Three engineered allogeneic porcine iPSs scaffolds after myocardial infarction in swine

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INTRODUCTION: Engineered constructs using induced pluripotent stem (iPS) cells to regenerate a myocardial scar may become true regenerative alternatives [1]. This work evaluates safety and efficacy of undifferentiated porcine allogeneic GFP+-iPS cells in three settings: embedded within an adipose pericardial graft (APG), an acellular pericardial scaffold (Scaffold), or both (APG-Scaffold) after acute myocardial infarction (MI) in swine.

METHODS: Porcine iPSs (p-iPS) were derived by transient expression of a single non-viral polycistronic vector in porcine skin fibroblasts [2]. Fifty seven swine underwent MI by coronary artery ligation, and distributed into three groups with biomatrices but p-iPS-free (APG n=6; Scaffold n=13; APG-Scaffold, n=7), and three groups with p-iPS-enriched constructs (p-iPS-APG n=9; p-iPS-Scaffold, n=11; and p-iPS-APG-Scaffold, n=11). Histopathology and cardiac magnetic resonance imaging (MRI) were used.

RESULTS: Histopathology confirmed: first, absence of teratogenesis at multi-organ level after 3 months of follow-up; second, no evidence of p-iPS by immunohistochemistry nor real time-PCR neither within the myocardial scar or biomatrices; third, similar infarct size in all studied groups. Pre-sacrifice cardiac MRI revealed no significant benefit in left ventricular ejection fraction, cardiac output, end diastolic volume, and end systolic volume.

DISCUSSION & CONCLUSIONS: Undifferentiated allogeneic p-iPS delivery within three different engineering approaches is safe. Nevertheless, p-iPS could not be identified upon sacrifice and do not seem to support functional benefit after MI in swine.

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Non-invasive evaluation of an engineered bioactive graft using impedance spectroscopy after myocardial infarction in swine

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INTRODUCTION: Cardiac tissue engineering, which combines cells and biomaterials, is promising for limiting the sequelae of myocardial infarction (MI) [1]. In this context, decellularized tissues offer a natural microenvironment, driving cellular attachment, survival, migration, proliferation, and differentiation [2-3]. We assessed myocardial function and scar evolution after implantation of an Engineered Bioactive Impedance Graft (EBIG) in a swine MI model.

METHODS: The EBIG was made of a scaffold of decellularized human pericardium, GFP-labelled porcine adipose tissue-derived progenitor cells (pATPCs), and a customized-design Electrical Impedance Spectroscopy (EIS) monitoring system (Figure 1). In a non-invasive manner, cardiac function was evaluated by magnetic resonance imaging (MRI), and scar healing by a customized-design EIS system incorporated within the implanted graft. In addition, infarct size, fibrosis, vascular density, and inflammation were also assessed.

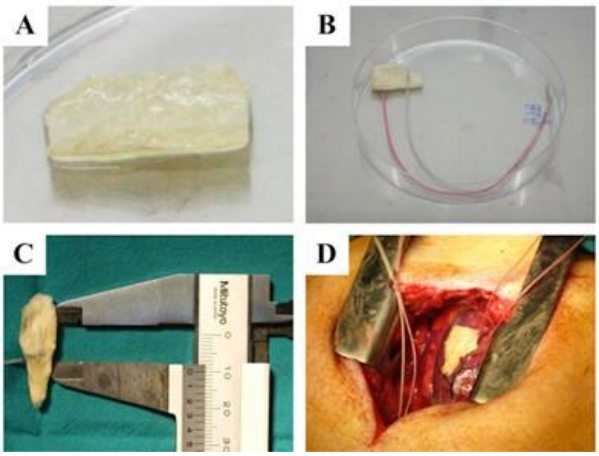

Fig. 1: Photographs showing the different steps of EBIG creation and implantation. (A) Human decellularized and rehydrated pericardium. (B and C) Metallic electrodes placement 1 cm spaced. (D) Image showing the EBIG implanted in a swine.

RESULTS: Upon sacrifice one month after the intervention, MRI detected a significant improvement in left ventricular ejection fraction (7.5±4.9% vs. 1.4±3.7%; P=0.038) and stroke volume (11.5±5.9 mL vs. 3±4.5 mL; P=0.019) in EBIG-treated animals. Non-invasive EIS data analysis showed differences in both impedance magnitude ratio (−0.02±0.04/day vs. −0.48±0.07/day; P=0.002) and phase angle slope (−0.18±0.24º/day vs. −3.52±0.84º/day; P=0.004) in EBIG compared to control animals. Moreover, in EBIG-treated animals, the infarct size was 48% smaller (3.4±0.6% vs. 6.5±1%; P=0.015), less inflammation was found by means of CD25+ lymphocytes (0.65±0.12 vs. 1.26±0.2; P=0.006), and a lower collagen I/III ratio was detected (0.49±0.06 vs. 1.66±0.5; P=0.019).

DISCUSSION & CONCLUSIONS: An EBIG composed of acellular pericardium with adipose progenitors reduced infarct size and improved cardiac function in a pre-clinical model of MI. Non-invasive EIS monitoring tracked differential scar healing in EBIG treated animals confirmed by less inflammation and altered collagen deposit.

ACKNOWLEDGEMENTS: 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-Sudirección General de Evaluación y el Fondo Europeo de Desarrollo Regional (FEDER).

First-in-human results of external ear reconstruction based on in vitro engineered human-ear-shaped cartilage

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INTRODUCTION: Generation of human-ear-shaped cartilage in nude mouse was an important milestone in tissue engineering [1]. Yet, its clinical translation remained unreported due to problems of cell source, shape control, cartilage regeneration stability, and shape maintenance. The current study addressed these issues and represented the first-in-human results of external ear reconstruction based on in vitro engineered human-ear-shaped cartilage.

