser (Alphalas, Gottingen, Germany) or bulk polymerization onto flat sheets. The resultant biomaterial was cut into desired structures/ shaped using a laser cutter (Epilog laser mini)

RESULTS: Laser etched and direct laser write of emulsion templated scaffolds are shown in *Figure 1 A* and *B* respectively. Both images show reproducible etched or polymerised regions of an emulsion templated foam. In Figure 1A, the outer surface of the laser ablated region shows an open morphology, whereas a polymerised woodpile structure can be seen in Figure 1B.

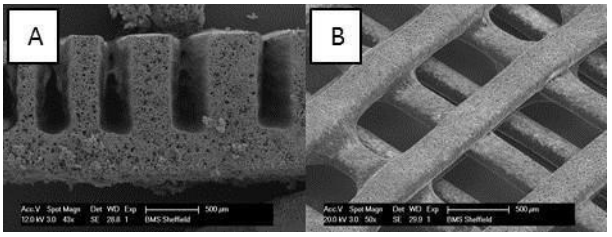

Fig. 1: A, Laser cut porous region. B, Direct laser write with emulsion templating

DISCUSSION & CONCLUSIONS: SEM images show an open interconnected morphology on the laser cut interface. While surface features and destabilisation can be seen on the side surface of the direct laser write method. In both cases the area of the etched or polymerised regions were highly dependent on the laser parameters used.

Laser etching produces features currently

The laser cut method is very useful for topographical features and scalability and direct laser write for complex 3D structures produced in a layer by layer fashion.

ACKNOWLEDGEMENTS:

The authors would like to thank the EPSRC for supporting this research.

Injectable, highly robust hydrogels towards cartilage regeneration

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INTRODUCTION: *In situ* gelling solutions are nowadays explored as alternatives to grafts and solid scaffolds for repairing soft tissues. Although it is already possible to mimic the mechanical properties of load-bearing soft tissues, a robust hydrogel system that combines the biocompatibility of natural polymers with injectability is yet to be developed [1]. Therefore, this work describes, for the first time, the engineering of cell-laden, injectable chitosan (CHI) hydrogels with enhanced mechanical properties that could sustain the loading efforts in the context of cartilage regeneration.

METHODS:

CHI hydrogels were synthesized using a double network (DN)-based method combining chemical and physical crosslinking mechanisms [2]. Two CHIs derivatives, namely low molecular weight methacrylamide CHI (L_{MW}-MACHI) and medium molecular weight CHI (M_{MW}-CHI), were mixed in an aqueous solution containing β-glycerolphosphate (β-GF) (Fig.1). Afterwards, two different crosslinking processes were employed by: (1) taking advantage of the methacrylic groups present on L_{MW}-MACHI, which occurs upon UV-light exposure in presence of a photoinitiator, I2959, (chemical mechanism) and (2) by placing the hydrogels at 37ºC to prompt the gelling process of the M_{MW}-CHI in presence of β-GF (physical mechanism). The mechanical characterization was carried out on the basis of compression tests employing a Universal Mechanical Testing Machine (INSTRON 5540). The viability of chondrocytes (ATDC5 cell line) encapsulated in CHI hydrogels was assessed by a Live/dead assay.

RESULTS:

By observing Figure 1, an optimal balance of the mechanical properties was obtained when the weight ratio of L_{MW}-MACHI/MMW-CHI was 5:2, yielding CHI DN hydrogels with an outstanding compressive strength of around 3.8 MPa. Actually, these hydrogels did not fracture even after ε as high as 90% of their initial height.

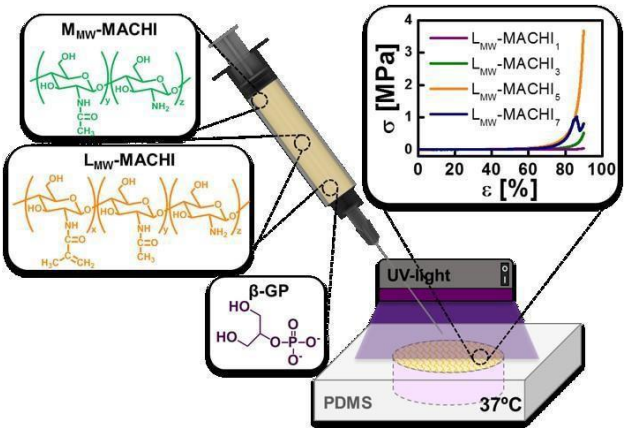

Fig.1: CHI hydrogels fabrication process and representative compressive stress-strain curve of CHI hydrogels with different L_{MW}-MACHI/M_{MW}-CHI ratios (the amount of M_{MW}-CHI in the hydrogel precursor solution was kept fixed at 2% (w/v)).

DISCUSSION & CONCLUSIONS:

The extreme mechanical properties of the L_{MW}-$MACHI_5M_{MW}CHI_2$ are even more remarkable when comparing with the respective single-networks (failure stress of 0.48 MPa at ε=36% and 0.12 MPa at ε=90% for L_{MW}-$MACHI_5$ and $M_{MW}CHI_2$, respectively). Worth mentioning, all the components were mixed together replacing the conventional multi-step process to produce DN-hydrogels. Moreover, all the process occurs at mild conditions and the chemicals used were proven to be cytocompatible. In sum, the resulting highly-innovative DN-hydrogels are able to (i) mimic the mechanical properties of the cartilage ECM, (ii) encapsulate chondrocyte cells with high viability rates and (iii) be placed in the body by a minimal invasive manner. Therefore, this system possess characteristics that could be useful for cartilage regeneration.

1

ACKNOWLEDGEMENTS: A.M.S.C. thanks Fundação para a Ciência e Tecnologia (FCT) for the financial support SFRH/BD/101748/2014.

Neo-cornea endothelium regeneration on glycerol modified silk fibroin film

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INTRODUCTION: Surface modulations of desired biological construct design for regenerative medical therapy is considered to be highly crucial for cell growth and the subsequent regeneration of biologically competent tissues. Corneal disorders/diseases leading to blindness affect millions of patients worldwide; hence require either entire cornea transplantation or only replacement of endothelial cell layer. However, lack of qualified donor corneas for transplantation and cell rejection after transplantation, has led to rapid development of various tissue-engineered strategies as an efficient alternative to the conventional corneal grafts. In this study, we fabricated stable, transparent, glycerol modified silk fibroin film (G/SF) as a potential substratum for the regeneration of corneal endothelial cells. Further, the G/SF film efficiency for mimicking native cornea was evaluated in rabbit corneal endothelial cells (rCEnCs) as models.

METHODS: Silk fibroin (SF) solution was prepared using our previously reported study [1]. In order to fabricate G/SF film, glycerol was blended with SF solution at varied glycerol ratios i.e. 1, 3, 5, 7 and 10%. The structural, morphological and *in vitro* biological characterizations were performed.

RESULTS: We observed a reduction of cracks formation on the G/SF film with an increased glycerol concentration (Fig 1). The contact angle analysis of G/SF film showed higher hydrophilicity as more glycerol was added. *In vitro*

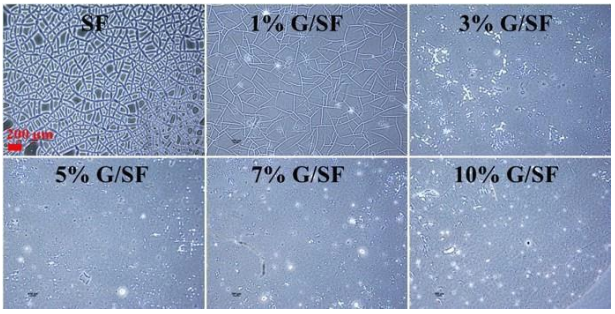

Fig 1. Microscopic images of G/SF films as a function of glycerol concentration.

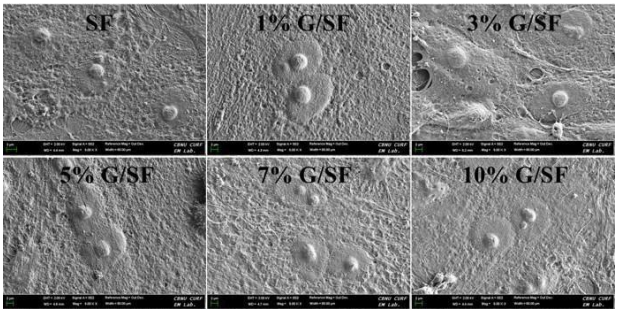

Fig 2. FESEM images of rCEnCs morphology on G/SF films.

biological studies showed no significant differences in the rCEnCs morphology in the experimental groups. Immunofluorescence staining of cultured rCEnCs on G/SF film showed well-maintained cell function (Fig 2).

DISCUSSION & CONCLUSIONS: Biocompatible and transparent G/SF film was designed to achieve enhanced rCEnCs adhesion and tissue specificity. Compared with bare SF films, the G/SF films displayed the requisite surface roughness, good hydrophilicity and showed no significant difference in transparency results. Next, cultured rCEnCs on G/SF films showed the characteristic polygonal shape of rCEnCs with enhanced initial attachment, proliferation and expression of mRNAs. Collectively, these results indicate that G/SF can be employed as a suitable alternative for high-quality corneal tissue expansion and transplantation.

ACKNOWLEDGEMENTS: This research was supported by Bio & Medical Technology Development Program of NRF funded by the Korean government (MEST) (NRF-2012M3A9C6050204).

Epigallocatechin gallate/silk fibroin films for cornea tissue engineering

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INTRODUCTION: Blindness resulting from corneal disorders/ diseases is immensely affecting millions of individuals worldwide. However, the worldwide demand for healthy donor corneas is greatly exceeding the present supply, the scenario becoming worse in an ageing society and with an increased number of young people undergoing corneal laser surgery. Therefore, tissue-engineering strategies have been adopted to develop various biological scaffolds/films as an alternative promising approach for cornea restoration [1]. In this study, we have designed a transparent, stable and insoluble epigallocatechin gallate (EGCG) blended silk fibroin films (EGCG/SF) as an alternative scaffold for bioengineering of cornea endothelium. Further, the EGCG/SF film potency for mimicking native cornea was evaluated in rabbit corneal endothelial cells (rCEnCs) as models.

METHODS: Silk fibroin (SF) solution was prepared using our previously reported study [2]. In order to fabricate EGCG/SF film, silk fibroin solution was blended with EGCG at different EGCG concentration and air-dried. The structural and morphological characterizations were performed using, FESEM, contact angle, water uptake behaviour, *in vitro* biodegradability, etc. *In vitro* biological potency of the films was assesses by MTT assay, mRNAs expression and histological analysis.

RESULTS: Compared to the SF film, EGCG/SF films were found to be less transparent, however the difference is not significant (Fig 1). The surface roughness was EGCG/SF films were higher than SF film. The FTIR spectrum of EGCG/SF films revealed the characteristic peaks of 3280 cm^{-1} for O-H group attached to the aromatic ring, also amide Ⅰ, Ⅱ, Ⅲ and 1236 cm^{-1} for O-C=O group shown for EGCG only. The proliferation of rCEnCs on 0.1mg/ml E/SF film was found to be the best among all experimental groups.

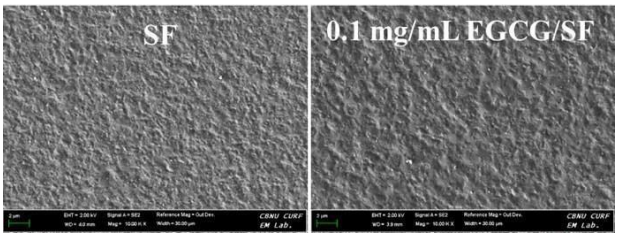

Fig. 1: FESEM images of EGCG/silk fibroin films.

DISCUSSION & CONCLUSIONS

We have designed a highly-compatible, transparent, biodegradable EGCG/SF film in order to incorporate specific functionality for enhanced rCEnCs adhesion, migration and proliferation, whilst limiting the probability of nonspecific interactions between the construct and the biological environment. *In vitro* results showed that the EGCG/SF film efficiently increases the initial rCEnCs adherence and maintains cellular migration, growth, cell phenotype, formation of cell junctions and gene expression required for functional rCEnCs. Thus, this bioengineered construct containing an expandable population of rCEnCs could be further used to address the present issue of shortage of corneas required for corneal repair procedures.

ACKNOWLEDGEMENTS: This research was supported by Bio & Medical Technology Development Program of NRF funded by the Korean government (MEST) (NRF- 2012M3A9C6050204).

Imageable polyisocyano-peptide based hydrogels

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INTRODUCTION: Hydrogels have emerged as important players in the field of Tissue Engineering, in particular, as promoters of new tissue constructs or grafts. However, monitoring its fate when implanted still remains as a challenge. To tackle this, we have designed a novel thermo-responsive hydrogel which is imageable by H-MRI technologies. This hydrogel is based on a new class of biomaterial, Polyisocyano-peptide polymers[1], decorated with Gd(III) chelate (Gd-DO3A).

METHODS: Polyisocyanopeptide polymers are synthetized by a nickel(II)-catalyzed co-polymerization. In order to add the imageable functionality, polymers were subjected to strain-promoted click reaction with DBCO-Gd-DO3A complex. Solutions of the resultant polymers were diluted in MilliQ water in order to prepare the hydrogel solutions. Quantification of Gadolinium content on the polymers was investigated via ICP-MS analysis. Mechanical properties of the hydrogel were determined by rheology. T1 and T2 relaxation times of PIC-Gd samples with different Gadolinium concentrations were measured with a 11.5T spectrometer in order to investigate the imaging properties.

RESULTS: Polymers with different Gadolinium concentrations were synthesized (1600, 900, 500, 200 and 100 ppbs). Solutions of 2mg/mL polymer concentration were subjected to a temperature sweep (5-50º) where the gels showed a liquid-gel transition temperature of 16º and 250Pa of stiffness at 37º. In vitro imaging demonstrated higher contrast on gels conjugated with higher amount of Gadolinium. In addition, T1 and T2 values were lower for samples with a higher degree of Gadolinium conjugation.

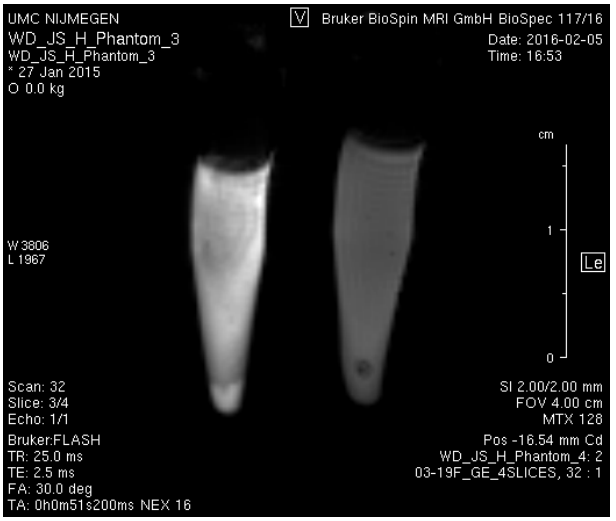

Fig. 1: MRI Images of PIC-Gd hydrogels with 0.1mM (left) and 0.5 mM (right) Gadolinium concentration .

DISCUSSION & CONCLUSIONS: We have synthesized a polyisocyanopeptide based polymer that we further decorated with different concentrations of a Gadolinium contrast agent (DO3A). Hydrogel solutions were prepared dissolving required amount of polymer into MilliQ water. There was no negative influence observed in the mechanical properties of the hydrogel due the incorporation of the Gadolinium complex on the polymer. Furthermore, our system yields relaxivity profile and enhanced contrast on a human MRI scanner. In addition, the previously demonstrated excellent properties as a 3D cell culture scaffold and its thermo-responsive behavior that allows its injectability makes this an excellent material for imaging in Tissue Engineering and Regenerative Medicine

ACKNOWLEDGEMENTS: The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement No. 607868 (iTERM).

Effect of e-beam sterilization on recombinant human collagen-phosphorylcholine implants for cornea transplantation

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INTRODUCTION: The sterilization of corneal implants composed carbodiimide crosslinked recombinant human collagen type III (RHCIII) and phosphorylcholine polymers (RHCIII-MPC) is constrained by the biochemical properties of RHCIII. Early human trials used 1% choloroform in 0.1M phosphate buffered saline (C-PBS), but require a stringent wash procedure with antibiotics to remove the chloroform. Irradiation with gamma or electron-beam (e-beam) allows a chemical-free sterilization method, but may result in crosslinking or denaturation. Here, electron-beam irradiation is evaluated as a sterilization method for RHCIII-MPC implants.

METHODS: Dose-finding study: RHCIII-MPC hydrogels were made as previously described [1]. Hydrogels were cast in round, 350µm thick, 12mm diameter molds for corneal implants and 0.5mm thick dumbbell-shaped molds for mechanical testing. The hydrogels received an irradiation dose of 17kGy, 19kGy and 21kGy or were stored in C-PBS, n=3 per group. The hydrogels were tested for sterility and endotoxin, optical and mechanical properties, biodegradation, free radicals, and cell compatibility.

Clinical evaluation in rabbits: RHCIII-MPC implants were e-beamed at 17kGy or kept in C-PBS. One implant from each group was implanted into the right cornea of each rabbit by deep anterior lamellar keratoplasty, n=4 animals per group. Animals underwent preoperative and 6-month post-operative in vivo confocal microscopy (IVCM) to check nerve count and ingrowth of keratocytes. Corneal grafts and controls were assessed via histology and immunohistochemistry.

RESULTS: Dose Finding Study: Hydrogels were sterile at all irradiation doses with no evidence of free radicals. There were no significant differences in optical or mechanical properties between the treatment groups and controls. All hydrogels supported cell growth. The 19kGy and 21kGy implants had high collagenase degradation for 21 hours until they stabilized, whereas the 17kGy and C-PBS implants had gradual degradation until 48 hours.

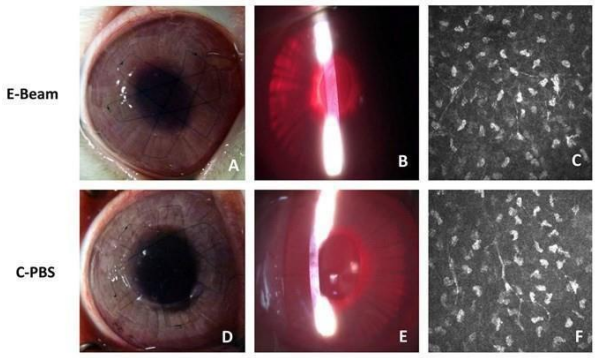

Fig. 1: Clinical results. Post-surgery (A,D). Six months post-operative (B,E.) Slit-lamp images (C,F), IVCM at a depth of 60-100µm.

Clinical Results: The rabbits did not experience post-surgical inflammatory reactions and full epithelial coverage of the implants occurred within the first week of surgery for all animals. Mild neovascularization occurred in all animals, but resolved by 6-month follow-up. A mild 0.5-1.0 grade subepithelial haze was observed in all rabbits, but the implanted grafts remained transparent. Re-innervation occurred in all grafts with no significant differences between sterilization methods (Fig. 1). All regenerated corneas were positive for cytokeratin 3 and 12 and both produced mucin. Grafted and control corneas were negative for macrophages and blood vessels.

DISCUSSION & CONCLUSIONS: E-beam sterilization is a safe and effective form of sterilization for RHCIII-MPC implants.

Cellular adhesion on surface-modified textile scaffold made of hydrophobized hyaluronan suitable for tissue engineering

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INTRODUCTION: Hyaluronic acid (HA) is biodegradable and biocompatible and is often used in different forms with advantage to faster heal injured tissue and support cells in relevant processes including survival, proliferation and differentiation. [1] Textile scaffolds made of HA-microfibers are one of the application forms, which can be used as an implantable surgical material. [2] This study aimed to physically and chemically treat surface of this scaffold to enhance cellular adhesion and proliferation of dermal fibroblasts and cardiopoietic tissue cells. Palmitoyl-HA based material, freeze drying and different biomolecules were used to reach the goal.

METHODS: Microfibers were formed by wet-spinning technology from palmitoyl-hyaluronic acid (p-HA, 250 kDa, substitution degree 55%). Textile stripes of 1 cm width were knitted on double needle bar warp knitting machine from yearns made of 3 monofilaments (*Fig. 1*). Toxicity of material was measured by MTT on 3T3 cells. Freeze drying of textile was used prior the surface treatment by fibronectin (FNC), fibrinogen (FNG), laminin (LAM), collagen IV (COL) and methacrylated gelatine (MGEL) in different concentrations. The homogeneity of surface cover was evaluated by confocal microscopy using fluorescent antibodies, HPLC analysis was used to determine decrease of biomolecules from sorption solution. Number of normal human dermal fibroblasts (NHDF) and cardiopoietic adipose derived stem cells (ADSC) after adhesion (1 day) and proliferation (2-7 days) was counted by ATP assay (Cell Titer Glo) and DNA assay (EvaGreen) and compared with a calibration curve.

RESULTS: Thanks to its hydrophobicity, p-HA fibers are non-adhesive. Surface treatment by FNC, FNG and LAM in concentration of 0,05 mg/ml in sorption solution supported NHDF adhesion and proliferation with maximum of 35 000 cells/cm^2. Analysis of surface showed uniformly covered fibers. MGEL cover got best results in concentration of 5 mg/ml and 15 minutes of UV exposition after sorption. The 30 000 cells/cm^2 was the maximum after 7 days of cultivation. HPLC analysis of COL treatment showed, that it didn't cover the surface of patch, so cells didn't adhere.

ADSC adhesion was tested on FNC treated surface (0,05 mg/ml of sorption solution). Cells uniformly covered fibers after 48 hours of cultivation (*Fig. 1*) with the maximum of 40 000 cells/cm^2.

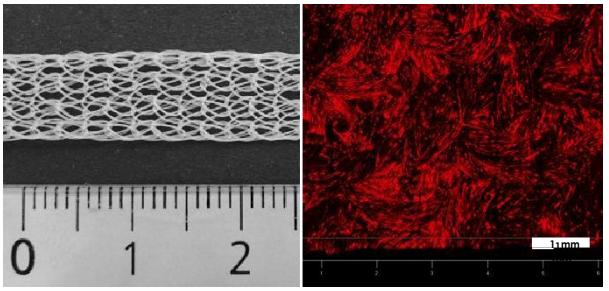

Fig. 1: Images of palmitoyl-HA textile scaffold in dry state (left) and ADSC cells (red) adhered on scaffold treated by fibronectin after 48 hours of cultivation (right)

DISCUSSION & CONCLUSIONS: Freeze drying and coating of p-HA textile by biomolecules improved cell-adhesive properties of material, especially in case of FNC, FNG, LAM and MGEL. Treating by COL wasn't successful probably because of its low interaction with fibres. Adhesion of cardiopoietic adipose derived stem cells on FNC-treated material showed very promising results which could take advantage in cardiac regeneration after myocardial infarction. To conclude it, the biocompatibility, non-toxicity, degradability, stability, flexibility and tensile strength make this material applicable in medicine as a cellular scaffold for tissue regeneration.

ACKNOWLEDGEMENTS: The research leading to these results has received funding from the European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreement n°604531 (AMCARE)

Development and evaluation of polypropylene nonwoven filters for blood filtration

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INTRODUCTION: Melt blown nonwoven membranes are used in specialized uses like fine filters, oil absorbents, noise insulation etc. The fine fibers in the substrate with some fibers less than one micron diameter provide for micro filtration of liquids and air [1]. Polypropylene (PP) is the most common raw material used for making melt blown nonwovens. One the other hand, the leucocytes present in red cell and platelet components have been implicated in several important immunological and infective complications of blood transfusion [2]. Leukocyte depletion of blood components may prevent or ameliorate some of these harmful effects. In this study, PP nonwoven filters with different thickness were fabricated by melt-blown process and efficiency of leukocyte depletion was investigated using a range of analysis techniques.

METHODS: PP nonwoven membrane was prepared by the melt-blown technique. The PP filter fabrics with different thickness were manufactured by thermal bonding of produced membrane using a heat-pressure plate system. The experimental condition and sample name are shown in Table 1. The morphological features of filter fabrics were analyzed using a scanning electron microscope (SEM). The mean flow pore diameter of the average pore size was determined by a porometer PMI.

Table 1. The experimental condition and sample names

Samples	Thickness (μm)	Thermal bonding condition	
S1	430	Temp.	120 °C
S2	740	Time	3 min
S3	820	Pressure	15 MPa

The leukocyte depletion experiment was operated as follows: the prototype filter fabric (diameter 3 cm, length 10 cm) was incorporated in a blood filtration module. To determine the leukocyte content, a sample of 10 mL was taken from both the pooling bag and the post-filtration collection

bag. Leukocyte counts were measured using a Nageotte hemocytometer.

RESULTS: Figure 1 shows representative SEM image of S1 filter fabric. The dense nonwoven filter fabric was successfully fabricated by thermal compression bonding process. The average pore size and efficiency of leukocyte depletion were 2.91 and 78.6 for S1, 2.47 and 83.2 for S2, and 2.28 μm and 92.9%, respectively.

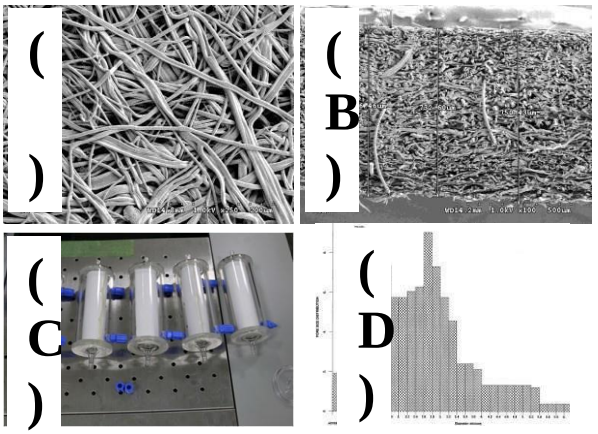

Fig. 1: SEM images of surface (A) and cross-section (B) of S1 filter fabric. Digital picture (C) of filtration module (C) and pore distribution graph (D) of S1 filter fabric.

DISCUSSION & CONCLUSIONS: In this study, the leukocyte removal rate increased with the decrease of pore size and increase of thickness of PP nowoven fabrics. This can be explained by the increase in surface area and narrow pore size distribution of produced PP filer fabrics.

ACKNOWLEDGEMENTS: This research was financially supported by the "Advanced medical new material (fiber) development program" through the Ministry of Trade, Industry & Energy (MOTIE) and Korea Institute for Advancement of Technology (KIAT) (R0000662).

Biophysical, biochemical and biological properties of nano-textured collagen fibres

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INTRODUCTION: Exogenous cross-links are required to produce stable constructs. Specifically in collagen-based scaffolds, chemical, physical and/ to control the biophysical, biochemical and biological properties of the produced devices or biological cross-linking methods are under investigation (1-3). However, biological and physical methods are not suitable for load-bearing tissues, due to the weak-induced stability, whilst chemical stabilisation methodologies are associated with foreign body response. Although the potential of multi-functional poly(ethylene glycol) systems as stabilisation and functionalisation agents for collagen hydrogels / sponges has been shown (4, 5), it is still unclear whether such agents can induce proportional stability to traditional cross-linking methods, without associated cytotoxicity. Herein, we hypothesise that 4-star poly(ethylene glycol) ether tetrasuccinimidyl glutarate (PEG) can produce cytocompatible collagen fibres for tendon repair and regeneration, with biophysical, biochemical and biological properties, superior to customarily used chemical approaches.

METHODS: Collagen fibres were fabricated and cross-linked with PEG of different concentrations; 0.5mM, 1mM, 2.5mM and 5mM (fig.1). Structural characteristics were assessed with Scanning Electron Microscopy (SEM). Thermal stability was assessed with Differential Scanning Calorimetry (DSC). The extent of cross-linking was assessed with ninhydrin and collagenase assays. Mechanical properties were assessed with tensile testing. *In vitro* cytocompatibility and protein expression were assessed with human skin fibroblasts after 3, 7 and 14 days in culture. Non-cross-linked and glutaraldehyde (GTA) cross-linked fibres acted as control.