METHODS: A 6-year-old female patient with right side microtia was recruited. A biodegradable scaffold containing a polycaprolactone (PCL) inner core with a shape mirror-symmetrical to the left auricle was generated via 3D-printed molds. In the first-stage surgery, a skin expander was implanted and microtia cartilage was harvested for chondrocyte isolation. During skin expansion, chondrocytes were expanded and seeded into the scaffold for in vitro cartilage regeneration. After 12 weeks, the engineered ear-shaped cartilage framework was implanted and covered with expanded skin flap, fascia flap, and skin graft for auricular reconstruction. The patient was followed up regularly to evaluate the reconstructed outcomes.

RESULTS: The scaffold achieved an exact shape replica of patient's auricle with high strength under the support of PCL inner core. Microtia chondrocytes proliferated robustly and showed good biocompatibility with the scaffold. After 3 months of in vitro culture, the cell-seeded construct formed immature cartilage-like tissue with pre-designed shape. After 1.5 years of implantation, the reconstructed auricle achieved satisfactory ear shape, skin tone, and elasticity, which was further supported by mature elastic cartilage regeneration as revealed by biopsies at 6 and 18 months post-implantation.

DISCUSSION & CONCLUSIONS: The current study may represent a significant achievement in the field of tissue engineering via translating the human ear-shape cartilage from a mouse model to human trial.

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Cell surface engineering with heparin conjugates using a short peptide-conjugated PEG-lipid for regulation of thromboinflammation in transplantation of human MSC and hepatocyte

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INTRODUCTION: Cell transplantation is an attractive therapy. However, the infusion of cells into human body is associated with large loss of transplanted cells, which was caused by immune responses termed as a thromboinflammation [1]. Thromboinflammation is an innate immunity attack triggered by complement and coagulation activation. Therefore it is important to protect the cell surfaces from the attack of thromboinflammation. To achieve this, heparinization of the cell surface was tried by a cell surface modification technique using poly(ethylene glycol)-conjugated phospholipid (PEG-lipid) derivatives. To this construct several heparin-binding peptides were conjugated to PEG-lipid for the immobilization of heparin conjugates on cell surfaces of human MSC and human hepatocyte. The hemocompatibility of heparinized cells were examined in human whole blood to measure platelet count, coagulation marker, complement markers and Factor Xa activity.

METHODS: We used heparin-binding peptides (HBPI: WQPPRARIC, HBPII: CWGGRARARARARARARA, HBPIII: CNSAHRTRGRQRS, control: GPQGPGGGWC) for immobilizing heparin conjugates (Corline AB, Uppsala, Sweden). The maleimide group of PEG-lipid reacts with SH group of cysteine in those peptides. SPR was used for the binding assay of those peptides for heparin conjugates and antithrombin. Human MSC and human hepatocyte were used in the whole blood experiments. Both cells were incubated with peptide-PEG-lipid (1mg/mL, in PBS) for 30min at RT, and then incubated with heparin conjugates (100 µg/mL in PBS). Those cells were then exposed to human whole blood for 60 min. Blood was analyzed for platelet count, TAT, C3a, sC5b-9, and Factor Xa.

RESULTS & DISCUSSION: The binding assay for heparin-conjugate and antithrombin showed that HBPII and III have the higher affinity compared to peptide I. When cells were treated with peptide II-PEG-lipid and heparin conjugates, the cell viability was much lower than the control and also depended on the ratio of HBPII, indicating the cytotoxicity due to the positive charge. On the other hand, no cytotoxicity was observed for cells treated with HBPIII-PEG-lipid and heparin conjugates. The binding of heparin conjugates on the cell surface depends on the ratio of HBPIII-PEG-lipid.

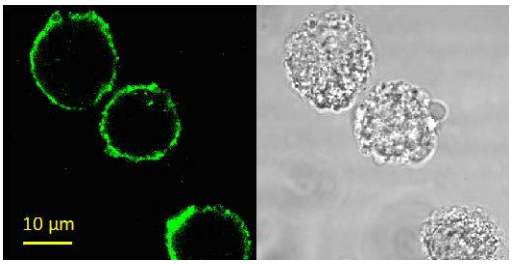

Fig. 1: hMSC coated with heparin conjugates. Cells were treated with HBPII-PEG-lipid and heparin conjugates.

For the whole blood experiments using HBPIII-PEG-lipid, there was no platelet aggregation in the heparin-conjugate-immobilized cells group in comparison to the control for both human MSCs and hepatocytes. In addition, TAT, C3a, and sC5b-9 levels were significantly lower in heparin-conjugate-immobilized cells group compared to the control, indicating a lower activation of coagulation and complement. Factor Xa analysis indicated that the activity of heparin conjugate was still detected on the cell surface during 24hr.

CONCLUSIONS: Heparinization of MSC and hepatocyte with PEG-lipids attenuated thromboinflammatory reactions in whole blood.

Nonlinear microscopy imaging of 3D-exoCollagen matrices for pancreatic β-cell differentiation

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INTRODUCTION: Collagen is known to be the most abundant extracellular matrix protein present around human pancreatic islets (hIslets) [1], and it can be imaged in a label-free way using non-linear microscopy [2]. β-cells are a hormone producing cell type in hIslets, which are responsible for the insulin regulation. hIslet-derived exosomes (exo) can enhance the epigenetic and/or hormonal signals that control native insulin release and regulation in differentiated stem cells.

We aim to develop an in-vivo like pancreatic 3D-model, and conclude about stem cell clusters-matrix interactions during in-vitro pancreatic β-cell differentiation.