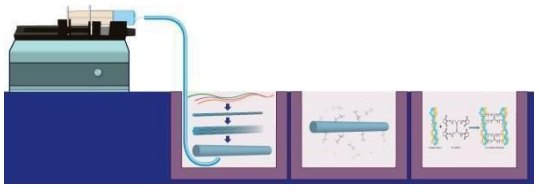

Fig.1: Fabrication of collagen fibres via an extrusion method.

RESULTS:

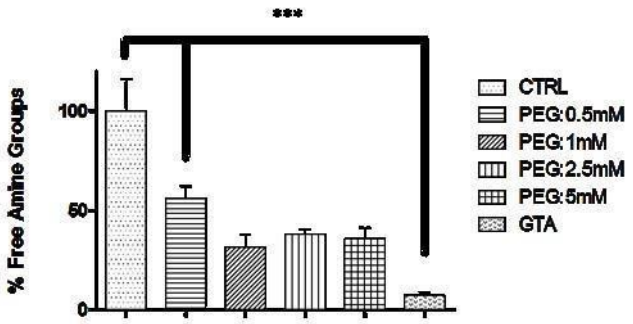

Fig. 2: PEG 0.5mM significantly reduces amount of free amine groups

DISCUSSION & CONCLUSIONS: PEG-based fibres were characterised by crevices and ridges running parallel to the longitudinal axis of the fibres. This surface topography induced bidirectional cell attachment, elongation and growth. PEG-based fibres had similar mechanical properties to GTA cross-linked fibres. Further, PEG-based fibres did not induce any cell cytotoxicity, whilst GTA fibres were found to be cytotoxic at the effective concentration. PEG fibres maintained tenocyte phenotype. Overall, our data clearly indicate the potential of these fibres for tendon repair and regeneration.

ACKNOWLEDGEMENTS: ReValueProtein Research Project (Exploration of Irish meat processing streams for Recovery of high Value Protein based ingredients for food and non-food uses, Grant Award No. 11/F/043) is supported by the Department of Agriculture, Food and the Marine (DAFM) under the National Development Plan 2007–2013 funded by the Irish Government and Science Foundation Ireland (SFI) Centre for Research in Medical Devices (CÚRAM) (13/RC/2073).

Stimuli-responsive elastin-like biomimetic coatings towards to tissue engineering

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INTRODUCTION: The interaction between cells and materials has been a key aspect for the development of biomaterials for biomedical applications, namely for tissue engineering [1]. To improve the integration of implantable devices, different strategies have been employed to biofunctionalize their surface [1]. Layer-by-layer (LbL) is a promising and versatile technique for biomaterials surface modification, and has allowed tailoring cell-material interactions [2]. In this work, LbL is used to construct biomimetic coatings using elastin-like recombinamers (ELRs). By using complementary clickable ELRs, we were able to construct multilayer coatings stabilized by covalent bonds, resulting from the Huisgen 1,3-dipolar cycloaddition of azides and alkynes [3]. The recombinant nature of ELRs provides the ability to introduce specific bioactive sequences and to tune their physicochemical properties, making them attractive for biomedical and biological applications [4].

METHODS: The build-up of ELRs multilayers was monitored using a quartz-crystal microbalance with dissipation (QCM-D). ELRs containing RGD and in the absence of this domain were used. The coatings were characterized by scanning electron microscopy (SEM), atomic force microscopy (AFM) and water contact angle (WCA) measurements. The C2C12 cell line was seeded on the multilayer coatings to investigate their biological properties regarding cell adhesion, proliferation and myogenic differentiation.

RESULTS: The effective construction of the multilayer coatings using two clickable ELRs was confirmed through QCM-D monitoring. While changing environmental stimuli as temperature and pH, we observed transitions in the wettability of the coatings and also changes in topography/morphology. C2C12 cell line adhesion was assessed by MTS and DNA quantification assays. Higher cell adhesion and proliferation was observed in the coatings prepared with RGD-modified ELR. These results were confirmed by fluorescence microscopy images. Furthermore, both biomimetic coatings seemed to promote myogenic differentiation.

DISCUSSION & CONCLUSIONS: The methodology employed constitutes an alternative to the use of polyelectrolytes that are assembled through electrostatic interactions, allowing to obtain multilayers linked by stronger covalent bonds. The results suggested that the ELRs multilayers prepared by precision chemistry routes have a pronounced potential as biomimetic coatings, targeting tissue engineering purposes. Furthermore, the multilayer responsiveness to different stimuli and the possible incorporation of bioactive sequences allows the construction of tunable systems that promote cell adhesion and directed cell responses.

ACKNOWLEDGEMENTS: This research has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No.646075. MP Sousa, MB Oliveira and RR Costa acknowledge the Portuguese Foundation for Science and Technology (FCT) the grants SFRH/BD/97606/2013, SFRH/BPD/111354/2015, and SFRH/BPD/95446/2013, respectively.

Tissue-engineering of acellular scaffolds – The significant influence of physical and procedural decellularization factors

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INTRODUCTION: Decellularized scaffolds offer an impressive potential for regenerative medicine. However, physical and procedural decellularization (DC) factors have been only poorly investigated up to now. By specific experimental settings we aimed to identify the individual influences of some of these factors on DC procedures.

METHODS: 72 porcine thoracic aortae (PTA) were divided in four procedures: Samples were exposed to a DC solution (0.5% SD, 0.5% SDS) for 4h or 8h, either continuously or in repeated cycles (each: 2h detergent exposure followed by 30min NaCl washing). PTA of group 1 (G1) were exposed to a jet induced whirl (10l/min). To evaluate supporting effects of sonication treatment, samples of group 2 (G2) were additionally placed in an ultrasonic bath (45kHz). PTA of the control group (GC) were rocked in an orbital shaker (90bpm), representing a standard DC process. After DC all scaffolds underwent 10 washing cycles (NaCl, 15min). Based on the results of these procedures, we investigated the increase of DC efficiency by combination of the different factors. Therefore, PTA (n=6; G3) were treated longer (24h DC) by a stronger whirl (20l/min), multiple sonication exposures (30min/processing step) and more DC cycles (3).

Samples were taken before and after each DC treatment. The efficiency of DC was equalized to the penetration depth (PD) of DNA eradication, measured by DAPI fluorescence and H.E. staining in paraffin embedded sections. PD was defined as shortest distance from inner (luminal) respectively external surface to the first DNA-dependent staining signal. For comparison of DC efficiencies, inner and outer PD were added to a total PD (tPD). Histological evaluation was assessed by Movat's Pentachrome and Picrosirius-Red staining. Picrosirius staining further allowed the examination of elastin (green autofluorescence) and collagen (rhodamine channel) by fluorescence microscopy. Finally, scanning electron microscopy (SEM) was used for topographic analysis.

RESULTS: DAPI fluorescence showed significant greater DC depths (61±7%; $p<0.001$) of outer compared to inner surface. Evaluation of continuous as well as cyclic incubation showed a highly significant time dependency ($p<0.001$) of tPD (Table 1) for all samples. However, tPD is associated to the treatment time in a logarithmic ($\log<1$) function. The slope of this function (=DC efficiency) is strongly dependent on the investigated DC factors. By cyclic treatment the DC efficiency was increased up to 30% ($p<0.001$). Furthermore, intensified flow by jet induced whirl could improve DC up to 18% ($p<0.001$) and the additional application of ultrasound led to a DC enhancement of up to 33% ($p<0.001$) compared to Gc. These findings and dependencies were further confirmed by results of G3. (Table 1)

Histologic assessment showed preservation of major extracellular matrix components. The typical tree-layered histology of (elastic) arteries was visible in all samples of all groups. SEM analysis revealed intact skeletonized fiber network after DC. Scaffold integrity was proven for all samples.

Table 1: Total penetration depth (in µm) of DNA eradication from inner and outer surface

	G_C	G_1	G_2	G_3
4h	372±34	440±18	496±76	---
4h cyc	485±33	530±27	614±59	---
8h	684±109	785±129	827±112	---
8h cyc	889±117	864±54	985±75	---
24h cyc	---	---	---	1392±85

DISCUSSION & CONCLUSIONS: This study highlights the potential of different DC factors and procedures. A significant influence of time, cyclic incubation as well as sonication could be demonstrated. Histologic evaluation proved that the procedures caused no tissue alterations. Therefore, we highly recommend considering the investigated factors in future DC protocols.

Revitalisation of a decellularised xenogenic meniscus implant by cells from human synovial membrane

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INTRODUCTION: Menisci in the knee joint play an important role in shock absorption and load transmission. Therefore, replacement materials need to fulfil special requirements. Ideally, they should offer long-term stability, support cellmigration and synthesis of specific extracellular matrix (ECM). Unpublished preliminary studies with bovine meniscus-fibrochondrocytes and the decellularised porcine meniscus matrix showed good revitalisation properties. In clinical practice acellular meniscus implants have to be revitalised by cells from surrounding tissue. So, the aim of this study was to investigate cell adhesion, migration, proliferation and ECM synthesis of cells from human synovial membrane.

METHODS: Porcine meniscus scaffolds were decellularised by a chemical multistep procedure [1]. These scaffolds were seeded with human synovial cells (hSC) and cultivated in a modified chondrogenic medium [2]. Cell growth, migration and ECM synthesis on and in seeded scaffolds were evaluated histologically by a combined alcian blue-hematoxylin staining (AB-H) and immuno-histochemically (IHC) by staining of collagen I, collagen II and aggrecan. Variations of gene expression were analysed by real-time polymerase chain reaction (rt-PCR).

RESULTS: AB-H staining demonstrated cell adhesion and proliferation of hSC on the scaffolds. After 14 d a homogeneous cell layer and synthesis of ECM could be observed. Cell migration along collagen fibres up to 200 µm into the tissue could be detected during the first 42 d of cultivation. Also, the layer thickness of the *de-novo* synthesised matrix increased up to 150 µm. IHC staining demonstrated strong collagen I, clear aggrecan and slight collagen II synthesis. The rt-PCR showed a significant increase of collagen I gene expression by a factor of 7.6 during cultivation (Fig. 1). Additionally, gene expression of collagen II and aggrecan increased by a factor of 4 and 2.5, resp.. However, the expression of both were relative slight.

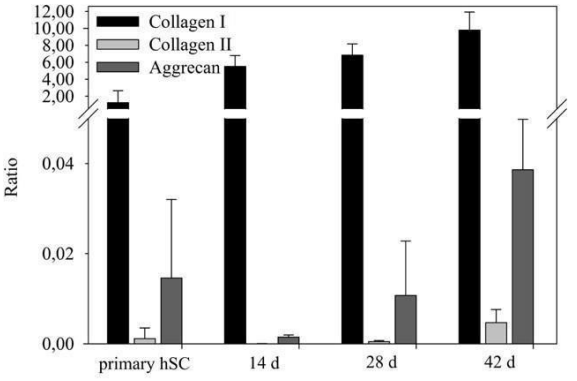

Fig. 1: Gene expression of collagen I, collagen II and aggrecan during cultivation referred to GAPDH.

DISCUSSION & CONCLUSIONS: Cells from synovial membrane are able to revitalise the decellularised xenogenic meniscus matrix. The cells synthesised meniscus-specific extracellular matrix and migrated into the tissue. Furthermore, rt-PCR analysis showed that the cells differentiated into a phenotype similar to meniscus cells, an important requirement for healing and natural function of the meniscus implant. Overall, the cultivation of decellularised porcine meniscus scaffolds showed promising results for revitalisation of meniscus implants by body's own cells from surrounding tissue.

ACKNOWLEDGEMENTS: Synovial tissue was kindly offered by PD Dr. Kolja Gelse from Universitätsklinikum Erlangen.

Vascular network formation within a cotton wool like bioactive glass scaffold using induced pluripotent stem cell-derived endothelial cells

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INTRODUCTION: Bone grafting is required for repair of congenital defects, traumas or diseases. Autologous bone grafts are commonly used as they contain vasculature, vital cells and growth factors that are essential for remodelling and osseointegration. However, due to the lack of donor sites, need of additional surgery and high costs, alternatives are being explored. One of such alternatives is bioactive glass, which is capable of bonding to bone, degrading within the body and releasing ions that have shown to stimulate bone regeneration. Once implanted, bioactive glasses are known to form a surface layer of hydroxyl carbonate apatite, which is similar to a bone mineral and is responsible for bonding with it. Recently, bioactive glass of the 70S30Ca (70 mol% SiO2, 30 mol% CaO) composition was successfully electrospun into cotton-wool-like fibres. This morphology favours cell infiltration and resembles the supramolecular structure of collagen bundles.

In order to prevent necrosis of the graft, autologous bone marrow aspirate is used in clinics to supply vital cytokine-secreting cells. Alternatively, this could be achieved through the use of induced pluripotent stem cell-derived endothelial cells (iPS-EC) and bone marrow mesenchymal stem cells (MSCs), which create a vascular network within the fibrous osteogenic scaffold.

METHODS: iPS-ECs were purchased from Axol Bioscience, cultured on gelatin (0.1%) coated plates and expanded in EGM-2 complete medium (CAMBREX). iPS-EC were characterised by Acetylated-low density lipoprotein (Ac-LDL) uptake, expression of CD31, von Willebrant Factor (vWF) and network formation on Matrigel. MSCs were purchased from Cambrex and characterised for their osteogenic and adipogenic potential. iPS-ECs and MSCs were transduced with lentiviruses to express Red Fluorescent Protein (RFP) and Green Fluorescent Protein (GFP) respectively.

Cotton-wool like bioactive glass of the 70S30Ca (70 mol% SiO2, 30 mol% CaO) composition were prepared through electrospinning as previously described[1]. For vascular network formation, iPS-derived ECs and MSCs were combined in a fibronectin containing collagen gel prepared as previously described[2] and infused into the interconnected fibre scaffolds. *In vitro* vessel network development was determined using confocal microscopy. Vessel length in the fibrous scaffold was quantified using the AngioTool and vessel network complexity was evaluated using MATLAB.

RESULTS: For the formation of a vessel like network iPS-derived ECs and MSCs mixed within a collagen gel added to the 70S30Ca cotton wool like scaffold were cultured for 4, 10 and 14 days. Fluorescent confocal microscopy demonstrated the formation of a strong and long lasting (14 days) capillary-like network connecting through the scaffold pore network via the pore interconnects. CD31 and vWF immunostaining revealed the formation of a vascular network by the iPS-ECs with lumen like structures. Advanced 3D imaging approaches that reveal the formation of the vessel network throughout the 3D structure, cell- fibre interactions will also be presented.

DISCUSSION & CONCLUSIONS: iPS-EC and MSCs combined in a collagen hydrogel successfully formed a robust 3D vessel network. Moreover, our results demonstrate that the 70S30Ca cotton wool like scaffolds can support the formation of vascularised 3D constructs with enormous potential for tissue engineering.

ACKNOWLEDGEMENTS: Dr G Poologasundarampillai would like to acknowledge funding by EPSRC.

Evaluation of osteoblast-like Saos-2 cells behaviour on nanotubes enriched with Ca^{2+}, $H_2PO_4^-$ or heat treated nanotubes

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INTRODUCTION: Nanostructured surfaces are believed to mimic the arrangement of bone tissues. It is not only the size or distance of the surface irregularities but also their charge and composition that are important for cell adhesion and growth. The aim of the study was to determine how modified nanotubular TiO_2 layer formed on Ti-6Al-4V alloy alters cell behaviour. The nanotubes with diameters were prepared by electrochemical oxidation in electrolyte containing ammonium sulphate and ammonium fluoride. The nanotubes were further modified with calcium, phosphate ions or were heat treated.

METHODS: Round specimens of Ti-6Al-4V ELI (ASTM F136) were polished sequentially with 320–2,500 SiC grinding paper, then degreased in an alcohol, rinsed with distilled water and dried. Three types of treatments were performed. Samples were placed into saturated calcium hydroxide solution at 60 °C. After 2 min, the specimens were taken out and washed with distilled water. Phosphoric acid was added in sulphate electrolyte to enriched nanotubes in phosphorus. A set of nanostructured samples was annealed in air atmosphere for 3 h at 500 °C. Temperature heating rate was 10 °C min^{-1}. For the cell culture experiments, the samples were sterilized in ethanol, inserted into 12-well polystyrene cell culture plates and seeded with human osteosarcoma Saos-2 cells, suspended in McCoy's medium with 15% foetal bovine serum and gentamicin. Each well contained 43 000 cells (i.e., approximately 12 000 cells/cm^2). The cells were cultured for 1, 3, and 7 days at 37 °C in a humidified air atmosphere containing 5% CO_2. Three samples were used for each experimental group and time interval.

RESULTS: The attachment, spreading and growth of human osteoblast-like Saos-2 cells was similar on samples subjected to all types of treatment. Type I collagen was significantly less formed on the substrate with calcium, but higher values were recorded for heat-treated nanostructured samples. Alkaline phosphatase (ALP) showed the lowest fluorescence intensity in samples enriched in phosphorus. The highest values were observed for cells in the thermally treated samples.

DISCUSSION & CONCLUSIONS: Type I collagen is an early marker of osteogenic differentiation. It is followed by mineralization, where calcium is incorporated into the extracellular matrix. The decrease in production of type I collagen on Ca-enriched nanotubes might be caused by the fact that the cells were able to detect calcium that was already present. This would stimulate the cells to act as mature osteoblasts and stop the production of type I collagen. It is known that Ca-containing structures, including calcium carbonate, support the maturation of osteogenic cells [1]. On the other hand, Ca-containing materials can also have adverse effects on cells, which is due either to release of Ca ions from the material and their cytotoxic action [2] or by depletion of these ions from the culture media [3]. Another important marker of osteogenic cell differentiation is ALP. Its efficiency is enhanced by alkaline environments. The phosphorus added to the nanotube structure is acidic, which can suppress the development and function of ALP. Cells on heat-treated nanotubes contained higher amounts of total ALP protein and showed higher ALP activity. The cells displayed the greatest alkaline phosphatase activity with anatase. These beneficial effects of crystal structure on cell colonization and cell phenotypic maturation were attributed to the appropriate orientation of hydroxyl groups. He at al. explains that it occurs with the hydrophilic character of the surface water drop contact angle of approximately 60° [4].

ACKNOWLEDGEMENTS: The authors acknowledge Grant Agency of the Czech Republic for financial support (grant No.15-01558S).

DISCLOSURES: The authors have nothing to disclose

Bioactive collagen-fibrin bead gel system, acting as bulking agents for the treatment of stress urinary incontinence

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INTRODUCTION: Stress urinary incontinence (SUI) is one of the most common medical conditions affecting people worldwide [1]. As a treatment modality for SUI, endoscopic injection of bulking agents has been widely used to increase bladder outlet resistance. However, currently used bulking material suffers from issues with long-term material stability and poor integration into host tissue. In this study, fibrin beads containing recombinant insulin like growth factor-1 (rIGF-1) have been produced using a microfluidic platform. These bioactive fibrin beads were combined with bovine collagen, acting as carrier and improving its injectability. Morphology and growth factor binding efficiency of fibrin beads have been investigated. Further the human smooth muscle cell interaction with the beads has been evaluated.

METHODS: Recombinant insulin like growth factor-1 (rIGF-1) conjugated fibrin beads were obtained using a microfluidics system, connected to computer-controlled syringe pumps (neMESYS, Cetoni, Germany) [2]. The morphological characterization of fibrin beads was performed using scanning electron microscopy (XLF30, Philips). Incorporation of rIGF-1 within fibrin beads was evaluated by immunohistochemistry. rIGF-1 conjugated fibrin beads and human smooth muscle cells (hSMC) were simultaneously added to bovine collagen solution after neutralization and incubated in cylindrical molds at 37°C for 30 min to allow complete collagen gelation. hSMC viability in the presence of fibrin beads, either conjugated or non was evaluated using Live/Dead assay. Further the migration and adherence of hSMCs were studied.

RESULTS: The obtained fibrin beads were very homogeneous in terms of size and porosity, showing highly dense fiber bundling (Fig.1A&B). The average diameter of the fibrin beads was around 140 μm. The recombinant insulin like growth factor-1 (rIGF-1) was homogeneously distributed within the fibrin matrix of the obtained microbeads (Fig.1C&D).

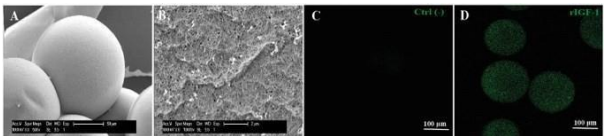

Fig. 1: Surface morphology and recombinant insulin like growth factor 1 (rIGF-1) binding capacity of fibrin beads. (A,B) SEM images of fibrin beads. Immunostaining of (C) non and (D) rIGF conjugated fibrin beads.

Cell viability was above 94% in all samples on day 1 (Fig.2A&B). hSMCs were attracted by the IGF-1 loaded beads and attached to their surfaces (Fig 2C).

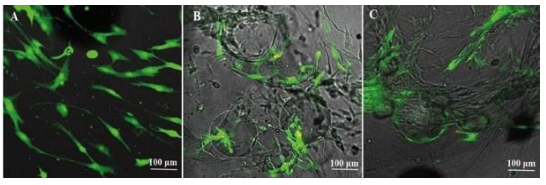

Fig. 2: Viability and behavior (Live/Dead staining) of human smooth muscle cells on day 1: (A) within collagen only (B) within bovine collagen and fibrin beads (control) and (C) migrated towards and attached on the beads.

DISCUSSION & CONCLUSIONS: rIGF-1 conjugated fibrin beads were produced using microfluidics, enabling rapid, reproducible, and size-controlled fabrication. Fibrin beads had high surface porosity with very small pore sizes. hSMCs were attracted by IGF-1 and attached on the bead surface. This bioactive collagen-fibrin bead gel system might potentially be used as a therapeutic bulking agent, inducing physiological regeneration of the compromised outlet resistance in urinary incontinence.

ACKNOWLEDGEMENTS: This study is supported by a CTI grant.

On the evaluation of long term *ex vivo* cultivation of pancreatic cancer in a 3D scaffolding system

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INTRODUCTION: Pancreatic cancer is the fourth leading cause of all cancer-related deaths in the United States, fifth in the UK and eighth worldwide. It is a highly lethal disease (the 5-year survival rate is only 6%) with poor prognosis. Additionally, while the mortality of other types of cancers is declined, pancreatic cancer's mortality is stable, showing limited progress on the development of efficient treatments. *In vitro* pancreatic cancer research up to date is mainly conducted in traditional 2D monolayer culturing systems that are far from being an accurate representative of the *in vivo* tumor microenvironment. Thus, in order to understand in depth the cell and tumor behavior and consequently, the response to treatment, it is crucial to mimic as close as possible *ex vivo* the *in vivo* tumor environment. The latter, is the main aim of our highly porous scaffolds[1]. These scaffolds aim to recapitulate the *in vivo* microenvironment in terms of structure, porosity and presence of microenvironmental niches. Their spatial arrangement provides better cell-cell interactions while coating with different extracellular matrix proteins (ECM) reassures more realistic cell-matrix interactions. The overall aim of this work is to evaluate the evolution (growth) of different pancreatic cell lines in highly porous polymeric scaffolds in presence of different extracellular matrix systems, i.e., different proteincoatings.

METHODS: ASPC-1, PANC-1 and BXPC-3 were cultivated for five weeks in 3D cubic polyurethane (PU) highly porous scaffolds (pore size of approximately 100 μm) as described in [1], as well as in classical 2D micro-tetri plates. Both the 3D and the 2D systems were either uncoated or coated with three different proteins, i.e., collagen Type 1 (Sigma Aldrich, UK), laminin (Sigma Aldrich, UK) and fibronectin (Sigma Aldrich, UK). Quantitative assessment of cell viability took place using the MTS metabolic assay (Promega, MTS ®). Moreover, at different time points of the experiment scaffolds were collected, fixed and sectioned and Scanning Electron Microscopy (SEM) was conducted in order to visualize the cell distribution and morphology. Experiments took place at least in triplicate and Analysis of Variance (ANOVA) was performed to evaluate whether there was a statistical difference/indifference between the experimental conditions.

RESULTS: All pancreatic cell lines successfully proliferated in the 3D polyurethane scaffolds for at least 4 weeks, something impossible in classical 2D cultures where confluency was observed for all conditions under study within the 1st week of culture. Overall, we observed interesting differences in the evolution of all pancreatic cell lines in presence of extracellular matrix proteins with Collagen type I showing the least effect on the cell proliferation in 3D.

DISCUSSION & CONCLUSIONS: Our findings show that all pancreatic cancer cell lines can be cultured for at least 4 weeks in the 3D scaffolds, following different kinetics depending on the type of extracellular matrix present. It should be mentioned that, to our knowledge, this is the first time that such a long term *ex vivo* cultivation of pancreatic cancer cells has been reported. The latter is of great importance when studying *in vitro* the response of pancreatic cancer to treatment as it allows long term monitoring of the response and potential development of resistance of the cells to chemotherapy as well as irradiation therapy.

ACKNOWLEDGEMENTS: This work was supported by an Impact Acceleration Grant (IAA-KN9149C) of the University of Surrey as well as an IAA-EPSRC Grant (RN0281J).

Pulsed electrodeposition of calcium phosphate coatings for drug delivery

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INTRODUCTION: Titanium and its alloys are widely use as material for scaffolds and implants. However, metallic biomaterials are not well suited to work as drug carriers, and they have some limitations related to bioactivity and bacteria colonization leading to infections.

One strategy to increase the bioactivity of titanium is by coating the surface with calcium phosphate (CaP) by electrodeposition. This technique presents several advantages, such as the low temperature of the process and a higher control of the thickness and chemical composition of the coating. During the process, however, hydrogen bubbles are produced at the vicinity of the cathode, acting as a barrier which lowers the ion throughput [1].

The present study proposes the application of pulsed current to reduce the production of hydrogen bubbles and to improve the ion concentration's uniformity from the bulk solution to the cathode. Furthermore, this CaP coating was tested as a carrier for drug delivery to avoid infections in the early phase afterimplantation.

METHODS: Electrodeposition was performed on titanium samples working as a cathode in an electrochemical cell. A platinum electrode was used as anode. The electrolyte was prepared by mixing $Ca(NO_3)_2 \cdot 4H_2O$ and $NH_4H_2PO_4$. Samples were processed for 30 min with different conditions of temperature (40-60ºC), polarity (unipolar-bipolar) and current density (2-5mA/cm^2). The coating was doped with Chlorhexidine Digluconate (CHX) by co-deposition (adding CHX to the electrolyte) or by adsorption (adding the CHX to the coating after its formation) [2].

Physicochemical properties were evaluated by SEM/EDS, FIB, XRD and white-light interferometry. The adhesion strength of the coating was analyzed according to ASTM F1147 standard. Release kinetics was studied by UV-Visible spectroscopy. *In vitro* assays were performed with a *S. aureus* bacterial strain to analyse the antibacterial activity. Cellular adhesion was studied with an osteoblast-like cellline.

RESULTS: Different morphologies were obtained varying the process parameters with good mechanical stability (Figure 1).

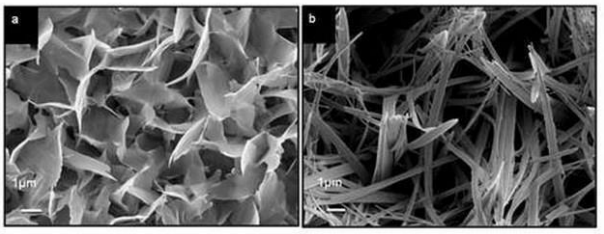

Fig. 1: Microstructures obtained at two different process conditions, a) Plate like morphology b) Whisker like morphology.