METHODS: Induced Pluripotent Stem Cells (iPS) were cultured in suspension to promote cell clustering. Human pancreas tissue (hPanc) and isolated hIslets were purchased. Collagen hydrogels were performed by mixing collagen type 1 (COL1) with Hepes buffer and NaHCO3. Cell clusters were encapsulated within the hydrogels. Conditioned media from h-islets was used to isolate exosomes, which were encapsulated within COL1 hydrogels. Second Harmonic Generation (SHG) was used to imaging collagen in a label-free way.

RESULTS: Label-free SHG imaging of hPanc collagen matrix showed a fibrillar network organization (Fig 1A). Typical fibrillar structure of collagen, in hydrogels, is concentration dependent. 1.5 mg/mL of COL1 hydrogels allowed cell cluster remodeling of the matrix, while 3 mg/mL entrapped the cell clusters (Fig. 1B). Furthermore, we functionalized COL1 hydrogels (1.5 mg/mL) with hIslets exosomes (Fig. 2). The paracrine behavior of these smart-matrices will be further explored on stem cell clusters differentiation towards pancreatic β-cells.

DISCUSSION & CONCLUSIONS:

SHG can be used for the label-free imaging of collagen, in-vitro and in-tissue. Collagen hydrogels can be used to encapsulate stem cell clusters as well exosomes. Thereby, we hypothesize that exoCOL hydrogels might help to promote the in-vivo like context necessary to drive cell functionality and phenotype.

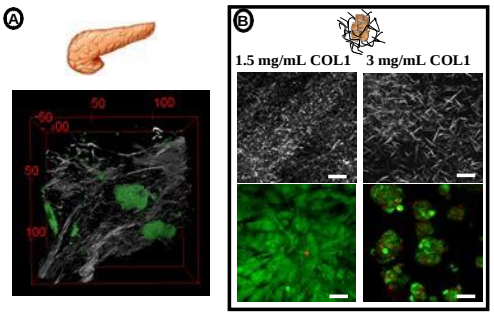

Fig. 1: Label-free imaging of collagen. A. *imaging of hPanc:* SHG *collagen (grey) and autologous cell auto-flurescence (green). B.* SHG *imaging of COL1 hydrogels (top; grey) and viability of encapsulated iPS clusters (Down;* ***live cells/dead cells****).Bar = 50 µm.*

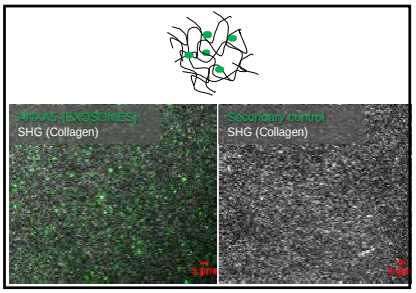

Fig. 2: SHG imaging of functionalized COL1 hydrogels (grey; 1.5 mg/mL) with hIslets exosomes (green).

REFERENCES: [1] J.C. Stendahl, D.B. Kaufman,

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Generation of stable endothelial networks in a human bioartificial cardiac patch designed for surgical implantation

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INTRODUCTION: One of the main challenges of tissue engineering is the vascularization of engineered tissues in order to increase their size whilst allowing survival of cells through exchange of nutrients and waste products. This paper reports current results in the generation of a large-scale tissue-engineered human cardiac patch that exhibits stable endothelial networks composed of cryopreserved cardiomyocytes (CMs) derived from human embryonic stem cells, human cardiac CD31-negative stromal cells (hCSCs), human cardiac endothelial cells (hCECs), and small intestinal submucosa (SIS).

METHODS: Wnt pathway modulation was used to differentiate hES3 α-MHCC1 into CMs. CM purity was determined by FACS analysis. Embryoid bodies were dissociated with an Embryoid Body Dissociation Kit and frozen in a solution with 90% (v/v) fetal bovine serum (FBS) and 10% (v/v) dimethyl sulfoxide. Atrial appendages from patients were minced into 1-5 cubic mm pieces and digested with a Neonatal Heart Dissociation Kit. The resulting cells were cultured in EGM2 medium. hCECs and hCSCs were isolated by MACS (CD31 MicroBead Kit) as a positive and negative fraction, respectively. hCECs were cultured in EGM2 medium and hCSCs in DMEM with 10% (v/v) FBS.

Porcine small intestine was decellularized mechanically and chemically (1% Triton X-100, 24h) to obtain SIS. Thawed CMs in combination with hCSCs and hCECs at different ratios were seeded on SIS and Matrigel/Collagen gel under static conditions and cultivated with different cell culture medium compositions. Cardiac patches were analyzed by vital staining and video recording during cultivation for 1 month. At the end of the experiment, the constructs were fixed and subjected to staining for further analysis.

RESULTS: In the patch composed of 50% hESC-derived CMs, 25% hCECs and 25% hCSCs all cell types attached on the SIS and survived. In culture medium with a reduced content of FBS (5%) and without growth factors, the cardiac patch showed spontaneous and coordinated global contractions and stable endothelial networks until the end of the experiment. In contrast, cultivation of the patch with the same cell composition in medium containing 20% FBS and growth factors resulted in less developed endothelial networks and absence of contractions. Additionally, hCECs survival and endothelial network formation was impaired in a cardiac patch with a decreased percentage of hCSCs (5%), and an increased percentage of CMs (70%) that was cultivated in culture medium with 20% (v/v) FBS and growth factors.

DISCUSSION & CONCLUSIONS: Stable endothelial network formation was shown to be feasible in human large-scale bioartificial cardiac patches with specific cardiac cell ratios and defined culture medium composition without affecting the functionality and viability of CMs and hCSCs. The results suggest that an unidentified cell population among the hCSCs provided support and stability to endothelial networks. In order to achieve further maturation, alignment and connectivity of the CMs, a bioreactor that applies electrical and mechanical stimulation needs to be used.