XRD characterization showed that the phases present in the layers were hydroxyapatite and brushite. In addition, the incorporation of CHX has no appreciable effects on the microstructure of the coated samples.

The *in vitro* studies revealed that the coated surfaces maintained or even enhanced cell adhesion. Coatings loaded with CHX either by adsorption or by co-deposition showed antibacterial effects in vitro against adhesion of *S. aureus* after 4 hours.

DISCUSSION & CONCLUSIONS: Calcium phosphate coatings were successfully created on titanium by an electrochemical process with good mechanical properties.

The coatings were loaded with an antibacterial agent with antibacterial effects without hampering cell viability.

ACKNOWLEDGEMENTS: The present study was supported by the Catalan Agency for the Administration of University and Research Grants (AGAUR FI-DRG) and by Fundación Ramón Areces.

Ascorbic acid and dexamethasone simultaneously releasing electrospun scaffolds for carriers and differentiation of human adipose stem cells

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INTRODUCTION: Bioactive scaffolds that can be loaded and used to deliver therapeutic agents, such as drugs, growth factors and genes, are considered to be promising platforms for tissue engineering. Amongst the variety of fabrication techniques available to date, electrospinning is considered a relative simple and versatile processing technique allowing convenient incorporation of drugs in a single-step process. In the current study, we aimed to simultaneous control the release of two osteogenic differentiation factors, dexamethasone (DEX) and ascorbic acid (ASB), using multimodal electrospinning method. The effect of ASB-DEX laden scaffold on the viability, morphology, proliferation and osteogenic as well as adipogenic differentiation of human adipose stem cells (hASCs) was investigated.

METHODS: The bioactivity of the released osteogenic drugs was studied by culturing hASCs from four donors on blank, ASB-loaded and ASB-DEX-loaded scaffolds under basic culture medium for 21 days, and compared to hASC culture on blank scaffolds under optimized osteogenic differentiation medium and under basic medium supplemented with ASB and DEX. Cell viability was studied using Live/Dead staining on days 3 and 14. Proliferation based on DNA amount and osteogenic differentiation based on alkaline phosphatase (ALP) activity were studied on days 7, 14 and 21. On day 21, Alizarin Red staining and quantification was performed to study mineralization. Cell morphology and adipogenic differentiation were evaluated based on combined Oil Red O and phalloidin staining on days 7, 14 and 21. In addition, gene expression of osteogenic and adipogenic marker genes were studied on days 7 and 14.

RESULTS: Released drugs did not affect the viability of hASCs. Instead, ASB-DEX releasing scaffolds enhanced proliferation compared to blank and ASB releasing scaffolds, reaching the same level as with supplemented media. Simultaneous release of ASB and DEX slightly increased the ALP activity of hASCs compared to blank and ASB releasing scaffolds. However, the chemical induction straight from the media had better impact on osteogenic differentiation of hASCs. Furthermore, the optimized osteogenic medium proved to be superior to induce deposition of mineralized matrix. In addition to osteogenic differentiation, simultaneous release of ASB and DEX enhanced adipogenic differentiation of hASCs.

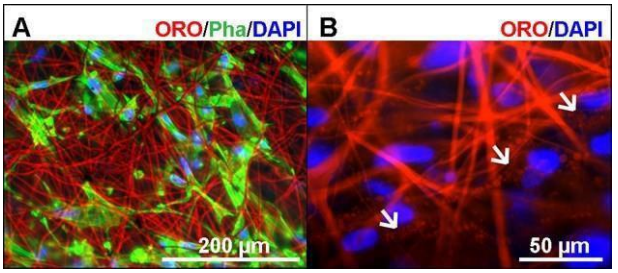

Fig. 1: hASCs cultured in ASB and DEX simultaneously releasing scaffold for 14 days. Actin cytoskeleton of the cells stained with phalloidin in green (A), nuclei with DAPI in blue and scaffold fibers as well as fat vacuoles (highlighted in image B) were stained using Oil Red O solution.

DISCUSSION & CONCLUSIONS: Our findings suggest that without any other chemical or physical stimulation, simultaneous release of ASB and DEX from electrospun scaffolds slightly supported osteogenic differentiation of hASCs *in vitro*. However, this multimodal scaffold can be exploited as a carrier for sustained release of drugs and also be combined with relevant biomaterials for soft tissue engineering applications *in vivo* and *in vitro*.

ACKNOWLEDGEMENTS: The authors thank Ms Anna-Maija Honkala, MSc Miia Juntunen and Ms Sari Kalliokoski for excellent technical assistance. The financial support from TEKES – The Finnish Funding Agency for Innovation (Project: AdipOS) and the Agency for Science, Technology and Research (A*STAR) (Project No: 102 129 0098) are greatly acknowledged.

DISCLOSURE: The authors declare no competing interests.

Extracellular matrix hydrogels from CNS and non-CNS tissues developed for neural tissue repair

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INTRODUCTION: Extracellular matrix (ECM) scaffolds prepared by tissue decellularization preserve the composition, mechanical integrity, and biological activity of the native ECM, suggesting that the molecular composition of these materials is an active factor in remodelling events. While ECM scaffolds are commonly used for various tissue reconstructions, including skin, muscles, bone, cartilage, trachea, lung or heart, there is little evidence of their use in CNS tissue reconstruction [1]. The aim of this study is to prepare injectable ECM hydrogels from CNS and non-CNS tissues and compare their physical, biochemical and biological properties *in vitro*.

METHODS: Four types of ECM hydrogels were prepared by the decellularization of porcine brain (BM), porcine spinal cord (SCM), porcine urinary bladder (UBM) and human umbilical cord (WJM). All materials were characterized in terms of their residual DNA concentration as well as their collagen and glycosaminoglycans content. The morphology of the hydrogels was characterized by a scanning electron microscope (SEM). The gel point and rate of shear modulus were derived from rheological testing. Turbidimetric gelation kinetics was determined spectrophotometrically. The viability and migration of human mesenchymal stem cells (MSCs) isolated from bone marrow, adipose tissue and Wharton jelly were evaluated using a WST assay and xCELLigence system. The neurotrophic properties of ECM hydrogels were assessed on dorsal root ganglion (DRG) explant cultures.

RESULTS: All hydrogels can be successfully solubilized and formed into an injectable gel with a three-dimensional porous fibrillar structure (Fig.1). Turbidimetric gelation kinetics showed a sigmoidal shape for BM, SCM and UBM hydrogels, whereas WJM showed an exponential shape. The time required to reach half of the final turbidity was the longest for UBM (43.70±0.83 min) and the shortest for WJM (11.30±1.29 min). In terms of composition, WJM hydrogels contained the highest concentration of sulphated glycosaminoglycans and collagen.

All types of ECM hydrogels promoted the proliferation and migration of MSCs as well as massive neurite sprouting out of DRG explants.

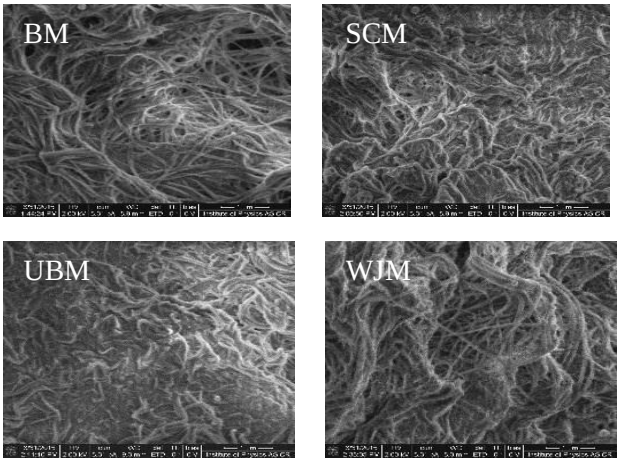

Fig. 1: SEM images of ECM hydrogels from porcine brain (BM), spinal cord (SCM) and urinary bladder (UBM) and human umbilical cord (WJM).

DISCUSSION & CONCLUSIONS: Although ECM hydrogels from CNS and non-CNS tissues do not reveal significant differences in their biological testing *in vitro*, dissimilarities in their composition, rheological characteristics and gelation kinetics can have possible effects on scaffold gelation in situ and lead to diverse tissue response after their injection into damaged neural tissue.

ACKNOWLEDGEMENTS: This project was supported by GAČR 15-01396S and LO1309 of the MEYS CR.

Tunable dynamic covalent crosslinked hydrogel for tissue engineering

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INTRODUCTION: Injectable hydrogels tailored using dynamic covalent-coupling chemistry (hydrazone and oxime) have been extensively used in regenerative medicine owing to its simplicity and versatility. However, these reactions possess slow reaction kinetics at physiological conditions, especially when keto substrates are used. This results in slow dissociation of dynamic covalent bonds leading to excessive swelling and fast degradation of hydrogel. Thus, developing dynamic covalent- crosslinking chemistry with excellent hydrolytic stability and fast reaction kinetics is extremely important to design robust biomaterial scaffolds for regenerative medicine applications.

METHODS: Hyaluronic acid was modified with carbonyldihydrazide (CDH) or adipicdihydrazide (ADH) using EDC coupling strategy[1]. HA hydrogels were developed by mixing equal amounts of HA hydrazide derivatives (6-8 % modification) and HA aldehyde derivatives[2].

Carboxylate activated oxime ligation kinetics: Oxime reactions were performed using a 10 mM of aldehyde/ketone, 1 mM (aminooxy)methane and an appropriate catalyst amount in 10 mM deuterated phosphate buffer saline at pH 7.0 (pD 7.4). The extent of oxime ligation was monitored by ^{1}H NMR.

RESULTS: HA-CDH gel exhibited superior hydrolytic and enzymatic stability than conventional HA-ADH gel (figure 1b). It also showed limited swelling ratio even in medium containing 10% fetal bovine serum. This HA-CDH gel was also shown to stabilize bone morphogenetic protein-2 (BMP-2) *in vivo* and form ectopic bone subcutaneously with oriented collagen [3]

Oximes possess higher stability than hydrazones due to inherent α-effect. We have discovered that oxime ligation proceed efficiently in presence of polycarboxylate polymers or small molecules bearing carboxylic acid functionality. Thus carboxylates were utilized to catalyze and activate oxime reaction at physiological pH with aldehyde and keto substrates. Combination of aniline and carboxylates resulting in a ~14–31-fold increase in reaction rate at pD 7.4 with keto substrates (figure 1c) [4].

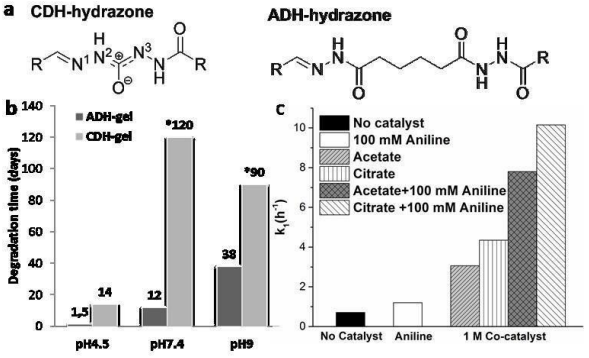

Figure 1. a) Structure of CDH and ADH-hydrazone; b) Degradation study of hydrogels at different pH, ∗ indicates no change in morphology and no disintegration observed throughout the study. c) Comparative pseudo-first-order oxime reaction kinetics.

DISCUSSION & CONCLUSIONS: The HA-CDH hydrogel possessing electronically stabilized bonds demonstrated superior swelling and degradation properties than conventional hydrazone crosslinking hydrogel. This superior stability is attributed to the delocalization of the N^2 positive charge distribution over the CDH hydrazone moiety (figure 1a). The in vivo evaluation of HA-CDH hydrogel revealed neo-bone with highly ordered collagen matrix mimicking natural bone regeneration. Similarly for oxime coupling, we discovered acetates efficiently activating oxime ligation at physiological pH. HA can be used as a polycarboxylate catalyst for developing oxime crosslinked hydrogels. These new hydrazone and oxime coupling strategies could be envisaged for linking other biomolecules under mild physiological conditions.

Extracellular matrix formation of chondrocytes on novel polycaprolactone-based composite scaffolds for cartilage tissue engineering

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INTRODUCTION: The need for cartilage grafts in reconstructive craniofacial surgery can be overcome with tissue engineered cartilage. To improve the applicability of FDA approved macroporous polycaprolactone-based scaffolds for cartilage regeneration, two different composite scaffolds were evaluated with special regards to extracellular matrix formation.

METHODS: Macroporous polycaprolactone-based scaffolds were combined with fibrin glue and decellularized extracellular matrix (DECM) derived from porcine septal cartilage to reduce porosity and create a more condense composite scaffold. DECM was minced and manually inserted into the voids of the polycaprolactone-scaffold. Fibrin glue yielded a more loose extracellular matrix with embedded chondrocytes whereas DECM led to highly condense scaffolds with chondrocytes seeded on top of the composite scaffolds. Both kinds of composite scaffolds were seeded with 1 x 10^7 human chondrocytes and cultivated with chondrogenic medium for 28 and 42 days respectively. Glycosaminoglycane content was measured with dimethyl-methylene blue assay and gene expression of aggrecan, collagen I, collagen II and versican was examined by reverse transcription polymerase chain reaction.

RESULTS: Scaffolds with fibrin glue showed a significant increase of glycosaminoglycane content after 28 days and a further increase after 42 days in culture, whereas scaffolds with DECM showed only a significant increase after 28 days in vitro. These results were reflected by a continuous increase of collagen II and aggrecan transcription in scaffolds with fibrin glue after 28 and 42 days in vitro. In contrast, collagen II and aggrecan transcription decreased in DECM-scaffolds with a concomitant increase of versican and collagen I transcription after 42 days in vitro.

DISCUSSION & CONCLUSIONS: To the best of our knowledge, this study is the first to analyse and characterise changes of chondrocyte differentiation on polycaprolactone-based scaffolds in combination with fibrin glue and with DECM. The results show a clearly distinct pattern of gene expression that is reflected by the overall extracellular matrix formation, more specifically the different amount of glycosaminoglycane after 28 and 42 days in vitro. Moreover gene expression analysis showed a significantly different pattern of gene activation after 42 days in cell culture. As both scaffolds share an identical macroporous polycaprolactone backbone, the observed differences can be ascribed to the added composite material. As the combination of polycaprolactone with fibrin glue [1] as well as uncombined DECM scaffolds [2] have been used for cartilage tissue engineering and proved to lead to a substantial formation of neocartilage in vitro and in vivo, further research will be necessary to identify the impact of fibrin glue and DECM on chondrocytes, when used as a composite material. Additionally, the combination of polycaprolactone-based scaffolds with DECM might resemble the tissue architecture of native cartilage better than the combination with fibrin glue. In previous studies we have shown that the regenerative potential of cartilage arises from the superficial layers [3]. Therefore the immersion of chondrocytes within fibrin glue does not reflect natural conditions.

In conclusion, the combination of polycaprolactone with fibrin glue or DECM significantly influences the gene expression of chondrocytes . Therefore further research will be necessary to optimize the differentiation of chondrocytes on composite scaffolds with DECM as this approach better resembles natural conditions than the combination with fibrin glue.

Elastin-like recombinamer covered coronary stents

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INTRODUCTION: Covered stents have been proposed as a solution to in-stent restenosis and stent thrombosis. The membrane covering the stent struts excludes the luminal surface from the blood and functions as a physical barrier to prevent cell ingrowth. Ideally, the membrane properties include: elasticity to allow for stent expansion, hemocompatibility to prevent stent thrombosis and capability to support endothelialization. Pertinent materials failed to fulfill these requirements when investigated in small-caliber stents [1]. Elastin-like recombinamers (ELRs) are recombinant polymers based on natural human elastin. They maintain their elastic behavior and low thrombogenicity. Consisting of aminoacids, they evoke no host immune response. Additionally, they have been functionalized with peptides to improve and accelerate endothelialization.

METHODS: This study aims at stent-vessel biointerface optimization by A) establishing the manufacturing of ELR-covered stents, B) assessing the hemocompatibility of ELR-covered stents, C) evaluating the feasibility of trans-catheter delivery of ELR-covered stents and D) characterization of membrane integrity maintenance during simulated implantation and biomimetic flow conditions. Coronary stents (3.0x15 mm, cobalt-chromium; n=5) were covered with ELR by a layer-by-layer dip-coating technique. Three stents underwent a simulated deployment procedure by balloon inflation followed by optical verification of the integrity of the membrane. Subsequently, the stents were placed in a biomimetic flow loop system and exposed to high shear stress (1.5 Pa) and pressure conditions (120/80 mmHg) for 24 hours to prove the mechanical stability of the layer. Membrane integrity was evaluated by dry weight measurement before and after exposure to mechanical stress. In two ELR-functionalized stents, a thrombogenicity assay during simulated use was performed. Stents were placed in PVC tubings by trans-catheter delivery. Tubes were filled with anticoagulated human blood (0.106 mol/L Tri Natriumcitrate) and the ends were connected to each other to create a Chandler-loop. After loop rotation for 60 minutes at 37°C and 1.5 Pa, platelet adhesion was assessed by scanning electron microscopy (SEM).

RESULTS: The layer-by-layer dip coating procedure resulted in a complete and uniform ELR membrane covering the coronary stents. Stents were expanded from their crimped diameter of 1.8 mm to a diameter of 3.5 mm (9 atm) without any void formation. Stent recoil was not observed. Membrane thickness after stent deployment was approximately 30 µm. Exposure of the ELR-functionalized stents to arterial flow and pressure conditions revealed a stable membrane, resulting in a dry weight difference before and after treatment of 0.5 % ± 0.4 %. As assessed macroscopically and by SEM after Chandler loop simulated use, neither blood clots nor platelet adhesion, leukocyte adhesion or fibrin network formation was evidenced. Successful fabrication of novel ELR-functionalized covered candidate stents was substantiated. Simulated delivery and biomimetic use procedures revealed a regular deliverability and expansion pattern as well as low thrombogenicity and complete maintenance of membrane integrity.

DISCUSSION & CONCLUSIONS: Taken together, these results advocate ELR-covered stents as a novel approach to reduce stent restenosis and, due to their inherent lack of thrombogenicity, stent thrombosis and thus hold potential for optional future rescheduling of dual antiplatelet therapy after coronary stent implantation.

Adaptations to RNA extraction protocols for peptide hydrogels

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INTRODUCTION: There are numerous groups investigating the use of peptide self-assembling hydrogels in the fields of tissue engineering and regenerative medicine. Many of the published outcomes of these investigations are in the form of images illustrating cell viability with very little quantitative data [1-3]. It is well known that techniques commonly used in 2D cell culture may lead to ambiguous and potentially misleading results when transferred to 3D [4]. To provide more useful biological data, molecular biology and genomics techniques need to be applied to experiments utilising peptide hydrogels. The current work outlines adaptations to standard RNA extraction methods required to obtain good quality, high yield RNA for further downstream reactions, exemplified by QPCR analysis.

METHODS: HEK293 cells (ATCC, CRL-1573) were maintained in DMEM (Sigma) supplemented with 10 % FBS (Sigma) in a 5 % CO_2 humidified incubator at 37 °C. Cells were suspended in gels (PeptiGelDesign) at a concentration of $4x10^6$ cells mL^{-1} and cultured in 24-well ThinCerts™ (Greiner). RNA was extracted using a) TRIreagent (Sigma), b) TRIreagent followed by RNeasy Mini Kit (Qiagen) or c) RNeasy Mini Kit. Pronase (10mg mL^{-1}) was added to cells encapsulated in gels for 5 minutes prior to RNA extraction in selected samples. Extracted RNA was analysed using NanoDrop (ThermoScientific) and TapeStation (Agilent Genomics). Contaminating genomic DNA in 1µg of RNA was degraded using Amplification Grade DNase 1 (Sigma). Synthesis of cDNA was carried out using High Capacity RNA-to-cDNA kit (Applied Biosystems). Expression levels of housekeeping genes were analysed using SYBR Green QPCR kit (Qiagen).

RESULTS: RNA was extracted from cell only controls using all methods (Table 1). RNA was extracted from encapsulated cells using all methods apart from TRIreagent followed by Qiagen columns. This method was not pursued any further. Although yields of RNA extracted using TRIreagent and Qiagen columns were equivalent, purity was observed to be lower in TRIreagent samples. Yields of RNA were lower than control samples when TRIreagent and Qiagen columns were utilised. Use of pronase to break down the gel prior to use of RNeasy columns resulted in equivalent yields of RNA compared to control cells. After DNase treatment equivalent amounts of cDNA were synthesised from RNA extracted using all methods. However, when expression levels of common housekeeping genes were analysed using QPCR only RNA extracted using Qiagen RNeasy columns, with or without pronase pre-treatment, showed equivalent results to cell onlycontrols.

Table 1. Concentration and purity of RNA extracted using various methods.

Method	Condition	Conc. (ng μL^{-1})	260/ 280
TRIreagent	Cells only	213.8	1.84
	Gel + cells	85.7	1.68
TRIreagent + Qiagen	Cells only	564.1	2.05
	Gel + cells	8.9	2.00
Qiagen	Cells only	107.5	2.08
	Gel + cells	84.1	2.07
Pronase + Qiagen	Cells only	486.5	2.07
	Gel + cells	537.3	2.08

DISCUSSION & CONCLUSIONS: Breaking down gels using pronase and subsequent use of Qiagen columns allowed good quality RNA to be extracted at an appropriate yield to carry out QPCR. This finding will allow more useful biological data to be collected from cells encapsulated within self-assembling peptide hydrogels.

1

ACKNOWLEDGEMENTS: The authors acknowledge EPSRC Fellowship EP/ K016210/1 for funding.

An osteoconductive and biodegradable composite implant for fracture fixation

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INTRODUCTION: Biodegradable polymer fixators such as polyglycolide (PGA), polylactide (PLA) and PLGA implants have been used widely in fracture management. However, insufficient mechanical property, inappropriate degradation time, non-radiolucency, and foreign body reaction during degradation process are the major concerns [1]. In addition, several studies showed that the degradation of biodegradable implants were not be accompanied by restoration of normal bony tissues [2]. Thus, the development of new biodegradable implants shall benefit fracture fixation.

METHODS: Poly(ε-caprolactone) (PCL) and calcium phosphate ceramic (CPC) were used to fabricate biodegradable PCL/CPC screws, and the physical properties of composite screws were characterized *in vitro*. The *in vivo* performance of the PCL/CPC screws were evaluated in a rabbit model. Animals were sacrificed after 3 months, and the retrieved samples were analyzed by using micro-computed tomography (μ-CT) and histology.

RESULTS: The PCL/CPC composites had good biocompatibility and mechanical properties (24.7±1.2 MPa). Under scanning electron microscope and X-ray diffraction examinations, CPC was transformed into hydroxyapatite (HAP) when the composites were immersed in simulated body fluid (SBF). No postoperative mortality or complications were noted in animal study. Biodegradation of the PCL/CPC screws and newly formed bony tissue around the degraded composites were found under μ-CT.

Fig. 1:Micro-CT inspections of retrieved femoral metaphyses.

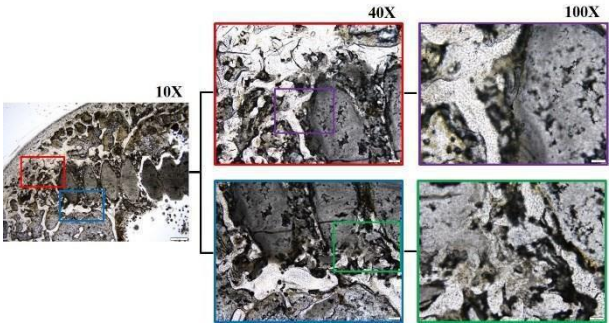

Fig. 2: Histological sections showed new bone formation around PCL/CPC screw.

The un-decalcified histological inspection revealed that new bony tissues formed around PCL/CPC screw, even in the tip of pitches.

DISCUSSION & CONCLUSIONS: PCL/CPC composite has good biocompatibility. HAP formed on the PCL/CPC composite when immersed in SBF. HAP can improve alkaline phosphatase activity and mineralize matrix synthesis in osteoblasts [3]. The HAP-coated bone screws had also been shown to increase stability in fracture fixation [4]. In animal study, no deaths or complications occurred postoperatively. Biodegradation of the composite screws and newly formed bony tissues around the degraded implants were found. The results indicate that the PCL/CPC composite can be used to fabricate biodegradable fixators for osteofixation.

ACKNOWLEDGEMENTS: This study was supported by Taipei Tzu Chi Hospital, The Buddhist Tzu Chi Medical Foundation, and the Ministry of Science and Technology, Taiwan.

DISCLOSURES: The authors confirm that there are no conflicts of interest.

Myoblasts-printed hierarchical scaffolds for inducing cell alignment and differentiation to regenerate skeletal muscle tissue

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INTRODUCTION: Skeletal muscle has a highly aligned hierarchical structure. As myoblasts grow and differentiate, cellular morphology changes from star-like shape to elongated confluent structure. [1] The cellular morphology is a critical parameter of myogenesis which can be guided by aligned fibers in terms of alignment, elongation, and differentiation. [2] In this study, we propose a melt-plotted polycaprolactone (PCL) hierarchical structure with partially aligned electrospun nano/microfibers and its influence on the cellular activities of myoblast.

METHODS: *Materials* PCL (M_w =45,000) used for melt-plotting system and PCL (M_w =90,000) used for electrospinning were purchased from Sigma-Aldrich (St. Louis, MO). For cell printing, LF10/50 alginate (FMC BioPolymer, Drammen, Norway), polyethylene oxide (PEO) (M_w =90,000) (Sigma-Aldrich St. Louis, MO), and calcium chloride ($CaCl_2$) (Sigma-Aldrich, St. Louis, MO) were used. The scaffolds were printed with C2C12 myoblasts (ATCC number CRL-1772, ATCC, Manassas, VA) encapsulated with the bio-ink.

Melt-plotting system A PCL pellet embedded in heating cartridge was used to melt PCL at 100 ˚C. A 3D robot (DTR3-2210-T-SG, DASA Robot, South Korea) moved the heating cartridge to draw a 3D structure.

Electrospinning The solution was prepared by dissolving 10 wt% PCL in methylene chloride (MC) and dimethylformamide (DMF) at 4:1 ratio. A syringe mounted on a syringe pump (KDS 230, NanoNc, Inc.) was used to eject the solution. A high-voltage power source (SHV300RD-50K, Convertech) applied high-voltage direct-current.

Cell printing For bio-ink preparation, 3 wt% alginate and 2 wt% PEO were mixed. As a cross-linking agent, 0.5 wt% $CaCl_2$ was added to alginate/PEO solution at 7:3 ratio. The bio-ink was created by mixing the solution and C2C12 myoblasts at 1×10^7 cells/mL, and dispensed on PCL struts.

RESULTS: To observe myoblast morphology, a scaffold with aligned fibres (AF) and two control scaffolds (NF and RF) were used. The NF scaffold was consisted of perpendicular PCL struts with no fibre, and the RF scaffold was consisted of the PCL struts with randomly arranged nano/ microfibers. As a result, myoblasts showed circular shape on NF scaffold and star-like shape on RF scaffold. In contrast, the myoblasts were aligned and elongated on AF scaffold.

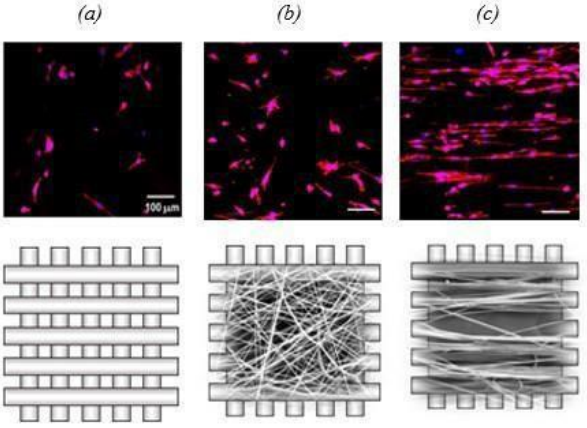

Fig. 1: Fluorescence images of cell-laden (a)NF, (b) RF, and (c) AF scaffolds after 1 day of culture (blue: nuclei; red: F-actin).

DISCUSSION & CONCLUSION: Myoblast morphology was distinct among different fibre arrangements. Myoblasts aligned and elongated better on AF scaffold. It suggests that the hierarchical structure with aligned fibers considerably contributes to myogenesis by guiding desirable cellular morphology.

ACKNOWLEDGEMENTS: This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and future Planning (NRF- 2015R1A2A1A15055305).

The synthesis and characterization of novel ionomer bio-scaffolds using different lactone co-monomers and layered double hydroxide initiator

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INTRODUCTION: Tissue engineering has become a viable option for the replacement of tissue and organ function. The substitutes require a three-dimensional, porous, biocompatible, and preferably biodegradable scaffold. The physical and chemical properties of a scaffold greatly influence a cell's bio-activity. By chemically synthesizing the materials of a scaffold, one can better simulate the natural extracellular environment to provide optimum conditions for cell adhesion and differentiation. Herein, we employ in-situ polymerisation to produce various 3D polylactone acid ionomer cell-scaffold structures using Mg/Al layered-double-hydroxide (CO_3^{2-}) as the initiator after the method first published by McCarthy et al [1]. The key product of this reaction is a polymer-based ionomer complex that is insoluble in methylene chloride and has a pore-morphology that enables its use as a biodegradable scaffold for osteoblasts.

METHODS: The initial hybrid product is prepared by the melt polymerisation of 95% L,D-lactide with ε-caprolactone or δ-valerolactone in different mass ratios with 5% LDH by mass at 150 °C for 24 hours. The ring structure of the lactone monomer can be opened by the cations of the LDH enabling anchoring and propagation of the resulting lactone acids. Then methylene chloride (CH_2Cl_2) is used as an extraction solvent with centrifugation at 8000 rpm for 10 minutes to isolate the insoluble ionomer phase. The insoluble component is dried in air for 12 h and then baked at 60 °C for 1 h to fully remove the solvent.

RESULTS: SEM images (Fig. 1) were taken to reveal the microstructure and morphologies of the porous-structured scaffold materials extracted from two hybrid polymerization products. In all images, a porous network structure is visible indicating that polymerizing lactone monomer with carbonate-intercalated hydrotalcite is a credible route to the chemical synthesis of a bio-scaffold.

Table 1 shows representative monomer conversions obtained via the use of ^1H nuclear magnetic resonance (NMR) spectroscopy. In the two copolymers, different final monomer ratios are produced. For example, in the Lactone:Valerolactone (L:V) 1:2 case (initial monomer mass ratio), LD-lactide is present in greater concentration than in the L:C 1:2 copolymer, with subsequent effects on network morphology.

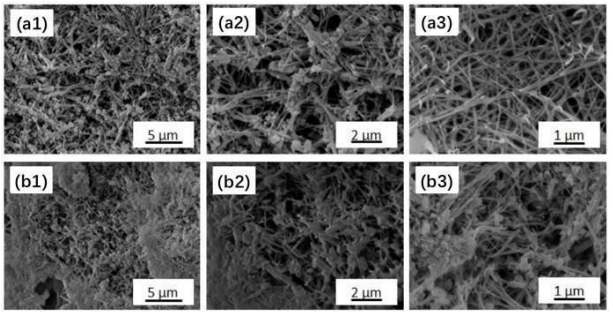

Fig. 1: SEM micrographs of (a) poly(lactide-co-caprolactone) and (b) poly(lactide-co-valerolactone) scaffolds with monomer mass ratio 1:2.

Table 1. Monomer conversions in (a) PL-co-CL and (b) PL-co-VL for initial monomer mass ratio 1:2 using 1H NMR characterization.

	Mass Ratios %	Mole Ratios %	Copolymer Conv. %
L:C 1:2	LA:CL 33.3:66.7	LA:CL 28.3:71.7	LA:CL 22.1:77.9
L:V 1:2	LA:VL 33.6:66.7	LA:VL 25.7:75.4	LA:VL 32.8:67.2

DISCUSSION & CONCLUSIONS: This study has demonstrated the production of various porous structures suitable for use as potential cell-growth scaffolds. These have been identified chemically as polymeric/magnesium or aluminium lactate complexes [1] by the aid of XRD. In the future, various bio-assays will be applied to these materials to confirm their biocompatibility. Then, an effective fabrication approach (e.g. 3D printing) will be investigated for scaled-up manufacture of these 3D structured scaffolds for tissue engineering applications.

Inhibition of epithelial-mesenchymal transition in cholangiocyte 3D culture by embedding alginate hydrogel beads

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INTRODUCTION: Regulation of microstructure in 3D culture is indispensable to induce organ features in vitro. Three dimensional (3D) culture, like forming of multicellular spheroids, is believed as useful tool to enhance cellular functions. However 3D aggregation has possibility to induce epithelial-mesenchymal transition. We reported that 3D aggregation without spaces decrease epithelial characteristics in fetal hepatocytes, and it was improved by embedding alginate hydrogel beads into the aggregates [1]. In this study, we tired to keep epithelial properties of a human cholangiocyte cell line MMNK-1 cell under 3D condition by embedding alginate hydrogel beads. We also surveyed the effect of cholangiocyte on hepatic functions in heterospheroids comprising human hepatoma cell line Hep G2 cells, MMMK-1 cells, and alginate hydrogel beads.

METHODS: We used a methylcellulose medium to form multicellular spheroids as reported before [2]. Alginate hydrogel beads were produced by an inkjet system. Spheroids were cultured for 2 days. Samples were fixed, sectioned and stained. Albumin secretin rate was calculated by normalizing secreted albumin with DNA amount.

RESULTS: On a 2D culture dish, MMNK-1 expressed an epithelial marker E-cadherin on the cell membrane. We also detected another epithelial marker CK19, but not a mesenchymal marker vimentin. However, under the 3D condition, E-cadherin protein was detected only in the nucleus of cells. Vimentin was detectable in the 3D cultured MMNK-1 cells, while CK19 expression was strongly supressed. These results showing that epithelial-mesenchymal transition (EMT) was occurred in the 3D condition. We then formed hybrid spheroids comprising same number of MMNK-1 cells and alginate hydrogel beads. Addition of hydrogel beads was expected to reduce cell-cell contact and cell density. The sections of these spheroids showed some spaces between cells, and the MMNK-1 cells maintained the normal localization of E-cadherin. CK19 expression was not so recovered but vimentin expression was suppressed. Next, we tried to reveal the effect of MMNK-1 cells on the hepatic functions of Hep G2 cell by forming spheroids consisting of Hep G2 cells, MMNK-1 cells, and alginate hydrogel beads. Compared with Hep G2-cell spheroid with alginate hydrogel beads, Hep G2-cell and MMN-1-cell spheroids with beads showed higher albumin secretion rates.

DISCUSSION & CONCLUSIONS: Embedding of alginate hydrogel beads suppressed EMT in MMNK-1 spheroid. It means that such culture system is better to keep epithelial features of MMNK-1 in 3D spheroid culture. One or some of epithelial features of MMNK-1 cells might increase albumin secretion in Hep G2 cells. Our next goal is to recapitulate the canal of Hering in the hetero spheroids.

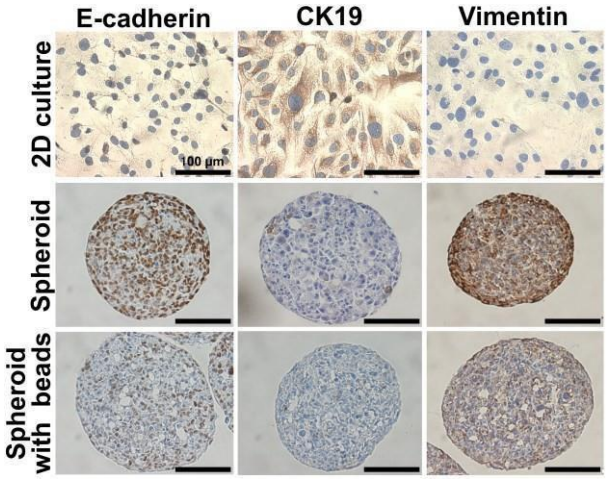

Fig. 1: MMNK-1 cells were cultured in 2D or 3D culture conditions. Cells were stained with E-cadherin, CK19, and vimentin antibody. Scale bars: 100 μm

ACKNOWLEDGEMENTS: This work was partly supported by Grant-in-Aid for Scientific Research on Innovative Areas "BioAssembler" from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

3D printing of biopolymer based core/shell structures – novel properties and applications in tissue engineering

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INTRODUCTION: Among the many additive manufacturing techniques, extrusion based 3D printing offers high flexibility and versatility for scaffold fabrication. For tissue engineering applications, many studies are focused on rendering a single function (e.g. cytocompatibility, mechanical stability or growth factor/cell delivery) to the fabricated scaffolds. Using multi-channel dispensing feature of 3D extrusion printers, multifunctionality could be rendered to the fabricated scaffolds by using two or more materials to build a single scaffold[1]. Based on a recently presented method for direct 3D plotting of hollow strands consisting of highly concentrated alginate pastes[2], a novel way of using two different materials as a single entity i.e. as core and shell was developed in the present work. These scaffolds were analyzed for their physical properties and release kinetics of proteins, when selectively loaded in core or shell. Further, selective *in situ* cell colonization of core/shell scaffolds was probed in order to study cytocompatibility of cell loading of the core.

METHODS: BioScaffolder 2.1 by GeSiM, Großerkmannsdorf with a 3 channel plotting unit and custom made core shell needles were used to fabricate core/shell scaffolds. 16.7 wt% of alginate paste was used as shell and a low concentrated biopolymer as core (3 wt% alginate, 1 wt% chitosan, 3 wt% gelatin, 1 wt% gellan gum or 0.6 wt% collagen). For protein loading - bovine serum albumin (BSA), lysozyme and vascular endothelial growth factor (VEGF) were mixed in respective core or shell material. After plotting, the scaffolds were stabilized in 1 M $CaCl_2$ solution (additionally in 1 wt% genipin when gelatin or collagen was used as core). Freeze dried scaffolds were characterized for their porosity, compressive strength and swelling behaviour. Release of the proteins was measured by Bradford assay or ELISA. Bioactivity of VEGF was evaluated by human dermal microvascular endothelial cells (HDMEC) proliferation assay. HDMEC were incorporated in the core, and cytocompatibility was analysed by live dead staining.

RESULTS: Porosity and compressive strength (in wet state) of the core/shell scaffolds was observed to be higher compared to conventional full strand scaffolds. The swelling ratio of core/shell scaffolds was found to be dependent on the biopolymer combinations. Also distinctive release kinetics of proteins was observed for each core/ shell combination. Thickness of the shell, type of protein and core material influenced the release kinetics.

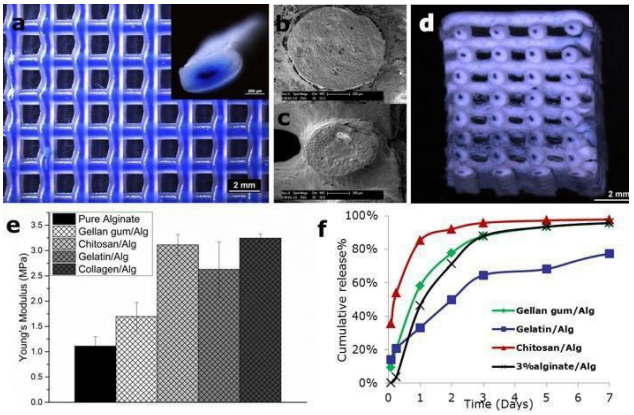

***Fig. 1**: **a**) core/shell scaffold of 2 layers; inset – single core/shell strand (blue: core; white: shell). SEM images of **b**) full and **c**) core/shell strand. **d**) 16 layered freeze dried core/shell scaffold. Young's moduli and release of BSA from different core/shell scaffolds (**e** & **f** respectively).*

HDMEC's encapsulated in the core have shown a viability of 66.2% at day 1 after plotting. An increase in the percentage of live cells was observed at the end of 21 days of culture.

DISCUSSION & CONCLUSIONS: Different biopolymers were successfully used for fabricating core/shell scaffolds which were found to have novel properties. Core/shell fabrication allows for easy and controlled integration of biological components rendering multifunctionality to the scaffolds.

ACKNOWLEDGEMENTS: This study is funded by German AiF and the Excellence Initiative by German Federal and State Governments.

Laser-microfabricated, biocompatible hydrogels based on hyaluronan

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INTRODUCTION: Hyaluronan (HA) is a major component in the extracellular matrix (ECM) and the basis of a large number of bio-inspired materials. The introduction of double bonds to the back bone of HA enables the production of photo-crosslinked, biocompatible hydrogels. Functionalization with (Meth)acrylates is not unfavourable due to inherent cytotoxicity. The low cytotoxicity of vinyl esters in combination with the superior photo-reactivity as thiol-ene system makes vinyl ester-functionalized HA (HA-VE) an interesting candidate for 3D laser-microfabricated biomaterial constructs for tissue engineering.[1] It this study the material system was optimized for cell encapsulation.

METHODS: HA-VEs were synthesized from HA with different molecular weights by lipase catalyzed transesterification with divinyl adipate (DVA).[1] Formulations with varied macromere contents were prepared and their reactivity was examined by photorheology. Additionally, the influence of the addition of dithiothreitol as thiol chain transfer agent was investigated. The slope of the storage modulus from rheometric curves was taken as a measure for the reactivity whereas the final storage modulus was used to calculate the mesh size of the hydrogels as reference value for the crosslink density. The swellability of ready-cured hydrogels was determined as complementary parameter. Cell compatibility of HA-VEs were assessed by metabolic as well as DNA assay. An exemplary HA-VE based formulation was used for the encapsulation of murine fibroblast cells by two-photon polymerization (2PP) based microfabrication.[2] The viability of the encapsulated cells was assessed by calcein staining.

RESULTS: HA with two different m.w. were converted to HA-VEs with different degrees of substitution (DS), hence six batches of HA-VE with varied macromere size and DS were obtained. Photo-reactivity increased with macromere content, macromere size, and DS. Crosslink density followed the same trend, while the trend for swellability was reversed. However, 80 mol% of thiol led to an optimized reactivity and final storage modulus. Metabolic as well as DNA assay indicated the same trend for the cytocompatibility of vinyl ester-modified HA. The impact of the number of vinyl ester-groups (DS) on the cytotoxicity of HA-VEs was negligible. As a proof of the concept murine fibroblasts were encapsulated in 3D hydrogel constructs and found to be viable after 24 h (*Fig. 1*).

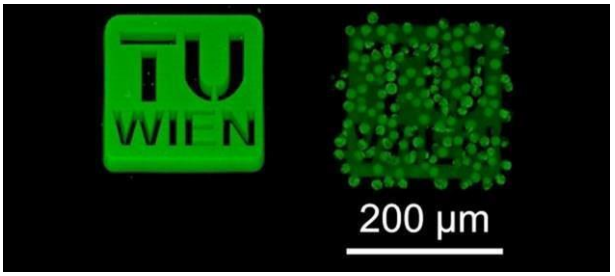

Fig. 1: Laser scanning microscope images of 3D microfabricated hydrogels without (left) and with encapsulated calcein-stained fibroblasts (right).

DISCUSSION & CONCLUSIONS: HA can be transformed to biocompatible HA-VE by lipase-catalyzed transesterification reaction with DVA in high yields. DS is easily adjusted by variation of reaction time. Macromere content, macromere size, and/or DS determine the material properties on demand. The cytocompatibility of the synthesized macromeres enables the fabrication of 3D hydrogel constructs with encapsulated cells by laser fabrication using 2PP. HA-VE were found to be a promising modular material platform to engineer 3D hydrogel constructs as environment for living cells.

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ACKNOWLEDGEMENTS: We kindly acknowledge the financial support by the Austrian Research Funding Association (FFG, Project Number 849787, S.B.) and the European Research Council (Starting Grant, Project Number 307701, A.O.).

Cryopreservation of cells encapsulated in hydrogel: evaluation of recovery and assembly after thawing

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INTRODUCTION: Cell encapsulation in hydrogels is a technique that offers a variety of applications, ranging from immunoisolation to biofabrication of three-dimensional scaffolds[1]. Assembly of cell-laden hydrogel aggregates aims to generate complex biological constructs by manipulating microscale units[2]. An important issue for the clinical implementation of this technique is the maintenance of a large stock of cryopreserved cell/hydrogel building blocks[3]. In this work we investigated and compared the ability of different cryoprotectants (CPAs) to preserve viability and functionality of alginate encapsulated cells after freezing. The integration of cryopreservation techniques with cell micro-scale encapsulation introduces a promising approach to the field of tissue engineering.

METHODS: MG63 cells were encapsulated in sodium alginate films. A stainless steel ring was immersed in the alginate solution containing cells (2M cells/mL), extracted and immediately immersed in a 200 mM $CaCl_2$ solution. This way the alginate film that forms within the ring gelates, entrapping the cells inside a thin disk (248±13 µm thickness). Afterwards, the cell-containing films were transferred to cryogenic vials and exposed to different cryopreservation media (10% DMSO or 10% glycerol in fully supplemented MEM). After 20 min the vials were slow-cooled up to −80 °C and plunged into liquid nitrogen (−196 °C) for 1 week. Longer storage was not estimated to affect the cell behavior since cells can be cryopreserved in liquid nitrogen for a period of time in the order of years[3]. Subsequently, the films were warmed rapidly in a 37 °C water bath and transferred in fully supplemented MEM. Viability (Calcein-am/PI staining) and metabolic activity (alamarBlue assay) of cells still entrapped in the alginate matrix and cells after releasing from the gel were evaluated and compared with those of cryopreserved cells.

RESULTS: We observed that cells frozen in presence of DMSO or glycerol remained viable after the encapsulation and cryopreservation process. According to alamarBlue analysis up to 2 weeks, cryopreservation decreased the metabolic activity of the encapsulated cells. However, from day 3 onwards we observed a rapid recovery of cell metabolism. Cells cryopreserved with glycerol recovered better than cells cryopreserved with DMSO ($p < 0.05$).

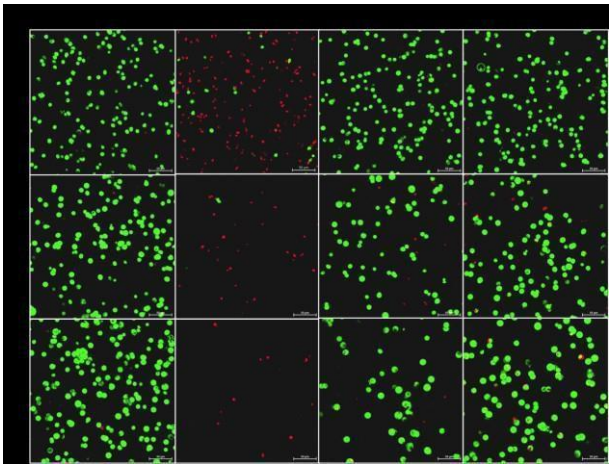

Fig. 1: live/dead analysis with confocal microscopy of MG63 cells encapsulated alginate discs and cryopreserved, at different time points after thawing. All scale bars are 50 µm.

DISCUSSION & CONCLUSIONS: In this study a comparison between different cryopreservation methods of cell/hydrogel constructs has been proposed and applied to the manufacturing of cell-laden alginate films. After being cryopreserved for 1 week in liquid nitrogen, cells were able to survive within the matrix with a reduced metabolic activity that rapidly increased after thawing. Cells cryopreserved with glycerol exhibited a higher recovery capability within 2 weeks. The proposed technique provides a scalable method to produce and store cell-laden hydrogel constructs and can be adopted as support for cell banking, expansion and release. Moreover, cryopreserved encapsulated cells can serve as building blocks for tissue assembly and biofabrication.

Polymer-supported membranes as models of the cell surface using a microphotopatterning technique to investigate 1D patterns in 3D cell migration

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INTRODUCTION: Developing biological membranes on solid supports links the biological field to the area of surface science and tissue engineering. Studying solid-supported membranes (SSMs) allow scientists to identify the parameters that are responsible for particular phenomena. Success in understanding cell behaviour and responses on extracellular scaffolds within physiological relevant 3D construct can aid the design of optimal bioactive tissue engineering scaffolds. To achieve this, reliable methods need to be developed for controlling the formation of SSMs and the deposition, incorporation and addressing of biological entities. We are investigating into polymer-supported membranes. Soft polymeric supports can act as spacers between the bilayer membrane and solid support that either 'cushion' or 'tether' the membrane, this minimizes contact between transmembrane proteins and the solid support.[1] The polymer support is generated through the incubation of poly-L-lysine conjugated to alkyl-chain-terminated poly(ethylene)glycol.[2]

METHODS: To synthesise the polymer support: The polymer support was synthesised through the incubation of poly-L-lysine conjugated to alkyl chain-terminated poly(ethylene)glycol on silica/glass using hexadecylamine. This was purified by 3 rounds of dialysis using dialysis cassettes.[2] Silicon wafers/glass coverslips were cleaned in piranha solution and incubated with a 1 mg/ml solution of PLL-PEG-HD in 50/50% (v/v) water/DMF for 30 minutes. They were then washed and used directly for bilayer formation. **Preparation of small unilamellar vesicles (SUVs):** A thin phosphatidylcholine lipid film was prepared by suspension in HEPES buffer saline to a 1mM lipid concentration. The suspension was subjected to probe sonication forming SUVs. A microfluidic flow cell with a continuous flow of the SUVs suspension was used to deposit the lipids onto the polymer support which formed a bilayer through rupture of adhered vesicles.

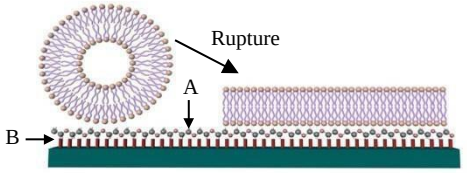

Fig 1. Representation of polymer-supported bilayer. A = hexadecyl alkane. B = PEG on a poly-L-lysine coated silica/glass support. Adapted from Ref. 2 by Tom Paterson.

RESULTS: Polymer supported membrane model has successfully been produced and characterized via atomic force microscopy, Fourier-transform infrared spectroscopy and X-ray scattering to probe the structural and dynamic properties of the supported membrane.

DISCUSSION & CONCLUSIONS: A microfluidic setup containing the polymer support with a continuous flow of the lipid suspension yielded a successful polymer-supported membrane model. We can now use hydrogels of polyethylene glycol as a coating on silica/glass to photoablate using a Ti:Sapphire laser.

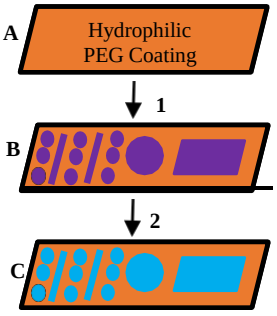

Fig 2. Generation of micropatterned PEG films. 1 – Ablation patterned generated by system software. 2 – Proteins only adsorb to ablated sites.

ACKNOWLEDGEMENTS:
Acknowledgements to the EPSRC for funding.

Self-assembly of bioactive silk to integrate cells into a 3D scaffold

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INTRODUCTION:

Tissues are built up of cells integrated in a composite material, called the extracellular matrix (ECM). The ECM provides physical 3D support and also specific sites for cell anchorage. We have recently used genetic engineering to develop a recombinant silk protein biofunctionalized with a motif from the ECM protein fibronectin (FN), which enhance the cell supportive capacity of FN-silk formed thereof[1]. A mild self-assembly process can be used to accomplish various formats of FN-silk scaffolds. Herein we show that dispersed mammalian cells can be added to the FN-silk protein solution before assembly, and thereby get integrated throughout the macroscopic silk-like material (Figure 1).

METHODS:

A collection of various mammalian cell types (from mouse and human) were integrated into FN-silk in the format of fiber, foam and film. Cells within the silk scaffolds were analysed using Alamar Blue and Live/dead viability assays and subjected to various imunostainings both directly in scaffolds and after cryosectioning. Dynamics of insulin release were studied by perifusion of glucose, and analysis using an insulin assay HTRF kit

RESULTS:

The proliferative capacity of the cells within the FN-silk scaffolds was maintained through more than two weeks, with some variability of when confluence was reached depending on cell type. Viability was high (>80%) for all cell types investigated, with confirmed viability in the innermost part of the scaffolds. Elongated cells with filamentous actin and defined focal adhesion points confirm proper cell attachment within the scaffolds. Cryosectioning was used to further confirm presence of cells within the deepest parts of the materials.

Separate experiments were performed in order to confirm that selected cell types maintain their specific native functions within the silk scaffold. For example, we have verified that a majority of the fibroblasts integrated into silk fibers produce collagen type I, aligned human skeletal muscle satellite cells within a silk fiber can be differentiated to form myotubes, and that endocrine cells within a silk foam secrete insulin upon stimulation with glucose.

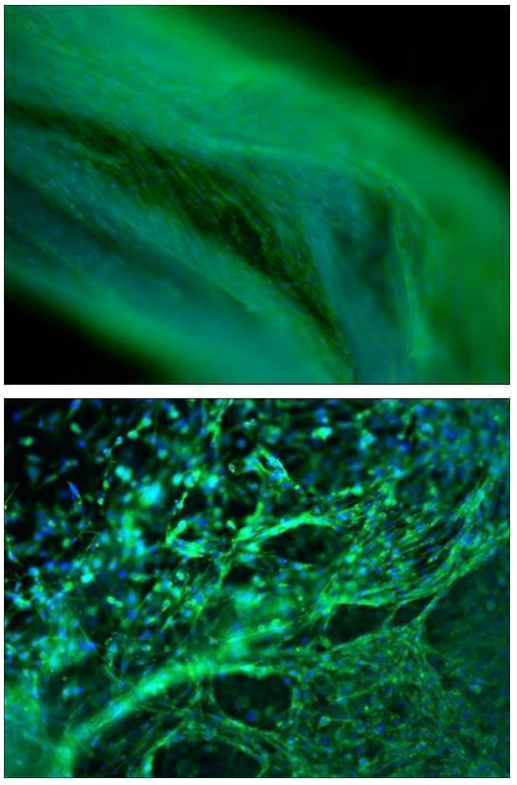

Fig. 1: F-actin and Dapi staining of mammalian cells within silk fiber and foam.

DISCUSSION & CONCLUSIONS:

Herein, a novel method for formulation of cell-containing silk scaffolds is described. Various 3D formats can be obtained to imitate different tissue types. The cells attach within the silk scaffolds and maintain high viability, proliferative capacity and specific functions. Moreover, with this method it is practically very easy to combine several cell types, to better replicate the native tissue, as long as they can be cultured in a similar media.

ACKNOWLEDGEMENTS:

Spiber Technologies AB is acknowledged for proving silk proteins.

Nanocellulose-based bioinks for 3D Bioprinting of human skin model

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INTRODUCTION: 3D Bioprinting is a revolutionary technology for the bottom up fabrication of human tissue and organs. The use of biocompatible inks in 3D Bioprinters enables the rapid manufacturing of 3D tissues and organs *in vitro* and the precise control over microarchitecture and functionality of cell-laden constructs. The printing versatility of bioinks together with living cells diminishes the risk for poor cell viability and cell accessibility throughout the 3D network. A typical bioink consist of cells mixed with hydrogels to generate a good distribution of cells inside the 3D scaffold.

Skin is the largest organ of the human body, functioning as an external physical barrier. At the outermost layer of the skin lies the epidermis, consisting of 90% keratinocytes and 8% melanocytes [1]. The intermediate layer is the dermis consisting of collagen and elastic fibres, which are proteins synthesised by fibroblasts.