ACKNOWLEDGEMENTS: This work was supported by the People Programme (Marie Curie Actions) from European Union [FP7/2007-2013] under REA grant agreement n° 317512, the CORTISS foundation, Deutsche Forschungsgemeinschaft (Project HA 13 06/9-1), the BMBF Project “AUREKA” and by Cluster of Excellence REBIRTH (DFG EXC62/1), German Ministry for Education and Science (BMBF; grant no. 13N12606), and StemBANCC (support from the Innovative Medicines Initiative joint undertaking under grant agreement n_ 115439-2, resources of which are composed of financial contribution from EU [FP7/2007-2013] and EFPIA companies’ in kind contribution).

Screening novel hydrogels in an *ex vivo* cartilage defect model

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INTRODUCTION: Cartilage defect treatments often result in fibrous tissue formation without mechanical stability. New cartilage therapies should achieve defect regeneration with functional repair tissue. Pre-clinical evaluation of novel cartilage regeneration therapies and material testing in a 3D osteochondral environment are necessary for effective therapy developing. We used the PhysioCart *ex vivo* culture platform developed by LifeTec Group that allows long term culture of osteochondral explants (OCE) to evaluate cartilage repair in novel materials. Our aim was to evaluate material-tissue-cell-interaction of novel hydrogels and compared cell loaded and cell free treatments for their regenerative potential in cartilage defects.

METHODS: Reproducible cylindrical 8mm OCE isolated from porcine condyles with 4mm full thickness cartilage defects and were filled with cell free or chondrocyte loaded hydrogels. Three hydrogels namely poly(ethylene glycol)/heparin (PEGh), PEG and (*N*-(2-hydroxypropyl) methacrylamide mono-di-lactate / methacrylated hyaluronic acid (MP/HA) and thiol functionalized hyaluronic acid / allyl functionalized polyglycidol (PG–HA-SH) were compared to the reference materials fibrin glue and collagen I (col I). OCE were cultured for 28 days in PhysioCart with separated media compartments for cartilage and bone, without supplementation of growth factors. Cell vitality was analysed by MTT staining. Cartilage matrix deposition of glycosaminoglycans (GAG) and defect regeneration were visualized histologically and quantified spectroscopically. Real-time polymerase chain reaction of chondrocyte loaded hydrogels were performed.

RESULTS: Chondrocyte viability in all hydrogels was maintained over 28 days. Fibrin and col I scaffolds shrank in height, whereas shape was maintained in all 3 hydrogels. In cell free approaches, migration of elongated, fibroblast like cells into hydrogel was observed for col I, fibrin, PEGh. Cell free MP/HA induced defect regeneration on calcified layer which was not present for PEGh and PG–HA-SH. In cell loaded approaches, embedded chondrocytes maintained their characteristic round phenotype. Cartilage like tissue formation could be achieved in all cell loaded hydrogels. GAG content in col I and MP/HA increased in 28 days to 2-3 fold, in PEGh to 7 fold of initial GAG amount at day 0 and remained constant in fibrin glue and PG–HA-SH. DNA content was doubled in col I, stayed constant in PEGh and slightly decreased in PG-HA-SH and MP/HA after 28 days.

DISCUSSION & CONCLUSIONS: The presence of subchondral bone stimulates cell migration into defect side supporting cartilage repair. Cell migration into cell free hydrogel is dependent on material indicating more favourable for softer hydrogels (fibrin, col I, PEGh). In cell free approaches, the absence of cell migration and reduction in DNA yield of chondrocyte loaded MP/HA and PG-HA-SH indicate cell outgrowth into OCE defect. This is in coincidence with the highest novel cartilage tissue formation observed with MP/HA on calcified layer. The increase of GAG in PEGh is explained by uptake of GAGs naturally released from OCE in culture media. This gel system has promising applications in attraction of cells due to its property to bind GAGs and other growth factors (TGF β). With regard to neo cartilage formation MP/HA is the most promising material.

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Angiogenic and osteogenic capacity of axially vascularized tissue engineered bone constructs after in-vivo high dose irradiation

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INTRODUCTION: Axially vascularized bone constructs are, due to their dedicated vascular supply, independent of the recipient vascular bed [1]. In order to introduce bone tissue engineering to the field of reconstruction after cancer, we are investigating for the first time the effect of various doses of ionizing irradiation on an in vivo axially vascularized bone construct.

METHODS: Silica-embedded nanohydroxyapatite (Nanobone®) in combination with mesenchymal stem cells and BMP-2 were used to create synthetic bone constructs that were implanted in the thigh of 32 Lewis rats. Each construct was axially vascularized through an arteriovenous loop (AVL) made by direct anastomosis of the saphenous vessels in the thigh. After 2 weeks, the animals received ionizing irradiation of 9Gy, 12Gy, 15Gy and 0Gy and were accordingly classified to groups I, II, III and IV respectively. The constructs were explanted either 2 or 5 weeks after irradiation where tissue generation, vascularity and apoptosis were investigated through micro CT, histomorphometry and real time PCR.

RESULTS: At 2 weeks after irradiation, the tissue generation and central vascularity were significantly lower and apoptosis was significantly higher in groups II and III in relation to group IV but without signs of necrosis or inflammation. After 5 weeks, the irradiated groups showed improvement in all parameters in relation to the control group and direct connections between extrinsic and axial vascularity could be noticed in micro CT images indicating a retained capacity for angiogenesis after irradiation (figure 1). Real time PCR results showed an enhanced expression of osteogenesis-related genes in all irradiated groups. Dense collagen formation and eventual woven bone was detected 5 weeks after irradiation indicating a retained osteogenic capacity after irradiation.

DISCUSSION & CONCLUSIONS: We were able to demonstrate for the first time that intrinsic axial vascularization through an AVL was capable of vascularizing and supporting a synthetic bone construct after a high dose of irradiation that is comparable to adjuvant radiotherapy.