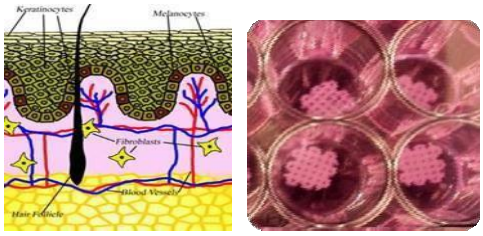

Fig. 1: Left: Schematic representation of skin's microarchitecture. Right: A scaffold printed with 3D printer.

Tissues in regenerative medicine have to be designed in a proper way for successful cell transplantation. The need of hydrogels with appropriate properties (e.g. viscosity, yield stress) in the 3D Bioprinters requires a comprehensive evaluation in regard to printability and cell compatibility. For instance, gelation, or the ability to encapsulate cells inside the bioink, is an important factor for these qualities. Nanocellulose inks have shown to have both printing fidelity and cell compatibility, and the inks can be solidified when they are mixed with crosslinkable polymers [2]. An accurate printed skin model using nanocellulose bioinks has the potential to be of great advantage in clinical research and testing of skin products and cosmetics.

In this study we evaluated and compared the feasibility of two different nanocellulose inks for the 3D Bioprinting of human skin model.

METHODS: Nanofibrillated cellulose (NFC)-based inks modified with the tripeptide Arg-Gly-Asp (RGD) (NFC-RGD) have been investigated and compared with NFC-Alginate ink in terms of 3D printing of the human skin model. The NFC-Alginate and NFC-RGD inks were mixed with alginate and Human fibroblasts and 3D Bioprinted in grid architecture with 3D Bioprinter INKREDIBLE. After bioprinting the hydrogels were crosslinked in a 100 mM $CaCl_2$ solution. Cell viability and morphology was investigated as well as gen expression.

RESULTS: The RGD peptides were coupled to the NFCs covalently. The formulations of the bioinks were evaluated in terms of reology, printability and cross-linkability. NFC-Alginate and NFC-RGD demonstrate analogous rheological properties when measuring shear rate-viscosity. After 7 days in the incubator NFC-RGD and NFC-Alginate both showed high cell viability results. The NFC-Alginate has high printability and cell compatibility for printing with human chondrocytes [2]. The RGD modification increased the Human fibroblastsproliferation.

DISCUSSION & CONCLUSIONS: RGD peptides were succesfully conjugated to the nanofibrils with various charge densities. The rheological performance and printability of the bioinks were ideal to be printed in 3D printer.

1

ACKNOWLEDGEMENTS: Nordic Innovation is acknowledged for funding project P-14031. The unmodified bioinks were kindly provided by CELLINK AB.

3D bioprinting stem-cells with solubilized tendon extracellular matrix (ECM) based hydrogel

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INTRODUCTION: 3D bioprinting aims to generate biological structures similar to natural counterparts such as tissues or organs in terms of their functions and morphology [1-2]. The major challenge with this technique is the choice of extra cellular matrix (ECM)-like biomaterial as a cell encapsulating agent. The objective of this research is to develop a composite hydrogel that would provide important biological cues to host cells. A new composite hydrogel system based on a mixture of natural ECM and agarose hydrogel is developed. ECM based hydrogel is derived from bovine native tendon by decellularization and enzymatic digestion procedures. Decellularized and solubilized tendon tissues contain important structural and bioactive extracellular matrix components such as collagen, which serves as anchorage sites for host cells.

METHODS: Decellularization and solubilization of bovine native tendon tissues are briefly explained in Fig. 1.

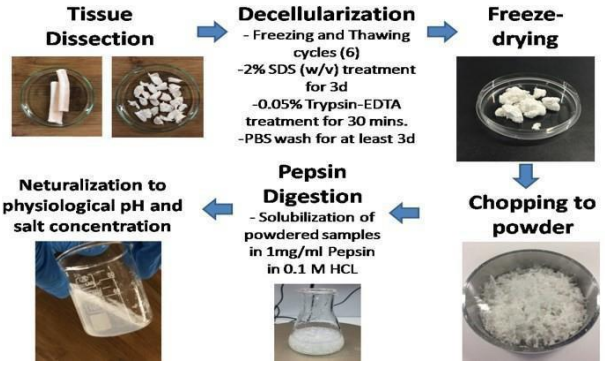

Fig. 1: Workflow to obtain decellularized and solubilized native tendon ECM.

Agarose (3%, w/v) and tendon decellularized extracellular matrix (dECM) solution were mixed with in a plate heater set to 38°C. E14 mouse embryonic stem cell suspension was introduced to agarose-dECM solution homogenously. The cell concentration in the pre-gel solution was set to 5 x 10^5 cells/ml. Extrusion of cylindrical 1 cm long cell-laden structures on pre-determined coordinates was carried out by Organovo Novogen MMX Bioprinter (Fig. 2).

RESULTS: The SEM images of tendon dECM powders indicating intact protein fibers with the absence of cells as shown in Fig. 3. High magnification images indicate collagen bundles with dense connection nodes. In addition, tile z-stack images of cell-laden structures are used to show the uniform distribution and increased viability of cells in agarose-dECM gel (Fig. 4).

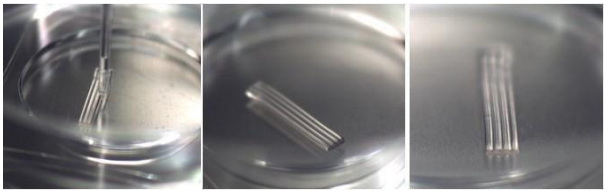

Fig. 2: Bioprinting process of cell-laden hydrogel structure. (Stripe lengths are 10mm)

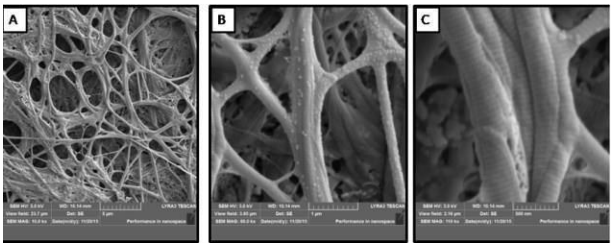

Fig. 3: SEM images of tendon dECM powders-magnifications A)10kX, B)60kX and C)110kX

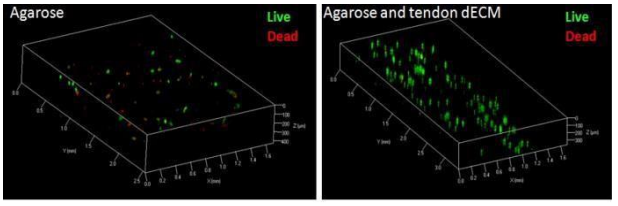

Fig. 4: Confocal images of cell-laden hydrogel constructs.

DISCUSSION & CONCLUSIONS: The results showed the biocompatibility of produced tendon dECM solution and the increased viability of MESC's encapsulated in agarose-dECM gel even after three days of incubation. Tendon dECM solution supported agarose hydrogel has a potential to be used as a cell encapsulating agent for 3D bioprinting applications.

Cell sheet as a bioink for 3D bioprinting

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INTRODUCTION: Cell sheet technology is a growing area in tissue engineering. It enables a sheet of interconnected cells which is enriched with cell-extracellular matrix (ECM) and cell-cell interactions. Poly (N-isopropylacrylamide) (PNIPAm) coating based thermoresponsive culture dishes are used as one of the advanced cell sheet technology methods [1]. It allows the surface to demonstrate temperature responsive wettability changes in aqueous environments. Different methods can be used to fabricate PNIPAm surfaces such as initiated chemical vapor deposition (iCVD) which offers a control of the polymer thickness [2]. In this research, we showed that thermoresponsive surfaces can create cell sheet which can be used as a bioink in 3D direct cell bioprinting [3]. The aim of this work is to show that cell sheets can be used to increase mechanical strength of bioink.

METHODS: 35 mm polystyrene culture dishes were coated by using initiated chemical vapor deposition (iCVD). The thickness of PNIPAm films were 30nm measured by using ellipsometry. Mouse embryonic fibroblast-like cells (NIH 3T3) seeded on PNIPAm coated polystyrene culture dishes with 100 cells/cm^2. Figure 1 shows how bioink is prepared during the proposed methdology.

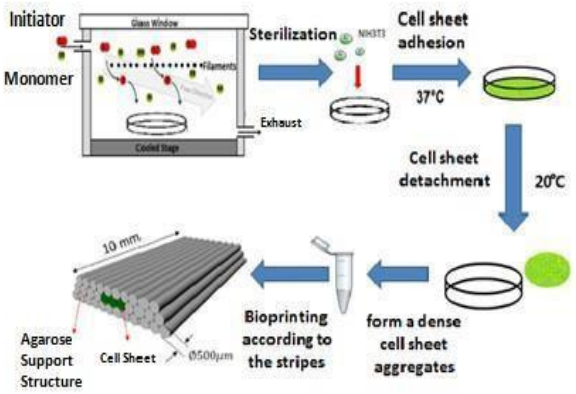

Fig. 1: The preparation of bioink

The prepared cell-sheet aggregates were 3D bioprinted using Novogen MMX Bioprinter according to the developed codes (Figure 2). The bioprinted constructs were incubated for 7 days so that the printed bioinks fuse together to form a tissue network. During the maturation period, the same culture medium was used. In order to visualize the fusion of cell sheet aggregates, they were stained with green or red membrane-intercalating dyes before printing.

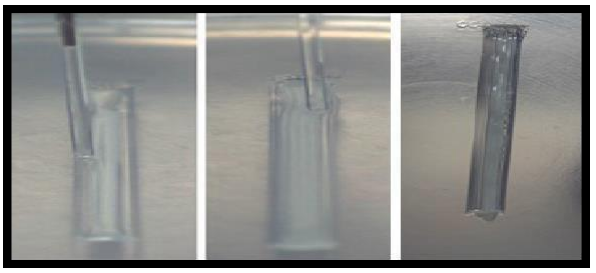

Fig. 2: The bioprinting process

RESULTS: After the printing process, the cell sheet aggregates fused within 0-7 days. The fusion of the printed cell sheet aggregates was examined at the first, third and seventh day after printing using a Zeiss LSM710 confocal microscopy. The fusion of alternate sequences of green and red cylinders is shown in Figure 3 and reveals fusion between the printed cell aggregates.

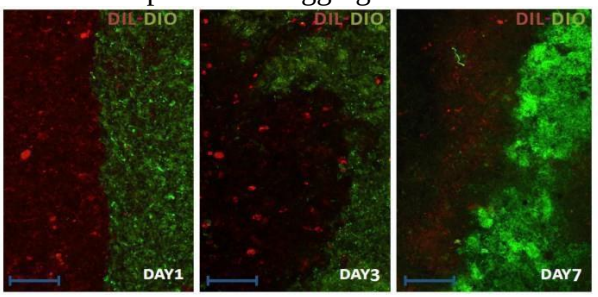

Fig. 3: Fusion evaluation of the printed cell sheets pattern (scale bar = 250 μm)

DISCUSSION & CONCLUSIONS: A novel cell-sheet based bioink developed for bioprinting. The developed bioink were used for direct cell printing. The results show that cell-sheet based aggregates can be bioprinted and fuse together. The results also showed that printed 3D structures have a better cell-cell and cell-ECM interactions, which is important for complex communication network of tissue constructs.

A method to fabricate multicellular spheroids containing various amount of extracellular matrix

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INTRODUCTION: Our laboratory is developing various methods to engineer inner microstructure of multicellular spheroid (MCS). In previous meeting TERMIS-EU 2014, we reported a method to fabricate microchannels within spheroids [1]. In that method, both cells and tiny spacer hydrogel beads were assembled by utilizing swelling property of a 3% methylcellulose (MC) medium [2]. In this study, we tried to use this system for assembling cells and extracellular matrix (ECM).

METHODS: Type I collagen and Matrigel were used as ECM. Hep G2 cells were suspended by the ECM solutions and injected into the MC medium. FITC-collagen and fluorescently labeled cells were used as necessary. Injected volume and cell number was 1 µl and 2000 cells, respectively. We kept solutions and pipette tips at a low temperature to prevent unexpected gelation. After 2 days from injection, MCSs were isolated from the MC medium, and incubated in normal culture medium for 24 h to measure albumin secretion rate. At the end of the assay, MCSs were lysed to decide DNA amount.

RESULTS: After injection into the MC medium, cells and ECM were rapidly gathered and formed spherical objects (Fig. 1). When we used undiluted ECM solutions, the objects became spherical gel particles including scattered cells. However, if we diluted the ECM solutions at 30 to 40 times, the objects turned to MCSs with thin-layered ECM (Fig. 2, lower). We prepared 5 different types of such objects (capsule or MCS) using Hep G2 cell and various concentration of Matrigel. Albumin secretion rate was different in each condition and we fond that MCS with thin-layered Matrigel showed higher function than control MCS (Fig. 2).

DISCUSSION & CONCLUSIONS: Our method was able to aggregate ECM as well as cells. Depending on the ECM concentration, we were able to control MCS structure as shown in Fig. 2. In addition, various types of macromolecules like polysaccharides or water-soluble polymers will be applicable to our method. We demonstrated that

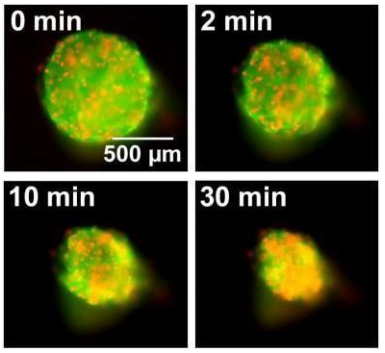

Fig. 1: Injected ECM solution suspending Hep G2 cells were condensed and formed spherical objects.

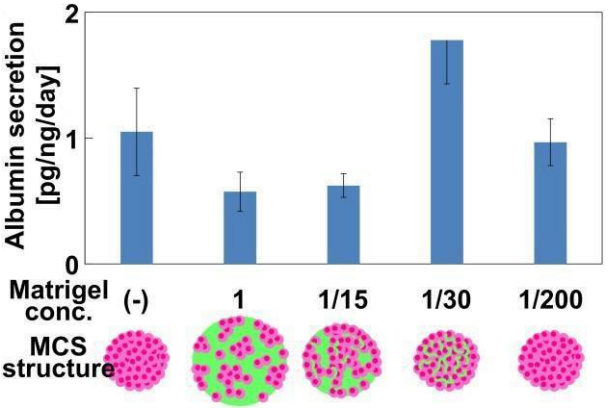

Fig. 2: Albumin secretion rates and structures of various types of MCSs.

the function of Hep G2 cells were changed by the type of MCS, indicating that microenvironment of the cell was tunable. This method will stimulate usage of MCS in pharmaceutical screening, tissue engineering, and regenerative medicine.

ACKNOWLEDGEMENTS: This work was partly supported by Grant-in-Aid for Scientific Research on Innovative Areas "BioAssembler" from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

3D-printing materials and medical coatings – a biocompatibility evaluation

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INTRODUCTION: Biomedical research often requires custom-made and highly specialized tools and testing devices. In regard to biocompatibility stainless steel and PMMA are among the most established materials for machining. We wanted to evaluate alternative production strategies to reduce production times, handling issues and costs while increasing flexibility. This can be achieved by using different base materials for machining and achieving biocompatibility by specialized surface coatings. A fast evolving alternative is to utilize additive manufacturing technologies. The aim of this research was to evaluate different surface coatings and 3D-printing materials in regard to their biocompatibility.

METHODS: Cylindrical plates (ø11.3mm) were used as sample geometry to fit in 24well-plates. Therefore, specialized holding rings were designed to allow easy handling of the samples in all test scenarios. To evaluate the surface coatings aluminium samples with beneficial machining properties (ALMg4,5Mn0,7) were prepared. Six different coatings (G1: Poliseal, Poligrat GmbH; G2: Anodizing, Zitt GmbH; G3: OF 69999, Wöhr GmbH; G4: FEP MKIII, Rhenotherm GmbH; G5: PTFE MKIII, Rhenotherm GmbH; G6: PFA MKIV, Rhenotherm GmbH) were applied. The 3D-printed samples were created solely from the evaluated material. Five materials from Stratasys Ltd. with different mechanical properties were chosen (G7: Tango plus; G8: ABS weiß; G9: Tango black plus; G10: Standard vero; G11: Med 610). Untreated samples of aluminium (G12) were treated as well.

Different scenarios were defined to evaluate the influence of the materials on cell vitality, genotoxicity, proliferation and cytotoxicity. In all cases endothelial cells (EC) were used, since these are very sensible to environmental factors. To evaluate the direct influence of the material surface on the cells, cells were seeded on the samples and the viability was evaluated via Live-Dead assay. The cell growth on the sample surface was evaluated by SEM analysis of the samples. Additionally, the proliferation of cells after 24h, 48h and 72h was assessed by WST-1 assay. To be able to evaluate long-term effects of santicizers or released ions, samples were placed in culture media for 24h, 96h, 30d and 90d. Thereafter, EC were cultured with these conditioned cell growth media. Again, proliferation and cytotoxicity was evaluated by WST-1 assay. Additionally, genotoxicity was evaluated via Ames tests. A total number of 1152 samples were evaluated to grant reliable results.

RESULTS: Significant differences between different surface treatments and 3D-printing materials were detected (table 1). The biocompatibility of surface coatings highly depended on the chosen procedure. Flexible as well as dyed printing materials generally showed inferior behaviour than rigid and colourless materials. Strong differences between short term (≤96h), mid-term (≤30d) and long-term (≤90d) results were identified.

	L/D	Proliferation	Cytotox (30d)	Cytotox (90d)
G1	+	+	0	0
G2	+	+	0	0
G3	---	---	---	---
G4	0	+	0	-
G5	-	0	-	-
G6	+	+	-	-
G7	-	-	-	-
G8	0	+	+	0
G9	-	-	-	-
G10	-	0	0	0
G11	+	+	+	+
G12	+	+	0	-

DISCUSSION & CONCLUSION: Surface coatings as well as printing materials with suitable properties could be identified. Even untreated samples prove suitable for short-term applications. The restriction to rigid and colourless materials for 3D-printing applications limits the use of this technique. However, many new materials are constantly developed and the possibility to produce rigid biocompatible geometries alone offers a multitude of new possibilities in biomedical research.

DISCLOSURE STATEMENT: The authors have nothing to disclose.

Enhanced cell viability in bio-printing process via piezoelectric transducer (PZT)-assisted 3D printing method for hard tissue regeneration

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INTRODUCTION: Three dimensional (3-D) printing has been developed and become a dominant technology in most fields due to its broad applications and advantages. 3-D bio-printing refers the 3-D printing technique used for human tissue and organ regeneration in tissue engineering. In the study, micro-extrusion printing method was used to fabricate the scaffolds, which is one among the three general printing systems in the bio-printing (inkjet, micro-extrusion, and laser-assisted printing) [1]. According to the shear thinning behavior of polymers, applying piezoelectric transducer (PZT) to the micro-extrusion printing process can be a solution of the limitation of the mechanism.

METHODS: *Preparation of bio-ink* Alginate was mixed with triple-distilled water to obtain the 1, 3, 5, and 7 weight % alginate solution. The cells (MG63; CRL-1427; ATCC, Manassas, VA, USA) were loaded in the alginate solution, mixed with 0.5% (w/v) $CaCl_2$ to complete the printable bio-inks. The prepared bio-inks were used to assess rheological properties before the printing process. *The fabrication and characterisation of scaffolds* The cell-laden scaffolds were fabricated with the inks by the computer-controlled 3-D printer, assisted with Piezo-electric transducer (PZT). The PZT vibration was applied as shown in Table 1. Two additional cross-linking processes were performed during and after the printing for the well-formed 3-D structure. The cell viability of scaffolds was assessed with live/dead assay staining after the printing process.

RESULTS: The bio-inks have shown the shear thinning behavior, which indicates that the viscosity decreases as the shear rate increases [2]. Due to the effect, the decreased well shear stress in the nozzle was analysed, which is highly related to the cell viability in cell printing [3]. The initial cell viability with PZT condition was higher than non-PZT condition, as shown in Fig. 1 (live and dead cells were stained in green and red, respectively). The initial cell viability with PZT of 5 weight % alginate was approximately 78.4%, while the cell viability with non PZT was averagely 51.7%.

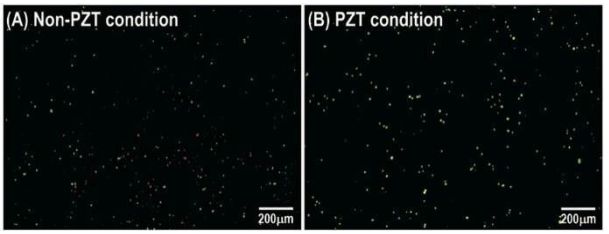

Fig. 1: Initial cell viability of cell-laden scaffolds with non-PZT condition (A) and with PZT condition (B).

Table 1. PZT vibration conditions by amplitudes and frequencies.

PZT	Frequency (Hz)				
1.5V			100	200	400
3V		50	100	200	
4.5V	25	50	100		

DISCUSSION & CONCLUSIONS: A new and simple method of enhancing initial cell viability by using PZT vibration to generate the shear thinning behavior of alginate and decrease the viscosity of bio-ink has been developed. The decline of the viscosity led to the decrement of the well shear stress in the printing nozzle. This method can be the solution to reduce the effect of the shear stress and increase the cell viability in bio-printing process.

ACKNOWLEDGEMENTS: This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and future Planning (NRF- 2015R1A2A1A15055305) and also was partially supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI15C3000).

A tailored three-dimensionally printable agarose-collagen blend allows encapsulation, spreading, and attachment of human umbilical artery smooth muscle cells

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INTRODUCTION: In recent years, novel biofabrication technologies have enabled the rapid manufacture of hydrogel-cell suspensions into tissue-imitating constructs. The development of novel materials for biofabrication still remains a challenge due to a gap between contradicting requirements such as three-dimensional printability and optimal cytocompatibility [1]. We hypothesize that blending of different hydrogels could lead to a novel material with favourable biological and printing properties. In this study, we combined agarose and type I collagen in order to develop a hydrogel blend capable of long-term cell encapsulation of human umbilical artery smooth muscle cells (HUASMCs) and 3D drop-on-demand printing.

METHODS: Different blends were prepared with 0.25, 0.5, 0.75, and 1.5 % agarose and 0.2 % type I collagen. The cell morphology of HUASMCs and the printing accuracy were assessed for each agarose-collagen combination, keeping the content of collagen constant. The hydrogel blend which displayed sufficient cell spreading and printing accuracy was then characterized based on swelling and degradation over 21 days and mechanical stiffness. The cellular response regarding cell attachment of HUASMCs embedded in the hydrogel blend was further studied using SEM, TEM, and TPLSM. Printing trials were fabricated in a drop-on-demand printing process using cooled perfluorocarbon as supporting liquid [2].

RESULTS: The hydrogel blend of 0.5 % agarose and 0.2 % type I collagen (AGR0.5COLL0.2) turned out to support both cell spreading and sufficient printing accuracy. The swelling and degradation evaluation of this blend showed an average of 20 % mass loss and less than 10 % swelling. AGR0.5COLL0.2 exhibited significant increase in stiffness, strength and breaking stress under compressions compared to pure agarose and type I collagen. In addition, columns of AGR0.5COLL0.2 three centimeters in height were successfully printed submerged in cooled perfluorocarbon, proving the intrinsic printability of the hydrogel blend.

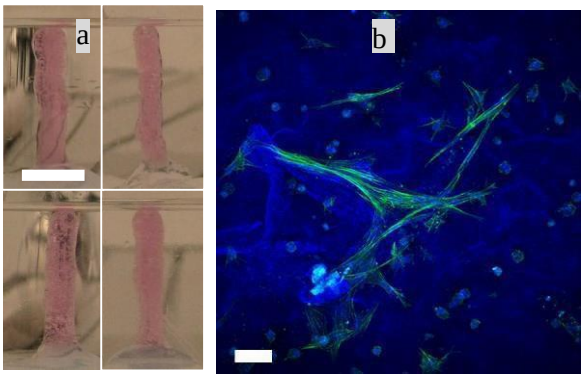

Fig. 1: By submerged printing a blend of agarose and type I collagen, columns of two to three centimeters in height were obtained which confirmed the intrinsic printability of the blend. Scale: 10 mm (a). Spreading HUASMCs encapsulated in the hydrogel blend were detected by TPLSM. Samples were stained for actin and collagen. Scale: 50 µm (b).

DISCUSSION & CONCLUSIONS: This study presents a three-dimensionally printable hydrogel blend of agarose and collagen which supports cell spreading and attachment of HUASMCs. This promising novel hydrogel blend could, for example, serve in the manufacture of *in vitro* 3D models. Based on these initial results, further studies should elucidate, if the hydrogel blend of agarose and collagen supports angiogenesis.

ACKNOWLEDGEMENTS: We gratefully acknowledge the financial support of the Deutsche Forschungsgemeinschaft DFG (grant FI 975/23-1).

polylactide short sub-micron fibers as reinforcement factor for bioprinted-laden chondrocytes alginate construct for cartilage regeneration

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INTRODUCTION: One promising tissue engineering approach for articular cartilage repair is the use of cell-encapsulated three-dimensional hydrogel scaffold to fill the defect. Hydrogels are attractive for cartilage repair as they are highly hydrated mechanically supportive three-dimensional environment that is often pro-chondrogenic. The objective of this research was to show the reinforcing effect of short sub-micron fibers in 3D printed hydrogel construct.

METHODS: Scaffolds for biomedical purpose must be mechanically sufficient with porous three dimensional architectures for efficiency cell proliferation. 3D bioprinting technique was used to fabricate regular, porous construction. Inspiration from the natural reinforced fibers occurred in cartilage prompted us to used short sub-micron fibers to enhance mechanical properties of hydrogel. Sub-micron fibers have been obtained from XanoShear technique from Xanofi. In this study, we evaluated the cartilage forming potential of different composites of alginate/short sub-micron fiber- polylactide (PLA) scaffolds when embedded human chondrocytes and cultured *in vitro* up to 14 days. The scaffolds were analyzed by scanning electron microscopy (SEM), mechanical testing and swelling study. For in vitro study: SEM, live/dead and immunofluorescent stainings were performed.

RESULTS: The fiber reinforced hydrogels exhibited four times higher compression modulus than pure alginate hydrogels. Investigation swelling degree of hydrogels indicates that the pure alginate absorbs huge amount of water, meantime addition of short sub-micron fibers decreases up to 75% swelling degree in case of addition PLA short sub-micron fibers. Scanning Electron Images as well as Live/Dead tests confirmed that PLA/alginate hybrid construct was proper solution for keeping the chondrocytes inside three-dimensional environment (Fig.1).

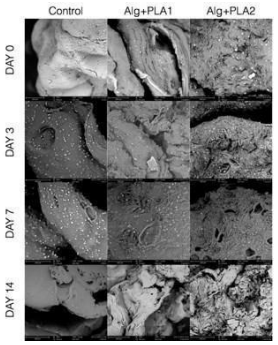

Fig.1. SEM images of microstructures with human chondrocytes cells for Control: 3.5wt.%alginate, Alg+PLA1: 3.5wt.%alginate+1%PLA and Alg+PLA2: 2.5%alginate+2%PLA. Scale bars 200 μm.

DISCUSSION & CONCLUSIONS: A composite hydrogel system reinforced with PLA short sub-micron fibers was successfully developed. Alginate reinforced with short PLA sub-micron fibers showed improved mechanical properties and construct stability as well as ell proliferation after 14 days' culture. Chondrocytes encapsulated in all gels retain their round morphology indicating that the fiber reinforced hydrogel system is suitable substrate for cell carriers and for mechanically supporting tissue structures for regeneration.

ACKNOWLEDGEMENTS: This work was financed by National Centre for Research and Development on the basic of a decision Pol-Nor/202132/68/2013.