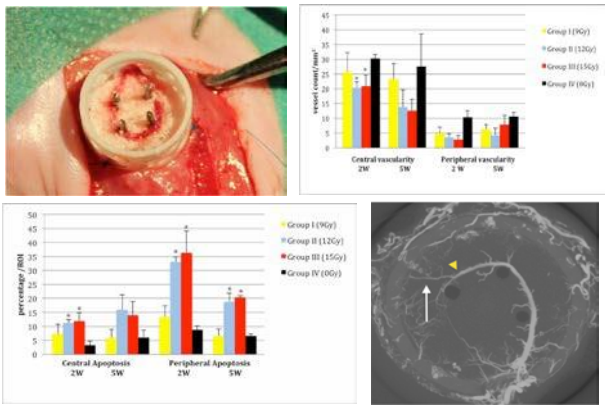

Fig. 1: Above left: The AVL dragged into the chamber. Above right: Average vessel count in the constructs in different zones at different time points. Below right: Micro CT images 5 weeks after 9 Gy irradiation showing angiogenesis sprouting from the AVL and connected to the extrinsic vascularity (white arrow). Arrowhead points to the AV anastomosis. Below left: Average apoptosis at different time points. Asterisk indicates significance $p \leq 0.05$, unpaired t test.

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non-invasive tracking of bone marrow mononuclear cell homing and engraftment to implanted biomaterials

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INTRODUCTION: Biomaterial scaffolds which regulate stem cell behaviour and differentiation have enormous potential for applications in tissue regeneration. However, the development of bioactive materials remains challenging given the precise microenvironments required to support stem cell function *in vivo*. In this study, we have developed a murine model to non-invasively track homing and engraftment responses of intravenously injected bone marrow mononuclear (BM-MNC) stem cells to implantable biomaterials.

METHODS: BM-MNCs derived from FVB-L2G donor mice, containing firefly luciferase and green fluorescent protein (GFP) reporter genes [1] were systemically injected ($3x10^6$ total cells) via the tail vein into wild-type FVB mice which received either subcutaneous wounds or implanted scaffolds. Over the course of 21 days, mice were administered daily i.p. injections of D-luciferin (250mg/kg) to induce BM-MNC bioluminescence for serial imaging of live mice by an IVIS Imaging System (Perkin Elmer). Bioluminescence (BLI) values were plotted as a function of time (days). Separate cohorts of mice were harvested at 2, 5, 8, 11, and 21 days for further histological analysis.

RESULTS: BLI curves of subcutaneous wounds showed that BM-MNCs homed and engrafted to wounds in two distinct phases: an early (days 3) and late phase (day 11). Complimentary histology revealed that each phase in the BLI curve represented the collective presence of distinct BM-MNC cell phenotypes at the wound site. In the early phase, the primary cell type observed were inflammatory cells, whereas in the late phase, remodelling fibroblasts were dominant. To further explore the utility of this model, we implanted two electrospun scaffolds, a control PCL and a blended PCL/Collagen implant. Histology showed that BM-MNCs were present in the periphery of PCL scaffolds, consistent with a traditional fibrotic response. In contrast, PCL/Collagen scaffolds showed BM-MNC infiltration into the body of the scaffold indicating a more favourable remodelling response (Figure 1B). More importantly, these differing BM-MNC responses were readily distinguished by their BLI curves which diverge from day 9 and show a 1.8-fold difference in max BLI (Figure 1A).

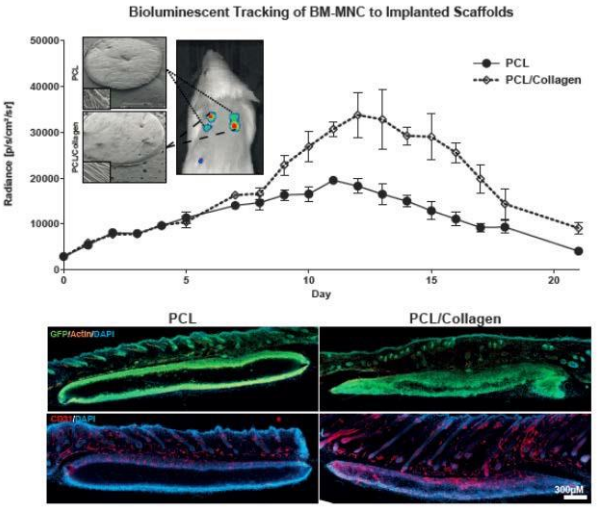

Fig. 1: A) BLI curves of implanted PCL & PCL/Collagen scaffolds; inset: SEM of scaffolds and BLI Image B) Histological staining of GFP$^+$ BM-MNC engraftment and CD31$^+$ capillaries

DISCUSSION & CONCLUSIONS: The findings of our study demonstrate a high-throughput screening model of homing and engraftment of stem cells to wounds and candidate biomaterials. A major advantage is the serial non-invasive tracking of injected BM-MNCs and the strong correlations with traditional histopathology analysis. Biomaterial engineering guided by the real-time imaging of BLI curves, may produce scaffolds which better direct stem cell behaviour and fate. Thus, our model has the capacity to advance both stem cell biology and biomaterial development while building a synergistic bridge between the two emerging fields.

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Building complex 3D biomimetic, biofunctional elastic structures with assembled human tropoelastin components

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INTRODUCTION: We have defined synthetic biology approaches that utilize the dynamics of individual human elastic protein molecules to give unprecedented control over spatial assembly, with the goal of building macromolecular 3D structures for tissue assembly and repair. The mechanical stability, elasticity, inherent bioactivity, and self-assembly properties of elastin make it a highly attractive and powerful technology for the fabrication of versatile biomaterials. Our unique ability to make and engineer the key elastin component, tropoelastin, drives our precise control of physicochemical and organizational performance, and further broadens the diversity of elastin-based applications.

METHODS: Neovascularization in vitro and in vivo was conducted in mice and pigs. Computer-based predictive biomaterials modeling has accelerated our design of these 3D protein constructs, and gives us control over higher-order cross-linking, efficient assembly and directed cell interactions [1].

RESULTS: Elastic fibers are integral to the extracellular matrix of vertebrate tissues such as blood vessels, skin and lungs, where they provide the structural integrity and elasticity required for mechanical stretching of these tissues during normal function [2,3]. We found that by incorporating the human elastic protein tropoelastin into a dermal regeneration template, we can promote angiogenesis in wound healing. In small and large animal models comprising mice and pigs, the tropoelastin biomaterial accelerates early stage angiogenesis by 2 weeks, as evidenced by increased angiogenesis fluorescent radiant efficiency in live animal imaging and the expression of endothelial cell adhesion marker. In the pig, a full thickness wound repair model confirms increased numbers of blood vessels in the regenerating areas of the dermis closest to the hypodermis and immediately below the epidermis at 2 weeks [4]. Tropoelastin in a dermal regeneration template enhances vascularization and promotes wound repair.

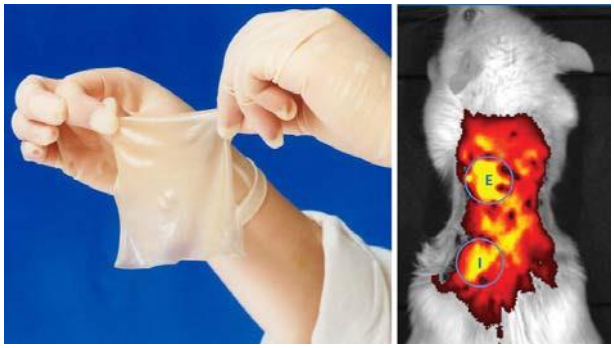

Fig. 1: L: Example wound sheet made from human tropoelastin. R: early stage angiogenesis at wound site (I) is enhanced (approximately doubles) with tropoelastin (E).

DISCUSSION & CONCLUSIONS: We are using these natural-based composites in diverse fabrication processes to manufacture 2D and 3D materials including fibers, gels, sheets and tubes. The extraordinary tunability of these elastin constructs means we can use them in a range of biomedical and tissue engineering applications that encompass cell encapsulation, vascular repair, nerve regeneration, wound healing and accelerated tissue repair.

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Biomaterial delivery of exogenous mir-29B to the infarcted myocardium yields improved myocardial recovery

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INTRODUCTION: Following acute myocardial infarction, damaged heart muscle is replaced by scar tissue. This scar tissue is non-contractile and imposes a greater hemodynamic burden on the remaining viable myocytes, contributing to heart failure development. Currently, targeted therapies for reducing fibrosis after heart injury do not exist. Micro-RNA (miR)-29B is downregulated in fibroblasts in the injured region and has been implicated previously in cardiac fibrosis [1]. We sought to determine whether a localised biomaterial based hydrogel delivery of miR-29B into the myocardium improves functional recovery following myocardial infarction, and how this treatment affects the organisation of newly formed extracellular matrix.

METHODS: Transient ischemia was induced in C57BL/6J mice via left ascending coronary artery occlusion / reperfusion. Following reperfusion, miR-29B (100 µg/animal) was delivered within a hyaluronan-based hydrogel (crosslinked using thiol-reactive poly(ethylene glycol) diacrylate) by intramyocardial injection at five infarct borderzone locations. miR-239B was employed as a negative control. Cardiac function was monitored over time using echocardiography and animals were sacrificed at 5 weeks post infarction. Cardiac tissue was then processed for immunohistological stainings, in situ hybridization and Raman microspectroscopy.

RESULTS: At 2 and 5 weeks following MI, miR-29B-treated mice showed significantly improved ejection fractions and fractional shortenings when compared with the non-targeting control of miR-239B. Histological and immunohistological analyses also revealed differences in extracellular matrix patterns particularly at the borderzone of the infarct at 5 weeks. A higher blood vessel density was detected within the infarcts of miR-29B treated mice and a significantly higher proportion of mature collagen fibres was detected at the borderzone of miR-29B treated infarcts when compared with the miR-239B controls. Additionally, Raman microspectroscopy detected statistically significant alterations in the molecular fingerprint of spectra, reflective of collagen orientation and structure, most profoundly at the borderzone of miR-29B treated infarcts.

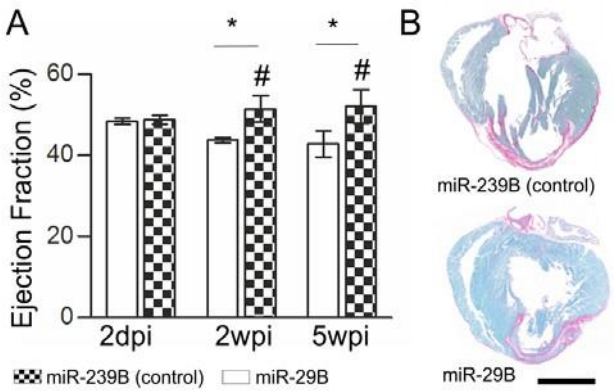

*Fig. 1: (A) miR-239B (control) or miR-29B were delivered to the borderzone of the infarcted myocardium within an injectable hyaluronic based hydrogel following myocardial infarction. miR-29B delivery elicited an improved ejection fraction at 2 weeks post infarction (wpi) and 5 wpi. * indicates a statistically significant difference between the groups indicated, # indicates a statically significant difference between the other time points in the data set. $p<0.05$. (B) Picrosirius staining of infarcted myocardium. Scale bar indicates 2 mm.*

DISCUSSION & CONCLUSIONS: These data highlight miR-29B as a potential therapeutic agent for ameliorating fibrosis after acute myocardial infarction and that delivery via an injectable biomaterial localised such effects to the borderzone of the infarcted tissue.