Development of three-dimensional printable hydrogel using hyaluronic acid/alginate mixture bioink Su

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INTRODUCTION: Numerous research groups have recently developed three-dimensional (3D) bioprinting techniques for tissue engineering and regenerative medicine. This 3D bioprinting technology can produce scaffolds without using solvents and can generate scaffolds with completely designed and controlled pore structures. The bioink used in 3D bioprinting is usually a combination of synthetic and natural materials.[1] However, synthetic materials have several limitations, such as low biocompatibility and long-term biodegradation in the body. Moreover, only a few materials can be used for printing thermoplastics, such as polycaprolactone, poly-L-lactide and poly-L-lactide-co-polyglycolide. Therefore, numerous studies have developed bioinks using natural materials. In this study, we prepared solutions with various ratios of hyaluronic acid (HA) and alginate without the chemical crosslinking agents of HA. We then evaluated the properties of these bioinks for their suitability in bioprinting. We specifically analysed their mechanical properties, such as viscosity, printability andbiocompatibility.

METHODS: HA of 2,500 kDa was supplied from Humedix. Co. (Korea). Alginate was purchased from Sigma-Aldrich. HA and alginate were dissolved in high glucose-Dulbecco's Modified Eagle's Medium with 1% penicillin/streptomycin and physically mixed in various ratios (w/v) with final concentrations of 2% (w/v) and 3% (w/v), respectively. The bioinks were prepared with six different HA/alginate (HA/A) volume ratios (0:100, 10:90, 20:80, 30:70, 40:60 and 50:50 and 50:50 without the alginate crosslinking agent, 1% $CaCl_2$). To test their viscosity, approximately 8 ml of each HA/A bioink was analysed three times by Kinexus (Malvern Instruments Ltd, United Kingdom) at 25°C. This study used a 3D bioprinting system (laboratory made system in KIMM). To assess printability and shrinkage behaviour, the bioink printed structures were measured for size variation before and after treatment with $CaCl_2$. To assess the biocompatibility of the inks, bioink/cell (NIH3T3 fibroblast cell line) printed structures were measured by live/dead assay and WST-1.

RESULTS: Although each group exhibited a similar pattern, the groups including HA were observed to have an injectable nature. Examination of the changes in size before and after the treatment of printed structures with $CaCl_2$ revealed that a higher content of HA led to less contraction owing to the crosslinking agent, and the structures maintained their size immediately after the printing step. In addition, based on the cell viability on the 1st and 4th days after the mixed printing of cells, the groups containing HA had higher cell proliferation than the groups including only alginate. However, the degradation analysis of the groups with different HA/A ratios of 20:80, 30:70 and 40:60 was restricted because the hydrogel loosened as the culture proceeded.

DISCUSSION & CONCLUSIONS: Alginate hydrogel has been extensively used as a bioink for 3D bioprinting. However, most previous studies have not mentioned the extent of reduction of printed structures because of treatment with a crosslinking agent.[2] This is a major weakness in 3D printing technique for production of tailored structures. These results suggest that HA/A hydrogel could be used to increase the printability of 3D bioprinting for tissue engineering.

ACKNOWLEDGEMENTS: This study was supported by a grant from the Korean Health Technology R&D Project, Ministry of Health & Welfare (HI13C1527) and Technology Innovation Program (10053020) funded by the Ministry of Trade, Industry & Energy (MI), Republic of Korea.

Bioactive grid-like hydrogel produced by bioplotting

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INTRODUCTION: Biofabrication provides free shape-designing and a precise positioning of materials and cells, enabling a close mimicking of the living tissues[1]. Hydrogels based on natural polymers becomes promising inks as they can be processed using cell-friendly encapsulation parameters such as mild temperature and shear forces, and they resemble the structure of the extracellular matrix[2]. The alginate-dialdehyde-gelatin hydrogel (ADA-GEL) has been bioplotted and showed to promote adhesion, proliferation, and migration of encapsulated cells[3]. Still, ADA-GEL lacks bioactive behaviour, which limits its application in the orthopaedic field. Bioactive glass nanoparticles (BGNPs) have been integrated into various biopolymers to promote a calcium phosphate layer deposition, which enhances osseointegration[4]. This work proposed to biofabricate bioactive hydrogels using BGNPs and ADA-GEL, blending the advantages of the polymeric and the inorganic constituents.

METHODS: ADA-GEL (5% (w/v)) with different BGNPs concentrations (0.1% and 0.5% (w/v)) were bioplotted to obtain a grid-like hydrogel construct. The BGNPs (SiO_2-CaO-P_2O_5 (mol.%) = 55:40:5) were obtained from the sol-gel route. Bioactivity tests were performed on simulated body fluid (SBF) until 7 days. Ibuprofen was incorporated in the hydrogel as a model molecule for drug delivery. Osteoblast-like MG-63 cells were encapsulated in the hydrogel, and their viability was assessed after 24h by live-dead assay.

RESULTS: EDS analysis shows that the BGNPs loading promoted the deposition of a bone-like apatite layer on the hydrogel surface. Moreover, the loading efficiency of ibuprofen into the hydrogel was found to be high and the drug released until 72 h. The biofabrication process also allowed the successful incorporation of MG-63 cells into the composite material. Cells distributed homogeneously within the hydrogels, and no difference was found in cell viability between ADA-GEL and the ADA-GEL-BGNPs, proving that adding BGNPs did not influence cell viability.

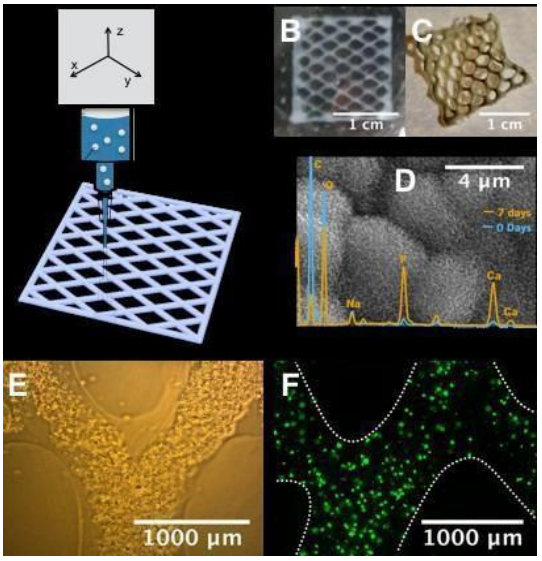

Fig. 1: (A) Model of the grid-like hydrogel. (B) and (C) depict the fabricated constructs of ADA-GEL-BGNPs in hydrated and dried forms, respectively. (D) EDS and SEM micrograph of a hydrogel soaked in SBF for 7 days. (E) Phase-contrast and (F) fluorescent microscopy images of the encapsulated MG-63 cells (green shows living cells and red shows dead cells).

DISCUSSION & CONCLUSIONS: The bioplotted hydrogel successfully induced the formation of an apatite bone-like layer and could sustainably release a drug model. The hydrogels and the biofabrication technique did not exhibit any negative effect on cell viability. Overall, the ADA-GEL-BGNPs hydrogel showed potential for bone tissue engineering applications.

ACKNOWLEDGEMENTS: Portuguese Foundation for Science and Technology (SFRH/BD/73174/2010), POPH/FSE program from QREN.

Polymer processing for 3D printing of tissue engineered heart valve scaffolds

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INTRODUCTION: Polylactid-co-glycolides (PLGA) in different percentual compositions were selected for 3D printing relating to their promising suitability for heart valve tissue engineering. Using an appropriate additive manufacturing process, i.e. 3D printing for selective laser sintering, custom-made scaffolds are to be fabricated for further seeding with vascular cells from human umbilical cords. PLGA compositions were processed by two different methods to evaluate the most suitable one and were analyzed for their suitability as potential scaffold materials.

METHODS: Currently, all primary materials are available only as granules which are not suitable for 3D printing due to their large particle size. Special grinding procedures in a cryogenic mill provide one possible method to generate the powder-like structure. Fabrication of polymeric microspheres is an alternative, promising method, but needs large amounts of toxic solvents. Molecular weight was analyzed before and after processing to elucidate the influence of the processing methods on the materials. Scaffold samples were produced using a custom-made contactless 3D structured light scanner and were analyzed for their cell-seeding capabilities. Vascular cells from human umbilical cords were expanded in a rotating bed bioreactor and were subsequently seeded onto the surface of the 3D printed PLGA. Longterm studies with regard to the pH and degradation as well as µCT analysis were performed before and after cell seeding.

RESULTS: Both processing methods are very extensive procedures resulting in a mostly flaked, rough shape after grinding in a cryogenic mill and in satisfying amounts of round microspheres using high amounts of solvents. Cytotoxicity assays showed low toxic influence of potential solvent residues on the cells compared to the medium. Molecular weight after the processing is similar to the molecular weight before processing. Polymeric flakes as well as microspheres were used to fabricate scaffolds by selective laser sintering. Human vascular cells attached to the scaffold surface with migration into the inner structure of the polymeric samples. Degradation and in part swelling were observed in unseeded samples. µCT analysis showed fusion and generation of sintering bridges.

DISCUSSION & CONCLUSION: Since appropriate powder-like resorbable polymeric materials for 3D printing of heart valve scaffolds are not available on the market, special processes such as grinding and fabrication of microspheres had to be evaluated. This study demonstrates the general suitability of PLGA for 3D printing and tissue engineering after extensive processing. For future experiments much effort needs to be devoted to establish the optimal material processing procedure with the aim of fabricating an adequate human heart valve scaffold.

Apatite-wollastonite scaffolds: a guide for osteogenic hMSCs differentiation

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INTRODUCTION: Apatite-wollastonite (AW) glass-ceramic has been considered for long time an attractive material for bone tissue repair and regeneration, due to its demonstrated bioactivity, biocompatibility and osteoconductive properties [1]. Additionally, more recently the possibility to produce custom–built microporous AW structures, which support the attachment, proliferation and differentiation of human mesenchymal stem cells (hMSCs) have been investigated [2]. Hence, this research study aimed to develop AW scaffolds with more complex architecture and assess their role in leading hMSCs differentiation.

METHODS: AW glass-ceramic, prepared according to Kokubo formulation[1], was processed to green parts using a Z Corp Z310plus 3D printer, and according to a novel designed architecture (*Fig. 1*). Subsequently, to consolidate the 3D printed structures, a sintering treatment up to 1150ºC was performed. The microstructure as well as the mechanical properties of the scaffolds were evaluated. hMSCs were harvested from femoral head bone biopsies taken from osteoarthritis patients. After *in vitro* expansion, hMSCs were seeded onto scaffolds and cultured for up to 14 days in either expansion media or osteogenic induction media. Fluorescent microscopy and SEM were used to assess the morphology of cells on the scaffolds. Additionally, osteogenesis was quantified using qRT-PCR and an ALP activity assay.

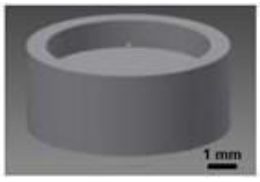

Fig. 1: CAD design of the novel developed AW geometry

RESULTS: Porous AW scaffolds were manufactured with a homogeneous interconnected 3D architecture. The bending strength of the sintered parts was found equal to 25.68 ± 2.5 (MPa). As expected, the cells seeded onto the AW scaffold had a more elongated morphology (*Fig. 2*) compared to those cultured on tissue culture plastic. The results for qRT-PCR showed that the osteogenicly induced scaffold seeded cell samples had higher levels of ALP RNA expression (18.89 ± 1.66 ΔCT) compared to the osteogenicly induced cells on tissue culture plastic (4.3 ± 0.56 ΔCT). Similarly, osteogenicly induced scaffold seeded MSCs had more ALP activity (1362.51 ± 191.76 pNNP µg/ml / hour) compared to the osteogenicly induced cells on tissue culture plastic (740.72 ± 72.79 pNNP µg/ml / hour) as reported in Fig.3.

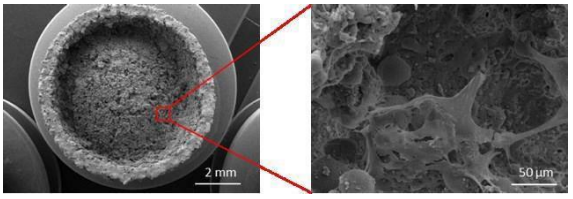

Fig. 2: SEM images showing the elongated morphology of hMSCs seeded on AW scaffold

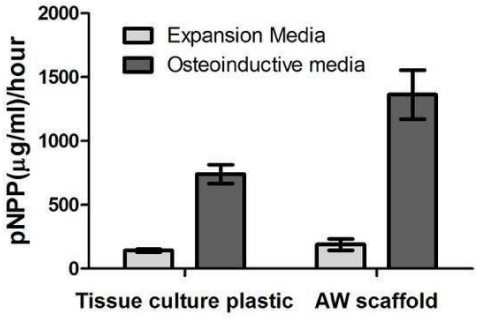

Fig. 3: ALP activity results of osteogenicly induced scaffold seeded hMSCs in comparison to osteogenicly induced cells on tissue culture plastic

DISCUSSION & CONCLUSIONS: This work shows the effectiveness of 3D printing technology to manufacture custom-design scaffolds. Additionally, it demonstrates that the osteogenic potential of hMSCs seeded onto the new designed AW scaffolds is stronger than hMSCs seeded onto tissue culture plastic.

1

ACKNOWLEDGEMENTS: The research has been supported by the Arthritis Research UK Tissue Engineering Centre.

*Authors contributed equally and should be considered co-first author.

3D bioprinted core-shell cellular fibres

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INTRODUCTION: Cellular fibres represent an innovative approach to the formation of 3D tissues in the field of regenerative medicine [1]. They resemble many native tissues such as tendons, nerves, muscles and blood vessels. Fabricated fibres must: 1) provide an extracellular-matrix-like environment to support cell growth, 2) be mechanically robust to withstand forces during *in vitro* manipulation and after implantation, and, 3) be spatially manipulatable to produce desired 3D tissue-like structures. In this study, we demonstrate a method to 3D print cellular core-shell fibres which are both mechanically robust and supportive for cell survival and functions.

METHODS: The fibres were printed into 3D structures using a commercial 3D printer modified with a custom-built co-axial needle for core-shell fibres extrusion. We have used a hybrid shell material consisting of alginate and poly(ethylene glycol) diacrylate (PEGDA). ECM-like materials were used to form the core. Tensile testing was performed to characterise the tensile properties of the shell material. Cell viability and function within the fibres were characterised. Furthermore, the release profiles of proteins and peptides from these fibres were examined.

RESULTS: Addition of PEGDA to alginate synergistically improved the mechanical properties of the hybrid hydrogel in a composition-dependent manner (Figure 1). We have also demonstrated the printability of the fibres into complex 3D structures with different core materials (Figure 2). Both cell lines and primary cells were encapsulated within the core of the fibres and demonstrated sustained cell viability for two weeks (Figure 3). The cells were also able to proliferate, indicating core stability and that the shell allowed sufficient nutrient diffusion.

CONCLUSIONS: We have 3D printed complex structures from core-shell fibres and shown compatibility with cells. Furthermore, we have demonstrated improved elasticity and strength of the hybrid shell material.

ACKNOWLEDGEMENTS: We would like to thank the EPSRC & MRC Centre for Doctoral Training in Regenerative Medicine for supporting Pritesh's PhD studentship. We also thank EU CASCADE fellowship scheme for sponsoring Ahmed's fellowship.

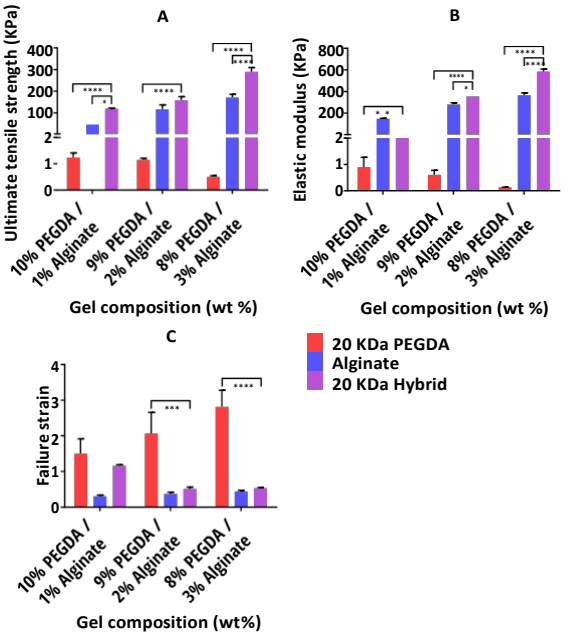

Figure 1: Mechanical properties of PEGDA, alginate and hybrid hydrogels showing tensile strengths (A), elastic moduli (B) and failure strains (C).

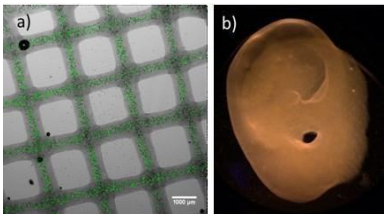

Figure 2: Core-shell fibres printed into 3D structures. A) A lattice with a core of 3T3 fibroblasts. B) An ear shape. Scale = 1 mm.

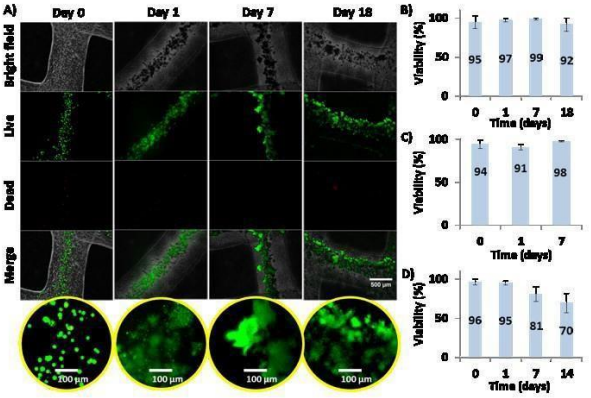

Figure 3: Cell viabilities in the core of printed fibres. A&B) Mouse embryonic stem cells in collagen. C) 3T3 mouse fibroblasts in methacrylated gelatine. D) Primary human mesenchymal stem cells in collagen.

Preclinical evaluation of 3D Bioprinted constructs with human chondrocytes and human bone marrow derived mesenchymal stem cells

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INTRODUCTION: Cartilage is an avascular and aneural tissue which has very limited ability for repair. Although several groups have attempted to engineer cartilage for clinical applications, no cartilage repair of larger defects is clinically available today. 3D bioprinting technology, with its ability to reproduce tissue on a cellular level and precisely control cell density and cell positioning, has a great potential to pave the path to bring cartilage tissue engineering to the clinic. Here we report, to our knowledge, the first successful *in vivo* cartilage tissue engineering utilizing 3D bioprinting.

METHODS: In this pilot study cell-laden nanofibrillated cellulose/alginate hydrogel (NFC-A) constructs were extrusion-bioprinted. Cell types used were human nasal chondrocytes (hNC; from a single male donor) and human bone marrow derived mesenchymal stem cells (hMSC; from a single female donor). Four different experimental groups were used: NFC-A w/o cells; NFC-A + hNC; NFC-A + hMSC; NFC-A + mixture of 20% hNC/80% hMSC. Lattice-structured constructs (5x5x1 mm) were implanted subcutaneously on the dorsal side of 7-week old female nude mice. Animals were sacrificed after 14, 30, and 60 days post-implantation.

RESULTS: First histological results from the hNC group as well as the hNC/hMSC group show a progression in formation of glycosaminoglycans (GAG) and thus chondrogenesis from Day 0 to Day 60. Also, cells are proliferating in these two groups, as indicated by the formation of cell clusters (Figure 1). However, formation of cell clusters was much more pronounced in the hNC/hMSC group than in the group with hNCs alone. Fluorescence *in situ* hybridization (FISH) for human chromosomes X and Y showed that it is the chondrocytes that proliferate and produce GAGs in the hydrogel, not the mesenchymal stem cells. The stability of the implanted grids was assessed by manual handling. Even after 60 days post-implantation, the surgeon was able to handle the constructs well and without special caution.

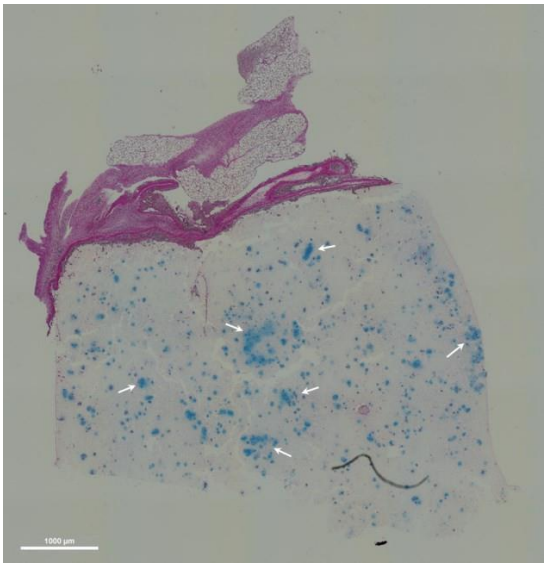

Fig. 1: Microscopic image of a histological section stained with Alcian Blue van Gieson (Magnification 4x). The image shows a printed hNC/hMSC construct after 60 days of implantation. GAG production (blue) and cell cluster formation (indicated by arrows) are observed.

CONCLUSION: This pilot study showed for the first time the *in vivo* chondrogenesis in 3D bioprinted human cell-laden constructs.
The promising results from this pilot study led us to conduct a consecutive larger study aimed to verify results from the pilot and to bring cartilage tissue engineering closer to clinical application.

ACKNOWLEDGEMENTS: This research was supported by Västra Götalandsregionen, Project 21250071 - 3D Bioprinting Center

DISCLOSURE: The authors declare no conflict of interest.

Alginate-sulfate nanocellulose bioinks for cartilagebioprinting application

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INTRODUCTION: The lack of available materials for biopritnting applications is currently what is holding this promising technology back. A good bioink should not only be biologically active but also have the proper rheological properties. A combination of materials is often superior over single materials and necessary to fulfil both the biological and rheological requirements. Here we present a combination of the biologically active biopolymer alginate sulfate, which was shown to be chondrogenic and induce cell proliferation and spreading [1], and the viscosity modifying agent nanocellulose (NC).

METHODS: Rheological measurements were performed to assess the flow behaviour and gelation properties of alginate sulfate when mixed with NC. Bovine chondrocytes were encapsulated in alginate and alginate sulfate with or without NC and the viability and cell morphology was investigated at day 1, 14 and 28. Cells were also printed using the alginate sulfate-NC bioink with different needle diameters and shapes and cell viability and morphology were evaluated. COMSOL simulations were performed to see if differences between the printed and the encapsulated cells arise from differences in maximum and average shear stresses occurring in the different printing geometries.

RESULTS: Rheological measurements revealed that, despite the lower viscosity of alginate sulfate solutions compared to unmodified alginate, the flow properties of the final bioink with NC were only slightly influenced. Mechanical properties of alginate sulfate-NC were equal to alginate NC after increasing the alginate sulfate concentration in the bioink. Cells showed a round morphology at every time point in the alginate and alginate-NC gels, whereas in the alginate sulfate and alginate sulfate-NC samples the cells started spreading after 14 days in culture. Only when the printing geometry was optimized was the ability of cells in alginate sulphate to spread and proliferate maintained. Simulations revealed differences in the average and maximum shear stress between conical and straight needles of different diameters which might explain the differences in the cells ability to spread or not in the printed samples.

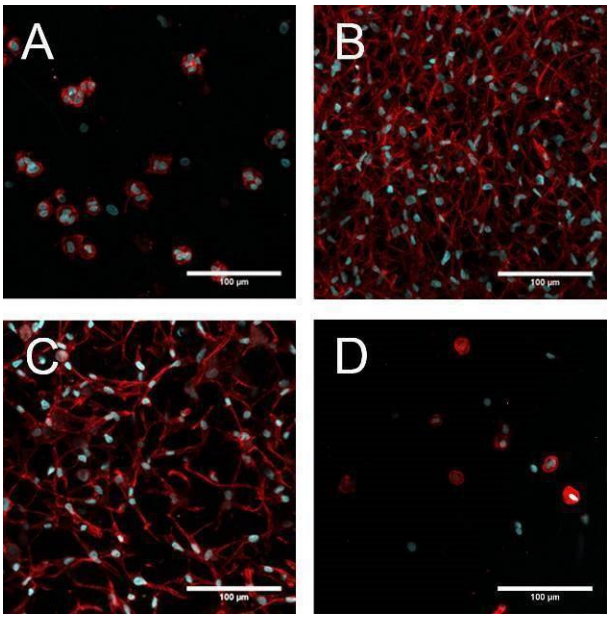

Fig. 1: Chondrocyte morphology at day 28 encapsulated in alginate-NC (A) or alginate sulfate-NC (B) or printed with alginate sulfate-NC with a straight 413 µm (C) or 159 µm (D) needle. Scale Bar is 100 µm.

DISCUSSION & CONCLUSIONS: Alginate sulfate maintained its mitogenic properties and effects on cell spreading in the presence of NC. The NC addition made alginate sulfate a printable material, but restrictions remain in regards to usable inner needle diameters and shapes for cells to survive and show the spreading behaviour.

ACKNOWLEDGEMENTS: This work was supported by ETH Research Grant ETH-23 14-1, FIFA/F-MARC and EU program Eureka and Vinnova.

In situ polymer-ceramic hybrid hydrogel for 3D printing applications

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INTRODUCTION: *In situ* hybrid hydrogel composed of polymers and ceramics would be one of choices in fabricating an ideal scaffold in 3D printing for engineering of both hard and soft tissues due to its advantageous properties such as outstanding mechanical properties from ceramics, delivery of bioactive molecules from mostly polymeric hydrogels as well as 3D printability. Polymer-ceramic hybrid hydrogel has been expected to be a scaffold for bone tissue engineering. While hyaluronic acid(HA), poly(ethylene glycol) have been employed as hydrophilic polymers for fabrications of hydrogel, hydroxyl apatite and tricalcium phosphate have been employed for bone tissue engineering. *In situ* hybrid hydrogel would be better in control of 3D printability of bone structures. We here report our system of 3D printer and then fabricated 3D polymer-ceramic scaffolds by using *in situ* hydrogels of polymers and ceramics.

METHODS: We built a 3D printer in house consisting of 5 channels for printing of hydrogel, biodegradable polymers and ceramics. The printing system composed of a rotary system of mechanical, drop-on-demand and inkjet. Printings of hybrid hydrogel composed of ceramics and HA were controlled by using the nozzles either with or without heating system. The *in situ* hybrid hydrogel was obtained by employing click chemistries for hydrogels of both polymers and ceramics. The polymeric *in situ* hydrogel was fabricated by using click chemistry between HA-phosphine and hyaluronic acid-acrylate. The *in situ* ceramic gel was obtained by mixing both tricalcium phosphate and citrate buffer. After mixing the solutions of polymers and ceramic compounds, it was moved to a syringe and then printed by a 3D printer in lattice forms of several layers. The hybrid scaffolds obtained were evaluated by diverse methods of physicochemical and *in vitro* biological assays. Extensive *in vitro* and *in vivo* evaluations were performed in our previous studies.

RESULTS: Printing the solutions of polymers and ceramics in buffer turned spontaneously into a hybrid scaffold in layers of lattices. Encapsulation of cells in the polymeric gel was possible and they were viable. The scaffolds, 1 cm x 1 cm, showed hybrid biomaterials of polymers and ceramics in 100 to 200 μm wall thickness as observed by SEM. While the ceramic column supported mechanically the scaffolds, the polymer hydrogel was located between the ceramic layers. The polymer gel showed encapsulation of cells and bioactive molecules as well as their controlled release. The cells in the hybrid scaffolds were viable. Further i*n vitro* cell culture of printed hybrid scaffold cell culture is under progress.

DISCUSSION & CONCLUSIONS: The hybrid gel was 3D printable in combined columns of polymers and ceramics. The *in situ* polymer gel showed encapsulations of cells and bioactive molecule. The 3D printed hybrid gel scaffolds could be applied as a scaffold for bone tissue engineering.

ACKNOWLEDGEMENTS:

This work was supported by the National Research Foundation of Korea (NRF) Grant (2014K2A2A7060928 & 2015R1A2A1A10054592).

Natural ECM bioink for the 3D printing of tissue shaped structure

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INTRODUCTION: The 3D bioprinting is used for manufacturing of scaffold which is customized patient-specific. However, it is difficult to find an available bioink for cell included 3D bioprinting. The hydrogel is found be biocompatible materials because of high content of water. The alginate which was regarded as representative example of hydrogels should not high laminating because of low mechanical property. Thus, the backbone should be there to support of hydrogel for cell printing. Synthetic polymer is mostly used for scaffold as backbone like a PCL material. However there are limitations that PCL is lower biodegradability than natural polymers. It is have long time to clearly biodegrade *in vivo.* On the other hand, natural materials are cell friendly and biodegradable and then can be more alternative with be generated ECM (extracellular matrix). Thus, this study is trying to develop the natural polymer bioink for the cell and material dual printing.

METHODS: As main backbone materials to support the alginate hydrogel, tissue derived ECM and silk fibroin composite material was utilized in this study. Experimental 3D printing groups were divided by the alginate only (group1), PCL/alginate (group 2) and ECM-silk/alginate (group 3). The stability and degradability of printed 3D structure and cell viability were evaluated in this study to verify the superiority of ECM-silk based natural bioink.

RESULTS: For the degradation test, all groups were incubated in 12.5 U/ml collagenase solutions and PBS. In the results, Group 1 did not maintain the original structure even in PBS. Printed alginate structure was swollen and then was completely dissolved in the end. Group 2 was also showed the swollen alginate layer between PCL layers. However, Group3 only appeared the well maintained alginate layer in the 3D structure. The volume change of hydrogel has been measured by uCT. Analysis of uCT confirmed the similar degradation tendency like gross observations. In addition, ECM-silk bioink showed the good printing resolution to imitate the specific tissue shapes for customized scaffold fabrication.

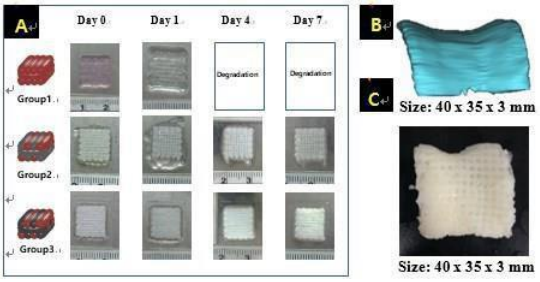

Fig. 1: (A), Degradation test of printed structures. (B), 3D image of patient damaged tissue. (C), 3D printed ECM-silk scaffold mimicked the tissue shape.

DISCUSSION & CONCLUSIONS: This suggested the ECM based natural bioink to fabricate the 3D structure which could support the maintaining of cell delivery hydrogel. In the future, this kind of natural bioink would be utilized for the implantable 3D organ fabrication.

ACKNOWLEDGEMENTS: This research was supported by the NRF-2013R1A1A1011040 project and Tech. development for disease overcome (HI14C2143) project by Ministry of Health and Welfare.

Clinical application in facial bone reconstruction: 3D printed patient specific absorbable implant

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INTRODUCTION: Management of significant facial bone defects or asymmetry are challenging. Conventional options include autogenous bone grafts or alloplastic implants. However bone grafts are limited by donor morbidity, unpredictable results and long operation time. Alloplastic implants are mainly non-absorbable and may cause foreign body reaction. We present the application of a 3D-printed patient specific absorbable mesh implant for reconstruction of bone defect caused by tumor resection.

METHODS: A 3D skull model and patient specific absorbable mesh implant using polycaprolactone(PCL) was produced based on CT images. Implants were designed with CAD soft ware and made by 3D printer with fused deposition mode.

(case 1) An 18-year-old male presented with facial asymmetry. He had undergone right medial maxillectomy and septectomy for chondrosarcoma of his right nasal cavity 8 years prior. Computed topographic(CT) findings revealed enophthalmos of the right orbit and bone defects involving the right medial orbital cavity, infraorbital rim and pyriform aperture. PCL implant inserted through the lower eyelid incision which fitted through defect perfectly (Fig 1).

(case 2) A 44-year-old male presented with facial asymmetry. He had undergone left radical maxillectomy and partial mandibulectomy 7 years prior. CT findings revealed severe bone defect involving the orbit floor, zygoma and maxilla. PCL implant also inserted through partial weber-ferguson incision (Fig. 2)

RESULTS Facial asymmetry improved dramatically after surgery (Fig. 3). There were no wound problem like foreign body reaction or inflammation. Implant volume maintained well until now.

DISCUSSION & CONCLUSIONS: 3D printed patient specific absorbable mesh implants may be the answer to complex bone defects of the facial region. We hope the subperiosteal location of this implant will induce osteogenesis and bony replacement. Long term follow-up and additional studies are the next step.

Fig.1. (case 1) PCL implant inserted through the lower eyelid incision which fitted through defect perfectly

Fig.2. (case2) PCL implant also inserted through partial Weber-Ferguson incision.

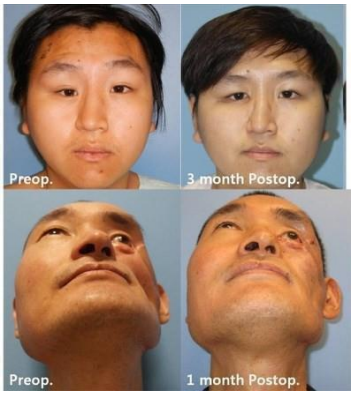

Fig.3. Facial asymmetry improved after surgery

ACKNOWLEDGEMENTS: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (No.2014K000163).

Effect of SCF and TPO on the bone marrow-like tissue

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INTRODUCTION: Because the bone marrow (BM) is located in the bone, it is very difficult to use analysis of the BM diseases and drug screening. If it is possible to reorganize the tissue have *in vivo*-like function and structure, it can be substituted for animal experiment. Because the BM tissue is almost composed of blood cells, it is difficult to aggregate or reconstruct with dispersed-state BM cells. In a previous study, we established the method to make a 3-dimensional (3D) aggregation state using methylcellulose (MC) medium [1], and we created 3D BM cells aggregates (BM-like tissue) from dispersed-state BM cells. Cells in the BM-like tissue could not maintain after 3 days in the previous method. In this study, we found the method to maintain the cells in the BM-like tissue after 5 daysculture.

METHODS: BM harvested from 8th-week-old C57BL/6NcrSlc mice of femurs and tibias. Hemolytic treatment was performed after the BM in the dispersion state by flushing using syringe and needle. The cell suspension was filtrated with a cell strainer, and collected cells by configuration. Using a micropipette, 1 µl of the medium containing 20000 BM cells were injected into the 3% MC medium and incubated in a CO_2 incubator. We use the Live/Dead assay to consider the cell viability in the BM-like tissues. We compared with normal MC medium and MC medium contained hematopoietic factor (stem cell factor (SCF) and thrombopoietin (TPO)).

RESULTS: The MC medium can make a 3D aggregation state rapidly regardless of adhesive property of the cells. When this method applied to dispersed-state BM cells, they gathered in 10 minutes and formed tissue-like spheroids within 24h. BM-like tissue at day 7 supplements with SCF and TPO were sustained the cells. Live/Dead assay showed cell expansion in the MC medium with hematopoietic factors.

DISCUSSION & CONCLUSIONS: BM-like tissue culture conditions contained hematopoietic factors were possible to maintain and expand the cells compared with control conditions, and expand the cells.

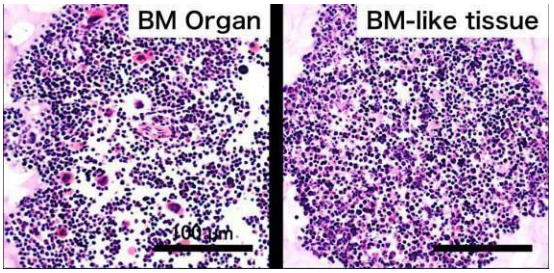

Fig. 1: Reconstruction of the 3D BM-like tissue from dispersed-state BM cells – HE staining of BM organ (left) and BM-like tissue of day 1 (right). All scale bars are 100 µm.

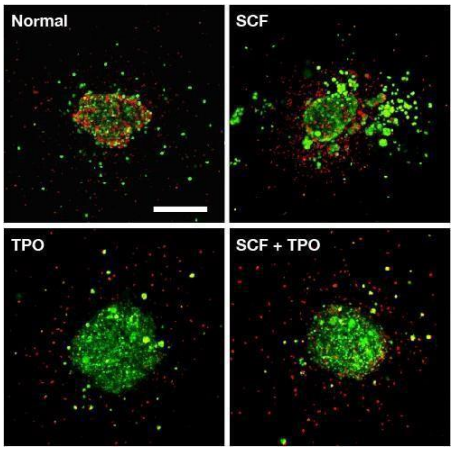

Fig. 2: Live/Dead assay images of day 7 BM-like tissues. Cells in the BM-like tissues were expanded in the MC medium contained with SCF and TPO. Scale bar is 100 µm.

ACKNOWLEDGEMENTS: This work was partly supported by Grant-in-Aid for Scientific Research on Innovative Areas "BioAssembler" from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Effect of mechanical stimulation on osteogenesis of self-assembled collagen-cell seeded microspheres

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INTRODUCTION: Mechanical forces and 3D topological environment can be used to control differentiation of Mesenchymal Stem Cells (MSCs. However, the effects of physical and mechanical cues of the microenvironment on MSC fate determination have not yet been fully understood. This study investigates and compares the effect of mechanical stimulations on soft cellular microspheres when subjected to dynamic fluid compression. It is hypothesised that dynamic compressive strains influence cell morphology and expression of osteogenic cell marker heMSCs.

METHODS: Microspheres were produced by gelation of bovine collagen type I with concentrations of 2 mg/ml and 1000-2000 hES-MP cells per 5µl droplet. Two loading conditions were studied: (1) 10% constant loading was applied by a Bose biodynamic bioreactor for 15 and 40 min/day for 5 and 10 days; (2) 10% adjusted loading was applied using Bose biodynamic bioreactor for 15 and 40 min/day for 5 and 10 days. Cell viability and proliferation, alkaline phosphatase activity and mineralization were compared with controls.

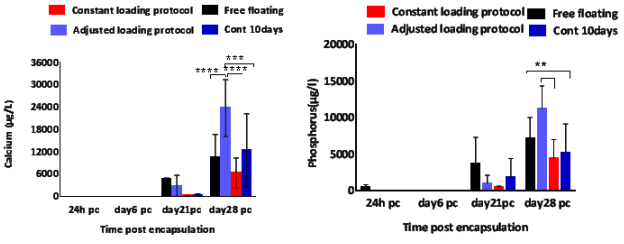

Fig. 2: Quantification of deposited calcium (left), phosphorous (middle), and zinc (right) in loaded samples versus controls over 28 days pc.

RESULTS: Viscoelastic properties and shear modulus of very soft collagen gel (0.2% of collagen/hydrogel) were measured as 0.005 and 0.02 kPa. Mechanical properties of PDMS (Young's modulus and Poisson's ratio) were calculated as 1000 kPa and 0.49 and were in line with the literature reports. Monitoring alkaline phosphatase level reported a significant increase in enzyme activity by day 14 in loaded samples of 40min/day loading protocol compared with other experimental conditions. Mineralisation was assessed by measuring calcium, phosphorous and zinc concentrations and intensity of H&E and alizarin red S staining and showed the highest minerals accumulation in the loaded samples on day 28 post en-capsulation (Fig. 2 and 3). Also, alignment of collagen fibres was observed in the adjusted loaded protocol samples on day 28pc.

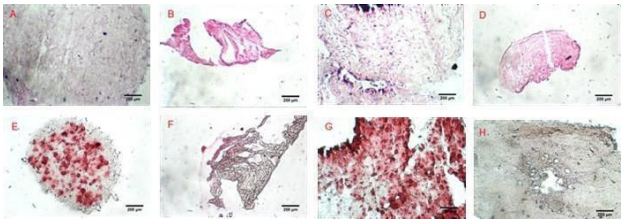

Fig. 1: H&E staining of microspheres cross sections on d28pc A, B: loaded samples Vs C, D: controls, ARS staining of samples cross sections d28 E, F: loaded Vs G, H: controls.

DISCUSSION & CONCLUSIONS: MSCs are widely implicated as a cell source for tissue engineering of skeletal tissue in cell based therapy. This study indicated that loading of very low cell number seeded on soft natural scaffold can encourage osteogenesis of cells by enhancing both early stage bone marker and mineralisation. Adjusted strain regime was superior in stimulating osteogenesis compared with other conditions. Self-assembled hES-MPs collagen microspheres present exceptional cell delivery model in bone healing and repair process and in addressing many inadequacies of the existing cell delivery approaches.

ACKNOWLEDGEMENTS: Financial support from the European Research Council (258321) is acknowledged.

DISCLOSURES: Include conflict of interest disclosure information here, or a statement that authors have nothing to disclose.

Bisphosphonate-functionalized hyaluronic acid hydrogel as three-dimensional printable bioink

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INTRODUCTION: Three-dimensional (3D) printing technology is promising for fabrication of engineered tissues and organs in regenerative medicine. A challenging task for 3D bioprinting is the design of printable and biocompatible materials, the "bioinks". Some natural polymers including alginate and gelatine have been developed as bioinks. However, some limitations of above inks including thermal-triggering gel formation and shape deformation hinder their further applications [1]. Hyaluronic acid (HA), a natural linear polysaccharide, was widely researched as a biomaterial due to its biocompatibility, biodegradability and non-immunogenicity. In this study, we modified HA with bisphosphonate (BP) groups to obtain a physical hydrogel, as a bioink, that can be printed both on a surface and into the same gel reservoir.

METHODS: HA was functionalized with BP groups using thiol-ene photoaddition reaction between thiolated hyaluronan and acrylated bisphosphonate following our previous publication [2]. A physical hydrogel with shear-thinning and self-healing properties was formed by mixing $CaCl_2$ and HA-BP solutions. Mechanical properties of the hydrogel were characterized by rheology. Different structures were printed using the physical hydrogel as a bioink, programming with software Cura 15.04.2. Photocurable analogue of HA-BP was also prepared, printed and then exposed to UV light to fix the printed construct. Cells viability was measured after C2C12 cells *in situ* encapsulation in the physical hydrogel and their subsequent 3D culture.

RESULTS: We observed formation of a physical hydrogel, which was attributed to the multiple coordination bonds between BP groups of HA-BP biopolymer and Ca^{2+} ions (Fig.1). Reversibility of the formed cross-links imparted sheer-thinning, self-healing and printable properties to the obtained network. Different structures both on surface of a glass side as well as inside a supporting gel medium were printed. The supporting gel was formed from HA-BP while the printed (extruded) one was formed from its photocurable analogue (bearing both BP and acrylamide groups). 3D encapsulation of cell in HA-BP hydrogel followed by 24 hours of culture resulted in 80% of the cells survival.

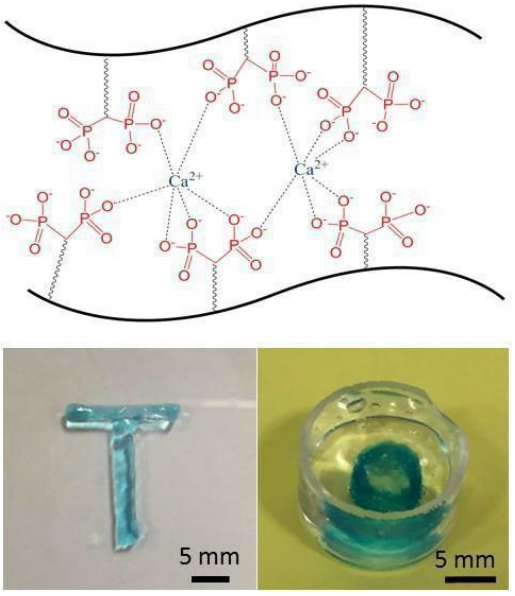

Fig. 1: Schematic presentation of coordination interactions between HA-BP and Ca^{2+}. Images of printed structures based on HA-BP•Ca^{2+} physical hydrogel: on glass slide surface (bottom left) and inside another HA-BP•Ca^{2+} gel (bottom right). Printed HA-BP contained alcian blue to contrast the printed structures.

DISCUSSION & CONCLUSIONS: New sheer-thinning and photocurable hydrogel was developed basing on hyaluronan-bisphosphonate derivative and exploiting coordination bonding between BP groups and Ca^{2+} ions. This physical hydrogel is promising as a bioink in construction of complex cell-laden hydrogels with complex and spatially defined structures.

ACKNOWLEDGEMENTS: The research has received funding from European Community's Seventh Framework Programme (BIODESIGN). L.S thanks the financial support from CSC.

In vivo implantation of novel photocurable PGSm 3D printed nerve guidance conduits for peripheral nerve repair

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INTRODUCTION: Three clinical approaches exist to aid peripheral nerve regeneration; suturing, autograft surgery or the use of nerve guide conduits (NGCs). NGCs are an attractive alternative to autografts, mainly because autografts lead to loss of function, pain and potential morbidity in the donor site. Both synthetic and natural material-based NGCs are commercially available. These products are often simple designs with material properties far stiffer than the native tissue. This leads to current NCGs being ineffective for anything other than very short nerve gaps.

To address this we have developed a novel methacrylated version of polyglycerol sebacate (PGSm). PGSm has mechanical properties similar to that of nerve, is biocompatible, biodegradable and can be structured by stereolithography. This allows for more complex designs of NGCs to be produced via CAD, maintaining the beneficial material properties of PGS, whilst avoiding some of the limitations (e.g. time for thermal curing, and the instability of the reagents in the similarly acrylated versions of the polymer[1].)

METHODS: PGS was synthesised through a polycondensation reaction of glycerol and sebacic acid. Methacrylate moieties were added to create, with the addition of a photoinitiator, a photocurable material. The material was characterized by GPC, FTIR, NMR, DSC, rheology, nanoindentation, tensile mechanical analysis, water contact angle measurements and suture tests. Several forms of PGSm were produced with a tuneable extent of methacrylation, to control over the material properties. Long term degradation studies and *in vitro* enzymatic degradation studies were performed on PGSm NGCs. Samples were evaluated *in vitro* using NG108-15 neuronal and primary Schwann cells. PGSm was then structured by stereolithography into NGCs (5mm long / 1.3mm internal diameter) and used to bridge a 3mm mouse common fibular nerve injury, with nerve graft as control.

RESULTS: Neuronal cell live dead analysis showed PGSm samples had a live cell percentage of 92% compared to glass controls (84%), confirming that PGSm samples were a good substrate for neuronal cell adhesion and had no neuronal toxicity. Through neurite analysis it was found that PGSm was able to support neuronal cell differentiation. Primary Schwann cell studies similarly confirmed PGSm as a good material for Schwann cell adhesion, and overall *in vitro* data indicated that PGSm was permissive for use as a novel NGC. *In vivo* mouse studies using Thy-1-YFP-H mice showed that over 3 weeks the nerve was able to fully regenerate from the proximal to distal stump [Fig.1]. Fluorescent axons from the mice were imaged and are currently being analysed.

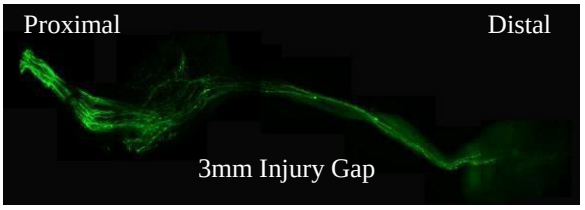

Fig. 1: Composite confocal image showing the regeneration of fluorescent axons through the PGSm NGC and across a 3mm injury gap over three weeks in an in vivo *Thy-1-YFP-H mouse.*

DISCUSSION & CONCLUSIONS: PGSm has tuneable material properties such as appropriate modulus values and degradation rates. It shows no neuronal toxicity, is a permissive substrate for differentiation and is easily structured using UV light-based stereolithography. This makes PGSm a good candidate biomaterial for peripheral nerve regeneration. Initial *in vivo* studies indicate nerve regeneration through 5mm NGCs to the distal stump of the nerve. Initial composite confocal images of the fluorescent nerve confirm that axons regenerate from the proximal stump, through the NGC and into the distal stump [Fig.1]. We are currently conducting detailed quantification of axon regeneration for comparison with that achieved following nerve graft repair.

Novel biocompatible two-photon initiator based on hyaluronan

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INTRODUCTION: Two-photon polymerization (2PP) technique enables free-form manufacturing with a resolution in the sub-micrometer range. It has been employed to produce precise 3D tissue engineering scaffolds.[1] 2D substrates used in traditional cell culture systems can lead to significant differences in structure, function or physiology compared to living tissue, whereas 3D scaffolds allow a more accurate reconstruction of the cells' natural environment. Especially attractive is the option of fabrication of hydrogels with embedded cells.[2] While specialized water-soluble two photon initiators (2PIs) have been developed,[3] there is still a demand for novel initiators with improved cytocompatibility. The present work aims at this via binding 2PI molecules to hyaluronan as a polymeric backbone, thus hindering diffusion through the cell membrane and restricting the 2PI to the extracellular matrix.

METHODS: An efficient 2PI chromophore based on dibenzylidene-cyclohexanone was prepared via aldol condensation and equipped with suitable amino linker groups in a 4-step synthesis. Subsequent covalent binding to hyaluronan via carbonyldiimidazole-mediated amidation lead to hyaluronic acid-bound photoinitiators (HAPIs) of varying chain lengths and degrees of substitution. Besides comprehensive characterization of HAPI and all intermediate compounds using various techniques (^{1}H- and ^{13}C-NMR, HR-MS, SEC, UV/Vis-spectroscopy and z-scan,...), the cyto-toxicity was evaluated and compared to 2PI references in a PrestoBlue assay. Finally, HAPI was tested in its designated application by 2PP cross-linking of methacrylated gelatin to encapsulate live MC3T3 cells, whose viability was assayed by live/dead-staining and laser scanning microscopy imaging.

RESULTS: Comparison of metabolic activity of MC3TC cells exposed to either HAPI or the water-soluble small-molecule 2PI references P2CK and E2CK[3] demonstrated a significantly reduced cytotoxicity for the polymer-bound 2PI. The first generation HAPIs based on 1.6 MDa hyaluronan showed acceptable water solubility on their own, but were incompatible with cross-linkable pre-polymers as precipitation occurred. Using degraded hyaluronan with a chain length of 56 kDa and methyl-β-cyclodextrin to aid solvation, 2PP cross-linked 3D hydrogel structures were successfully obtained. MC3T3 cells encapsulated in the gelatin-based hydrogel were kept viable for several days, and proliferated in the cavities of the structure (Fig. 1).

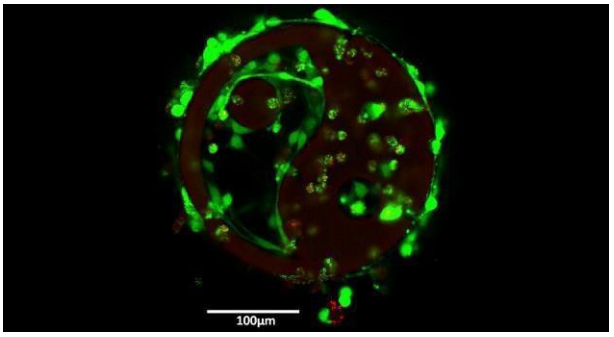

Fig. 1: Laser scanning microscope image of live/dead-stained MC3T3 cells encapsulated in 3D microfabricated hydrogel 5 days after structuring.

DISCUSSION & CONCLUSIONS: A 2PI precursor was developed and covalently linked to hyaluronan to hinder diffusion from the extracellular matrix into cells, thus limiting cytotoxicity and photodamage by the obtained HAPI. Cytotoxicity testing proved superior biocompatibility of HAPI compared to reference water-soluble 2PIs. 2PP of 3D hydrogel structures was achieved by cross-linking methacrylated gelatin with HAPI. MC3T3 cells were successfully encapsulated and viable cells were observed over a period of 5 days using a live/dead-staining assay, indicating a low phototoxicity of HAPI.

ACKNOWLEDGEMENTS: Financial support by Austrian Science Funds (FWF, Project P27555) and European Research Council (Starting Grant- 307701, A.O.) is kindly acknowledged.

Effect of 3D bioprinting on cell viability of human stem cells

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INTRODUCTION: 3D bioprinting technology has gained due attention in several tissue engineering strategies for its ability to spatially control the placement of cells, biomaterials and biological molecules. Consequently, it offers endless possibilities to the future of tissue and organ regeneration, basic research and drug screening. However, further development of hydrogel bioinks with good printability and bioactive properties that guide cellular fate processes is needed to facilitate the translation of this promising technology into the clinic. In this study we evaluated the biological functionality of a printable bioink, composed of nanofibrillated cellulose and alginate (NFC-A), in 3D culture.

METHODS: Lattice-structured constructs ($n \approx 120$) laden with human bone marrow-derived mesenchymal stem cells (hBM-MSC; RoosterBio Inc.) were 3D bioprinted using two bioprinting technologies, inkjet and pneumatic-driven extrusion, to evaluate cell viability during and post-bioprinting. The 3D bioprinted constructs were cultured after crosslinking (with 100 mM $CaCl_2$) for up to 14 days in growth medium (high performance media; RoosterBio Inc.). A bioink composed of 4% alginate and non-printed constructs served as controls.

RESULTS: Our results showed a decrease in cell viability by about 26% in the inkjet bioprinted groups with NFC-A and 4% alginate bioinks, compared to non-printed constructs. In contrast, the extrusion bioprinting method showed to be less harmful to the hMSCs, decreasing the cell viability by only 3.5% directly after bioprinting. During the *in vitro* culture period, cell viability of hMSCs was significantly higher in the extrusion bioprinted groups compared to the inkjet ones for both bioinks. Furthermore, cell morphology in both bioinks was similar for both printing methods. Cells mainly had a round morphology up until 7 days of culture. After 14 days, cell clusters were observed primarily in the NFC-A bioink and cell proliferation was observed *(Figure 1).* However, total DNA analysis showed a decrease in cell number for all of the groups after 7 days of culture.

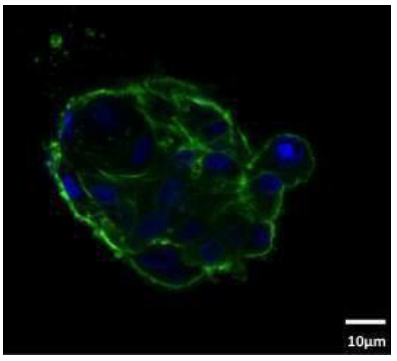

Fig. 1: Confocal microscopy on day 7 post-bioprinting of an inkjet printed MSC-laden NFC-A construct. To visualize the nuclei and the actin fibers, samples were stained with DAPI (blue) and Alexa Fluor™ 488 (green).

DISCUSSION & CONCLUSIONS: This is the first study demonstrating that NFC-A bioink supports viable hMSCs, while offering excellent printability in a biologically relevant 3D environment. We suppose that the increased shear stress on the cells generated in the inkjet technology, compared to the extrusion, is the main processing parameter responsible for the difference in cell viability in our study. In order to study the shear stresses on the cells during the bioprinting process, we aim to develop a biofluid dynamic model to simulate the different printing conditions. This model can help us to understand how printing conditions affect the cellular membrane and thus eventually how to optimize the bioprinting parameters to enhance cell viability. It is important to keep in mind that cell viability during bioprinting is also dependent on the cell type used. Our group is also working with human induced pluripotent stem cells (iPSCs), which express a different gene profile than MSCs, in order to establish cell viability-optimized bioprinting parameters for each cell type. One potential application of this technology is bioprinting of reproducible 3D models for stem cell research.