A high-glucose diet affects tendon healing in a rat model

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INTRODUCTION: Metabolic disorders are frequently associated with tendon degeneration and impaired healing after acute injury [1]. However, the underlying cellular and molecular mechanisms remain largely unclear. We have previously shown that human and rat tendon cells produce and secrete insulin upon glucose stimulation [2]. Therefore, we hypothesize that nutritional glucose uptake affects tendon healing in a rat model.

METHODS: In female rats, unilateral full-thickness Achilles tendon defects were created. Immediately after surgery animals were randomly assigned to three groups receiving different diets for 2 weeks (Diet C: control diet, n=25; Diet G: high-glucose diet, n=25; Diet F: high fat/low glucose diet, n=20). Gait analysis (Catwalk, Noldus) was performed at three time points. In addition, tendon thickness measurements, biomechanical testing and immunohistochemical analysis were conducted. Subsequently, gene expression analysis, comparing cDNA pools (n=5) prepared from repair tissues of the Diet C and the Diet G group was performed.

RESULTS: The repair tissues of both treatment groups were significantly thicker compared to the control group ($p<0.001$) and one and two weeks p.op. Intermediate Toe Spread (ITS) was highest for animals receiving Diet G ($p<0.001$), indicating less pain. Repair tissue of animals receiving Diet G was significantly stiffer compared to the control group ($p<0.05$), whereas maximum tensile loads were comparable. The proportion of Ki67+ cells in the repair tissue was 3.3% in the Diet C group, 9,8% in the Diet G group and 8.4% in the Diet F group, indicating an increased cell proliferation ($p<0.001$). Finally, gene expression analysis revealed chondrogenic marker genes to be upregulated (Fig.1).

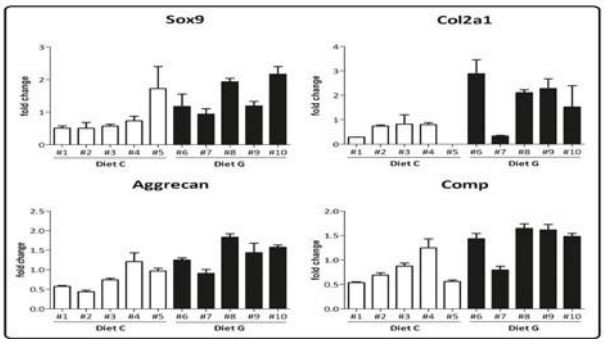

Fig. 1: Quantitative PCR analysis shows an upregulation of the chondrogenic marker genes Sox9, Col2a1, Aggrecan, Comp. Results shown for individual animals.

DISCUSSION & CONCLUSIONS: A high-glucose diet improves gait pattern and tendon biomechanics, influences tendon thickness and cell proliferation. Gene expression analysis reveals an upregulation of chondrogenic marker genes. The molecular mechanisms underlying these effects on cells and extracellular matrix are currently under investigation, potentially revealing targets for developing a dietary intervention scheme to support tendon regeneration after trauma or tendon disease.

ACKNOWLEDGEMENTS: Sparkling Science (SPA-05/058) – a program of the BMWFW, Austrian Cluster for Tissue Regeneration, Lorenz Böhler-Fond, Marianne&Hermann Straniak Foundation, Red Bull GmbH, Rauch Fruchtsäfte GmbH and Rexam PLC.

The clinical application of BMP for reconstruction of alveolar cleft

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INTRODUCTION: Orofacial clefts are one of the most common birth defects with an incidence of 1 in every 600-700 live births. Autogenous bone graft is the gold standard for the reconstruction of alveolar cleft which is associated with cleft lip and palate. However, harvesting of bone graft is associated with well documented morbidities including pain and swelling [1]. Therefore, researchers worldwide are seeking an alternative approaches, based on tissue bioengineering, to avoid these morbidities. Based on a series of preclinical studies [2-4] carried out by our team a clinical trial on the application of BMP-7 for the reconstruction of alveolar cleft.

METHODS: This study with conduct as prospective phase II clinical trial on 11 cases, 9 unilateral and two bilateral alveolar clefts. In all the cases the alveolar cleft was reconstructed with BMP-7 (Osigraft, OP1, Stryker Biotech, UK). This was reviewed and approved by the local ethics committee, regulatory authority (MHRA) and research & development. A standard exposure of the cleft area was performed (Figure. 1). For each case a vial of Osigraft® had 3.5mg of eptotermin alfa (a recombinant human osteogenic protein 1"Op-1") in bovine collagen (a bioresorbable scaffold) was applied at the alveolar defect. A putty additive (Stryker Biotech) containing carboxymethyl cellulose was added to the Osigraft® and this mixture was reconstituted with 2 to 3 ml of sterile 9 mg/ml sodium chloride solution (0.9% w/v) prior to use. The graft was then packed locally into the cleft region. The reconstituted product was administered by direct surgical placement in the alveolar cleft site in contact with the bone surfaces (Figure 1). Patients were examined clinically to assess the magnitude of facial swelling, discomfort and any signs of infection following surgery. Immediate post-operative radiographs were taken to assess the shape and appearance of the grafts and the position of the associated impacted. At three months the healing pattern and eruption of the adjacent teeth was assessed. Six months following surgery, the patients underwent their final radiographic scan which was used for the assessment.