ACKNOWLEDGEMENTS: This research was supported by Västra Götalandsregionen, Project 21250071 - 3D Bioprinting Center

DISCLOSURE: H. Martínez-Ávila declares his affiliation with CELLINK

Molecular framework of multifunctional peptide: Towards corneal regenerative therapies

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INTRODUCTION: Due to very inadequate efficiency of the standing anti-infective agents for the treatment of ocular infections and a simultaneous limited success rate of corneal grafting during infectious keratitis and its sequela, underlines the need of the hour for an alternative approach for infectious corneal diseases. New-fangled antibiotics are being tested but unfortunately those are becoming increasingly resistant. Notwithstanding the medical prerequisite, the quantity of newly approved drugs continues to decline. Numerous unconventional approaches are currently being under investigation and one such alternative is biotherapeutics. But induction of latency during anti-viral therapy is seriously limiting the efficacy of the approach.

METHODS: We have established a structural study based system for the development of molecular framework of multifunctional peptide. We are harnessing the primary structure of body's own cathelicidin antimicrobial peptide LL-37 by computing ESP maps on their Connolly surface, hydrophobic burial score, electrostatic complementarity to find a set of selective peptides. The selection peptides are then being investigated for their anti-viral activity with HSV infected HCEC for corneal regeneration.

RESULTS:

We have proposed N-terminal cork-screw model (Fig. 1) with cork docking hypothesis to find a selection peptide that is non-toxic to corneal cells but have anti-viral and regenerative potential.

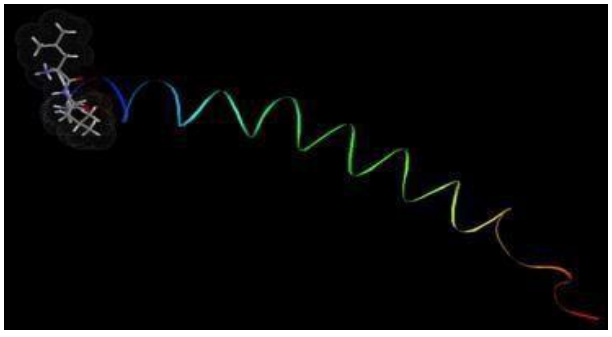

Fig. 1: *The crock-screw model of the developed framework for the antimicrobial peptide.*

The optimized peptides are less prone to (a) self-aggregation even at high therapeutic concentration during the event of membrane perforation and (b) will dock to one of the possible 'negative' surface grooves of the viral capsid protein via its N-terminal cork and showed its anti-viral activity (Fig. 2) and regenerative potential (Fig. 3).

Fig. 2: Relative size and nature of the individual infected viral foci estimated before (A) and after treatment (B) with the selection peptides and determined by fluorescence microscopy.

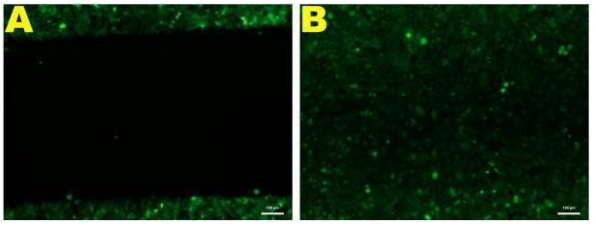

Fig. 3: Regeneration (A to B) of the human corneal epithelial layer after scratch wound.

DISCUSSION & CONCLUSIONS:

A structural molecular framework approach has been proposed for multifunctional platform from which we can develop new regenerative therapeutic strategies including target selection, manifesto development, engineering for function optimization, management of toxicity, enhancement of effectiveness, and scalability to treat infectious corneal disease.

Enhanced chondrogenesis of mesenchymal stromal cells by intermittent PTHrP: role of pulse duration versus pulse frequency

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INTRODUCTION: Avoidance of premature hypertrophy and the formation of abundant hyaline extracellular matrix are still the prime challenges for the generation of phenotypically stable, articular chondrocytes from mesenchymal stromal cells (MSC). We demonstrated recently that intermittent administration of parathyroid hormone-related protein (PTHrP) enhanced hyaline matrix production and concomitantly reduced undesired hypertrophy [1]. Mechanistic analyses suggested a central role of signal timing in the decision between anabolic versus catabolic effects on chondrogenesis. We therefore examined here the role of pulse duration, frequency and total exposure time with the aim to unlock the full potential of intermittent PTHrP.

METHODS: Human bone marrow-derived MSC were subjected to in vitro chondrogenesis for six weeks. From day 7-42, cells were additionally exposed to 2.5 nM PTHrP(1-34) pulses or left untreated (control). Pulse frequency was increased from three times per week (3x6h/week) to daily, thereby maintaining either pulse duration (6h/d, total 42 h/week) or total weekly exposure time (2.6h/d, total 18 h/week).

RESULTS: Daily PTHrP application significantly increased proteoglycan deposition regardless of pulse duration and suppressed alkaline phosphatase activity by 87%. A PTHrP pulse duration of 6h was necessary for a significant reduction of IHH expression, but 2.6 h and 6 h pulses suppressed MEF2C and IBSP mRNA levels. COL10A1, RUNX2, PTH1R and MMP13 mRNA levels were not significantly influenced by PTHrP treatment. Decreasing SOX9 levels during 6h of PTHrP exposure and significantly reduced cell proliferation on day 14 indicated delayed chondroblast formation and hypertrophy. Decreased IGFBP-2 expression suggested IGF-related mechanisms behind intermittent PTHrP actions.

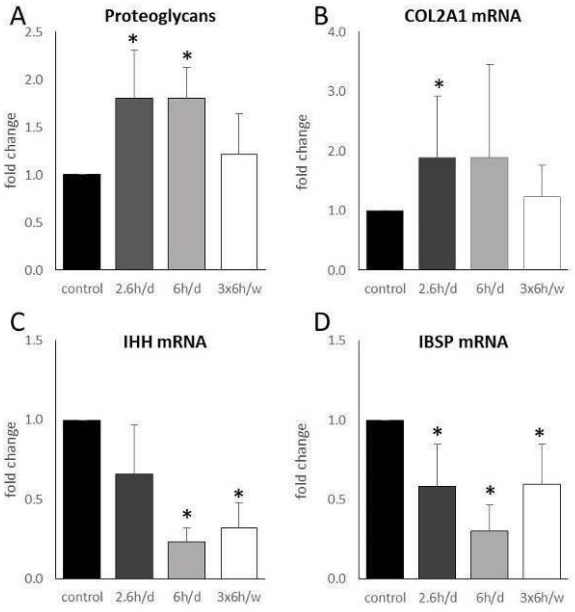

Fig. 1: Pellets were cultured under standard chondrogenic conditions (control) or received an additional intermittent PTHrP treatment from day 7-42. (A) Proteoglycan deposition, (B) COL2A1, (C) IHH, and (D) IBSP mRNA levels on day 42. n=5 independent experiments.

DISCUSSION & CONCLUSIONS: The adaptation of PTHrP pulse protocols demonstrated the vast potential of PTHrP to enhance the chondrogenesis of MSC and simultaneously suppress hypertrophy, with daily PTHrP exposure of 6h/d being the most effective protocol tested. IGF-related stimulatory and SOX-9-related inhibitory effects are suggested as potential molecular mechanisms. Overall, daily intermittent PTHrP administration should be considered as a supportive treatment for clinical, MSC-based cartilage regeneration strategies.

ACKNOWLEDGEMENTS: This work was supported by funds of the University Hospital Heidelberg.

Effective methodology for the long-term preservation of decellularised tissues in oesophageal tissue engineering

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INTRODUCTION: Tissue engineering of the oesophagus is a therapeutic alternative when oesophageal replacement is required. The use of decellularised scaffolds is increasing in tissue engineering because of their ideal architecture and composition derived from tissue-specific extracellular-matrix (ECM). However, appropriate preservation may significantly affect scaffold behaviour and it is necessary to identify a storage methodology that could allow anticipatory preparation of the scaffold coupled with appropriate biobanking. Here we aim to establish an innovative method for the storage of decellularised oesophagi that we developed for clinical application of regenerative medicine.

METHODS: Rabbit oesophagi were decellularised using our established detergent-enzymatic treatment (DET) [1] and evaluated at 2 and 4 weeks and 3 and 6 months of storage. Four storage methods were compared: SCM (slow cooling in medium with 10% DMSO at -1°C/min, then stored in liquid nitrogen), SF (snap-freezing in liquid nitrogen, then stored in -80°C), FD (freeze-drying, then stored in -20°C) and 4°C (phosphate-buffered saline solution at 4°C). Structural and functional analyses were performed prior to and after each storage condition.

RESULTS: Efficient decellularisation of rabbit oesophagi was achieved after 2 cycles of DET as evidenced by histology and DNA quantification, with optimal preservation of the ECM. Among all the methods, only the SCM maintained the architecture, layer arrangement and biomechanical properties of the scaffold up to 6 months. On the contrary, all other methodologies failed long-term preservation of the original structure. In particular, SF oesophagi displayed irreversible tissue collapse, FD samples were impossible to rehydrate 3 and 6 months post-storage and the 4°C option led to a progressive distortion of the tissue architecture.

DISCUSSION & CONCLUSIONS: In this study we generated a decellularised scaffold from a large animal model and investigated the effect of different storage conditions on ECM component and structure preservation with the aim of identifying the best technique for long-term storage. Here we show for the first time that slow cooling in a DMSO/medium solution and liquid nitrogen, commonly used for cell storage, leads to reliable long-term preservation of the structural and mechanical properties of the decellularised scaffold, essential for clinical translation.

DISCLOSURES: The authors have nothing to discloser.

Family medicine residents perceptions on tissue engineering health education

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INTRODUCTION: **D**evelopment of tissue engineering in the last decades and the potential impact in medicine has created great expectation not only for patients but also for the general public. To prevent the risks of misinformation about the therapeutic possibilities of tissue engineering, appropriate health education in this field should be implemented and family physicians should play an important role in this activity.

In this work we investigated the attitudinal perceptions of family medicine residents about how health education on therapeutic tissue engineering should be carried out. This would contribute to better knowledge and patients decisions in order to reduce health risks[1].

METHODS: This study was performed at the National Health System Facilities in the province of Jaén, Spain. The sample consisted of 30 family medicine residents enrolled in the residency national program of family medicine. The average age of the participants was 29.2 years. The male/female ratio of students enrolled in the study was 11/19 individuals. All participants agreed to participate in the study, which was approved by the Ethics and Research Committee of the Medical School of the University of Granada, Spain.
A questionnaire was used to evaluate the perceptions of the residents about the role of hospitals, primary health care centers, primary and middle schools, universities and mass media as agents to provide health education in tissue engineering. The responses were evaluated with a Likert-like scale by which residents indicate their level of agreement or disagreement for each option. Each participant rated each option on a five-point scale from 1 to 5, with each score corresponding to the following level of agreement: 1 = strongly disagree; 2 = disagree; 3 = neither agree nor disagree; 4 = agree; 5 = strongly agree. Comparisons were done for all participants as well as for each gender separately. The Kruskal-Wallis and Mann-Whitney tests were used to identify the family medicine resident priorities.

RESULTS: Family medicine residents gave highly positive scores to the need for health education in tissue engineering, although they differed on the role of the possible agents involved in such education. Providing tissue engineering health education in primary health care centres is much better perceived that doing it in primary and middle school ($p<0.022$). Non-significant differences were observed between education in primary health care centres and other agents such as hospitals, universities and mass media. Statistically significant differences were found between male and female residents. Specifically, scores were higher in males for education in hospitals ($p<0.003$) and education in mass media ($p<0.029$).

DISCUSSION & CONCLUSIONS: The introduction at different educational levels of an adequate literacy in tissue engineering applied to health problems solving has increased in recent years [2]. Although it has been recently emphasized the importance of education on tissue engineering in children [3], the perception of family medicine residents, with some differences between males and females, is that the primary health care centre is more appropriate for health education in this topic that primary and middle schools. Knowing the attitudes and priorities of family medicine residents on this subject can be of interest to plan future education programs not only to promote better informed choices, but also to avoid false therapeutic expectations.

ACKNOWLEDGEMENTS: Supported by Grant 15-36 from Secretariado de Innovación Docente, University of Granada, Spain, and Tissue Engineering Group CTS-115, Junta de Andalucía, Spain.

A combinatorial approach towards in vitro skin-regeneration and in vivo biocompatibility of polyurethane/gelatin composite scaffold

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INTRODUCTION: An in vitro approach to cellular regeneration using a composite scaffold made up of polyurethane (PU)/gelatin as a tissue regenerative vehicle. The scaffold has been fabricated by freeze drying and fibroblast growth factor is encapsulated in it which is employed for skin tissue engineering after confirming in Fourier transform infrared, scanning electron microscopy. The scaffold will induce regeneration due to its unique properties like induction of cell adhesion and migration through extracellular matrix-ligand interaction, cell migration etc. The scaffold has 15-30µm pore size which favors fibroblasts to proliferate and 90% porosity, PU being the primary tensile load bearing component in extracellular matrix and gelatin will aid in attachment of the cells to RGD peptides. It is known that regeneration is favored when tissue growth occurs at the same rate as that of scaffold degradation thus scaffold will degrade approximately in 30 days, thus in vitro degradation has been performed as well. NIH3T3 cells are successfully seeded on the artificial scaffold; cytotoxicity, proliferation investigated by MTT assay, blue alamar assay. Immunogenicity of the scaffold is verified in terms of change in level of tumor necrotic factor (TNF) comparing with the uncoated polystyrene tissue culture plate. In order to get its in vivo biocompatibility, scaffold has been implanted into the subdermal epithelial layer of whister strain female mice. Upon scaffold retrieval, SDS PAGE for adsorbed protein and histopathology of control and treated tissue is done.

METHODS: Mouse fibroblasts have been seeded onto the scaffold and cultured in DMEM to determine percent viability by MTT and alamar blue assay.Determination of tumor necrotic factor (TNF) has been done using kit protocol (Elabscience, India). Cell supernatants collected after 3h added to antimouse TNF antibody pre-coated 96-wells, biotinylated secondary antibody against TNF added to all the wells and incubated for 2h at room temperature. In vivo studies, after 24h, protein pulled out, investigated for checking any alteration in protein has been taken place or not through SDS PAGE.

RESULTS:

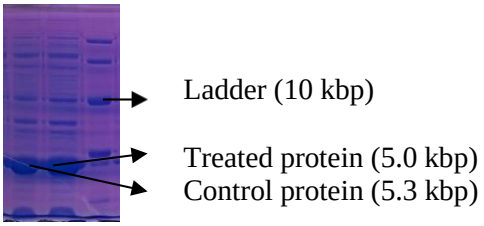

Fig. 1: Protein expression profile in SDS PAGE

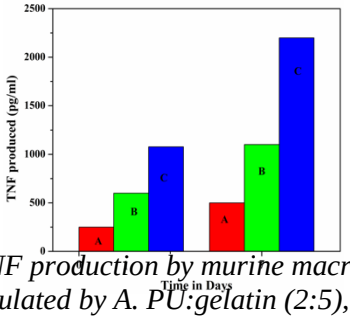

Fig. 2: TNF production by murine macrophages Cells stimulated by A. PU:gelatin (2:5), B. PU:gelatin (2:7), C. PU:gelatin (2:9)

DISCUSSION & CONCLUSIONS: The cytocompatibility is confirmed by both MTT and alamar blue assay. In both the cases, it has showed appreciable percent viability, greater the gelatin content, greater is its percent viability. Mechanical stability is also appreciable for composite, thus it has a promising future as a fibro-inductive vehicle.

ACKNOWLEDGEMENTS: We acknowledge Council of Scientific and Industrial Research (CSIR) for providing fellowship to the first author.

Developing a novel bioink for 3D bioprinting of adipose tissue models

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INTRODUCTION: There is a strong interest in engineered adipose tissue constructs for *in vitro* and *in vivo* applications. 2D cell cultured adipocytes have a different expression profile compared to native adipocytes [1] and factors such as restricted spatial growth and interaction, nutrient and gas diffusion and altered cell-cell communication makes 2D adipocyte culture an inferior research model [2]. Therefore, methods for 3D culture of adipocytes as research models of metabolic diseases such as obesity and diabetes are warranted. We have previously cultured adipocytes in 3D sponges of bacterial cellulose successfully [3], but manual seeding of cells cause batch variations and are time consuming. 3D bioprinting is a rapidly emerging technique with the ability to deposit cells, biomaterials and bioactive cues with high resolution and reproducibility, and allows for more complex structures to be engineered. Previously we used 3D bioprinting to print human chondrocytes in a cellulose-based bioink [4], and here we aim to 3D bioprint adipose constructs.

METHODS: C3H10T1/2, a mouse mesenchymal stem cell line was used as cell source. To induce adipogenesis, a cocktail of IBMX, indomethacin, dexamethasone and insulin was used. Nanofibrillated cellulose (NFC) produced by mechanical refinement and enzymatic treatment was provided by Innventia AB (Sweden). The NFC was mixed with either alginate or hyaluronic acid (HA) to obtain bioinks. Harvested cells were encapsulated in the bioinks and printed using a 3D Discovery from regenHu (Switzerland). HA was crosslinked with 0.001% H_2O_2 and alginate was crosslinked with a 100 mM $CaCl_2$ solution. As 3D control cultures, cells were encapsulated in either alginate, HA or collagen I was used.

RESULTS: The C3H10T1/2 cells were mixed with the cellulose/alginate based bioink and 3D bioprinted. Cell viability was 64% at day one and 95% at day seven. However, the cells showed no signs of adipogenesis after two weeks of culture in adipogenic medium. We therefore compared adipogenesis of cells cultured in casted 3D gels of either alginate, HA or collagen I. Both HA and collagen I proved superior to alginate in promoting adipogenesis and cell proliferation. Due to its ability to be crosslinked using HRP and H_2O_2, HA was mixed with NFC and evaluated as a bioink. The peroxide reaction was faster compared to the ionic crosslinking of alginate, and resulted in a uniform crosslinking throughout the 3D structure. The NFC-HA ink needed a higher concentration of HA to be able to match the stiffness of the NFC-alginate scaffolds but both inks had similar rheology properties when measuring shear rate-viscosity. After mixing with the NFC-HA bioink, the cells were able to differentiate into adipocytes with very high lipid accumulation after 3D bioprinting, figure 1.

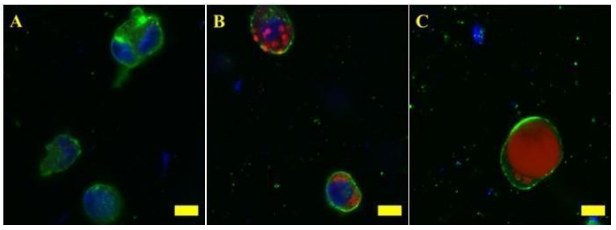

Fig. 1: Adipogenic differentiation after 3D bioprinting. At day 1 the cells showed no signs of lipid accumulation (A), but at day 7 numerous small lipid droplets were observed (B), and at day 14 large lipid droplets occupied the better part of the cytosol (C). Cell nuclei visualized with dapi (blue), cytosol with actin green and lipids with Lipidtox (red). Scale bar 10 µm.

DISCUSSION & CONCLUSIONS: We conclude that 3D bioprinting can be very useful for 3D culture of adipocytes. Future studies will include human cells and a vascularized network.

ACKNOWLEDGEMENTS: This research was supported by Västra Götalandsregionen, Project 21250071 - 3D Bioprinting Center

Self-assembling floating hydrogels for tissue engineering on air-liquid interface

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INTRODUCTION: Hydrogels, formed through crosslinking of hydrophilic polymer chains, represent a water-rich class of materials that present widespread applications. Still, pristine hydrogels are often insufficient to achieve extended use, imposing the functionalization of the hydrogels. Inspired by "liquid marbles", here we propose that coating the hydrogels surface with hydrophobic microparticles, structures with hydrophilic interior and hydrophobic exterior could be construct. These hydrophobic hydrogels (Hhydro) could free-stand, but also float on the surface of aqueous media. We present a potential application for the Hhydro in tissueengineering.

METHODS: Hydrophobic microparticles (PFDTS -DE) were obtained by chemical modification of the diatomaceous earth with a fluorosilane [1,2]. Photocrosslinked hydrogels (gelatin methacryloyl – GelMA [3]) with the desired shape (using a stencil mask) were obtained by exposing GelMA solutions at 4, 6, and 8 w/v% containing photoinitiator to UV light for 15, 30, 45 and 60 s. Then the obtained hydrogels were coated with a uniform layer of PFDTS-DE by rolling the crosslinked hydrogels over the microparticles. On the hydrogels both human dermal fibroblast and umbilical vein endothelial cells were encapsulated.

RESULTS: The Hhydro presented a high water repellence (water contact angle of 145°) at its surface and the ability to float in water for up to 5 days. Floating hydrogels with complementary shapes self-assembled spontaneously and could be manipulated to move directionally by using weak forces, such as electrostatic forces. Encapsulated cells on the Hhydro were cultured for up to 10 days, due to the intrinsic biocompatibility of the core hydrogel. On that basis, biomimetic 3D multi-tissue constructs could be produced. As a proof of concept, a structure mimicking a vascularized skin construct floating at the air-liquid interface could be easily manipulated to assemble.

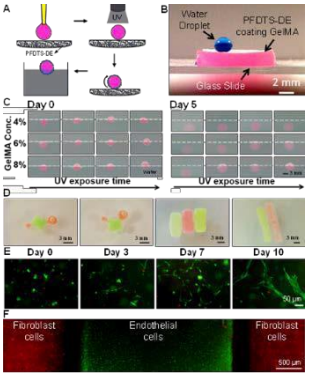

Fig. 1: (A) Schematic illustration of the method to produce the Hhydro. (B) A water droplet on top of Hhydro. (C) Floating ability of Hhydro prepared under different conditions at time 0 and 5 days. (D) Floating hydrogels self-assembling. (E) Live/dead of encapsulated cells at days 0, 3, 7, and 10. (F) Micrograph of floating assembly hydrogel blocks encapsulating fibroblasts and endothelial cells.

DISCUSSION & CONCLUSIONS: We report a novel class of hydrogels that could float on aqueous liquids. The self-assembly feature among complementarily shaped floating hydrogels demonstrated provides a potential strategy to engineer tissue constructs in air-liquid interface.

ACKNOWLEDGEMENTS: Office of Naval Research Young National Investigator Award, the National Institutes of Health (EB012597, AR057837, DE021468, HL099073, R56AI105024) and the Presidential Early Career Award for Scientists and Engineers (PECASE). Portuguese Foundation for Science and Technology (SFRH/BD/73172/2010), POPH/FSE program from QREN.

Ex vivo engineering of vascularized heart tissue model

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INTRODUCTION: Creation of 3-D tissue model similar to a biological tissue using tissue engineering method are expected as an alternative approach to 2-D culture or animal test in research for developing a new drugs. We have already developed functional 3-D heart tissue with perfusable blood vessels in vitro using cell sheet based tissue engineering approach.[1] The present study assessed the possibility as 3-D tissue model by performing drug evaluation in the engineered heart tissues.

METHODS: Neonatal cardiac cells co-cultured with endothelial cells were harvested as cell sheets from temperature-responsive culture dishes. Triple-layer cell sheets were overlaid on the vascular bed with an artery and a vein in vitro. Layered cardiac cell sheets were maintained in custommade bioreactor system with culture medium. After neovascularization within the first triple-layer tissue, the second triple-layer cardiac cells sheets were overlaid on the first graft after cultivation for 3 days and then the whole construct was further perfused for an additional 3 days. After 6 days cultivation, isoproterenol or doxorubicin was administrated to the perfused engineered heart tissues from artery side. The sympathetic and toxic effects were measured by in vitro bioluminescence imaging, electrical potential, and blood pressure.

RESULTS: In the case of isoproterenol administration, bioluminescence imaging (BLI) demonstrated that viability of the heart tissue improved. In addition, electrograms demonstrated increasing action potential and sphygmomanometer showed decreasing arterial pressure. In case of doxorubicin administration, BLI showed that the viability of the heart tissue worsened after 24 hours. It is now possible to analyze the pharmacological effects such as metabolism and the blood pressure as well as action potential by using our vascularized heart tissue model.

DISCUSSION & CONCLUSIONS: We believe that in vitro vascularized 3-D tissue model will give new possibilities for the developing a new medicine.

tissues with perfusable blood vessels. (2013) *Nat Commun*. 4:1399.

ACKNOWLEDGEMENTS: This work was supported by JSPS KAKENHI Grant Number 26350517.

DISCLOSURES: Tatsuya Shimizu is stakeholders of CellSeed Inc. Tokyo Women's Medical University is receiving research funding from CellSeed Inc.

The use of enzymatic bioful cell system without the power plant for improvement of human ESC-derived cardiomyocytes function

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INTRODUCTION: Especially, glucose is included in the media as a 0.08nA current is generated when glucose contacts this conductive enzyme system. This system is advantageous because it is user-friendly and does not require a power unit to improve cardiomyocyte contractility and synchronization, which can enhance processes behind drug screening and action potential measurements.

METHODS: Electrochemical characterization of enzymatic biofuel cell (EBFC): The anodic or cathodic electrode was used 15-mm-long, 2-mm-width, each active area was 30 mm^2, in this study. A two-electrode electrochemical cell coupled to a CHI 600B potentiostat (*Austin, TX, USA*)) was used for the open circuit potential (OCP) and i-t technique. 80 µg/mL enzymatic biofuel stimulation of cardiomyocytes: Purified cardiomyocytes were allowed to adhere to gelatin-matrigel coated dishes in DMEM high-glucose with 2% FBS. After two days of culture, 80 µg/mL was applied to cardiomyocytes culture dishes.

RESULTS: Contracting clusters were isolated through dissection techniques and re-plated to culture for 2 weeks following single-cell dissociation

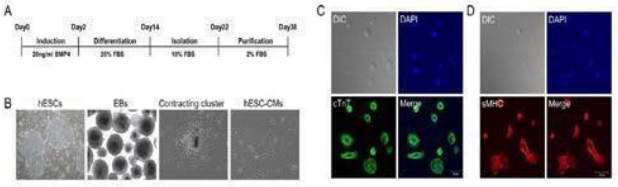

Fig. 1: hESC-derived cardiomyocyte characterization

This enzymatic charge is meant to generate an oxidative reaction of glucose. Therefore cardiomyocytes were plated on matrigel coated dishes, and the battery was submerged under media to ensure reactivity.

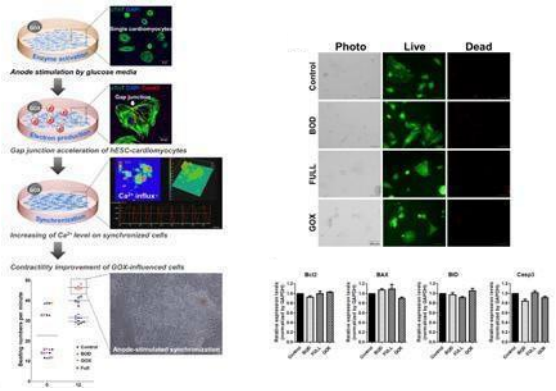

Fig. 2: 80 µg/mL alteration of contracting cardiomyocytes through enzymatic conductivity

DISCUSSION AND CONCLUSIONS: Notably, the GOX group showed a marked increase in calcium channels. The sarcoplasmic reticulum in cardiomyocytes functions to store and release calcium, thereby controlling intake and uptake levels. For this reason, significant increases in Gap junction protein Cx43 for the GOX group allowed for active contractility which subsequently increased calcium channels.

ACKNOWLEDGEMENTS: This research was supported by a grant (HI14C-2755-020014) of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), (PJ.00995602) from the Next-Generation BioGreen 21 Program of Rural Development Administration funded by the Korean government and (10047890) from the Industry Sources Development Project and funded by the Ministry of Trade, Industry and Energy.