RESULTS: The mean age of patients at operation was 10.4 years ranging from 8.8 years to 11.6 years. Clinically, postoperative complications were minimal; the surgical site healed well, all the patients were discharged from the hospital the next day after surgery. No critical incidents were reported. All the cases resumed the postoperative orthodontic phase of treatment. The extended of the bone formation in the unilateral alveolar cleft was scored as grade 1 (>75% in fill) in all the cases except one which scored grade II (50-70% bone infill). The bone showed normal trabeculation. None of these cases required further grafting. In the bilateral alveolar clefts, good bone formation was noted in one side only. Impacted teeth erupted in the newly generated bone.

Fig. 1: Images of the alveolar cleft defect and the application of rhBMP-7 (Osigraft).

DISCUSSION & CONCLUSIONS: In this study the donor site morbidity was completely eliminated by using rhMBP_7 for the reconstruction of the alveolar cleft. In addition, the duration of the surgical procedure was reduced and stay in the hospital was limited to one day. Radiographic assessment gave an objective and reliable indication on the quality and the quantity of the newly formed bone. In bilateral alveolar cleft cases, bone formation was limited to one side due to volume of the injected material, increase of the dose of the applied BMP was not an option due to the hypothetical potential risk of excessive bone formation. This was a major concern in this particular age group of patients. In conclusion, the high rate of success (89% of unilateral alveolar cleft cases) in this study should encourage a larger scale clinical trial and facilitate phase III comparative studies in comparison with autogenous bone graft.

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Cartilage degeneration is exacerbated by pro-inflammatory macrophages, but not inhibited by anti-inflammatory macrophages *in vitro*

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INTRODUCTION: Macrophages are present in many tissues, including the synovial lining in joints, and play a role in the progression of osteoarthritis (OA). They can acquire a phenotype that may range from pro-inflammatory to anti-inflammatory [1-2]. Depending on this phenotype, macrophages produce a repertoire of cytokines that can either stimulate or inhibit the regeneration of tissues, such as (engineered) cartilage. The aim of this study was to evaluate the effects of macrophage phenotypes on human articular cartilage by using phenotype specific macrophage conditioned medium (MCM).

METHODS: MCM was prepared by isolation of $CD14^+$ monocytes from 6 buffy coats (61±11Y). Monocytes were pooled and cultured with IFNγ+TNFα to obtain pro-inflammatory M(IFNγ+TNFα) macrophages, or with IL-4 or IL-10 to obtain anti-inflammatory M(IL-4) and M(IL-10) macrophages. After 3 days, stimuli were removed and macrophages were cultured for 24h to obtain MCM. Cartilage explants from OA patients were pre-cultured for 24h in DMEM, followed by culture in 50% MCM (*n*=6, 68±7Y), or a combination of 50% M(IFNγ+TNFα) plus 50% M(IL-4) or M(IL-10) MCM (*n*=2, 65±2). To simulate acute inflammation, cartilage (*n*=3, 61±4Y) was pre-stimulated for 24h with 10 ng/mL IFNγ and TNFα, followed by culture in 50% MCM. Explants cultured in unconditioned medium were included as controls. To assess the effects of the MCM, the cartilage was analysed for genes encoding inflammatory proteins: Interleukin-1β (*IL1B*), *IL6*, tumour necrosis factor-α (*TNFA*), suppressor of cytokine signaling-1 (*SOCS1*) and *SOCS3*, genes encoding for matrix degeneration enzymes: matrix metalloproteinase 1 (*MMP1*), *MMP13*, A disintegrin and metalloproteinase with thrombospondin motifs-4 (*ADAMTS4*) and *ADAMTS5*, and genes encoding extracellular matrix components: aggrecan (*ACAN*) and collagen type II (*COL2A1*). Nitric oxide (NO) production was measured as an indication of inflammation and release of glycosaminoglycans (GAGs) as indication of cartilage degeneration.

RESULTS: In OA cartilage, M(IFNγ+TNFα) MCM enhanced *MMP13* and *ADAMTS5*, NO production and GAG release, while *ACAN* and *COL2A1* were decreased. M(IL-4) did not affect any of the genes of interest, NO production or release of GAGs. M(IL-10) increased NO production to some extent and did not affect the genes of interest or GAG release. NO production by the cartilage was increased when cultured in M(IFNγ+TNFα), M(IFNγ+TNFα)+M(IL-4) and M(IFNγ+TNFα)+M(IL-10) MCM compared with cultures in DMEM. No effects were seen on gene expression levels when the cartilage was cultured in any of the MCM combinations compared to M(IFNγ+TNFα) only. Combined, these data suggest that M(IFNγ+TNFα) exacerbated cartilage degeneration, while M(IL-4) and M(IL-10) were unable to protect cartilage degeneration. To further investigate the potential effects of M(IL-4) and M(IL-10), the cartilage was pre-stimulated with IFNγ+TNFα to enhance inflammation. M(IL-4) MCM reduced *ACAN* of the stimulated cartilage, while NO production and GAG release were unaffected. M(IL-10) had no effect on genes of interest, NO production or GAG release of the stimulated cartilage.

DISCUSSION & CONCLUSIONS: M(IFNγ+TNFα) MCM exacerbated cartilage matrix degeneration, inhibited matrix production and induced inflammation, while M(IL4) and M(IL10) were unable to protect the cartilage from these processes, also after application of additional inflammatory stimulation. This suggests that pro-inflammatory macrophages may play a more prominent role in cartilage degeneration than anti-inflammatory macrophages. Pro-inflammatory macrophages should therefore be considered as important targets for strategies that are aiming to improve regeneration of (engineered) cartilage tissue.

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Modulating the behaviour of macrophages activated by biomaterials: an *in vitro* study

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INTRODUCTION: Biomaterials are commonly used in orthopaedics, gynaecology, cardiology, and reconstructive and general surgery. Moreover, the frequency in which bioengineered tissues are being implanted is expanding rapidly. After implantation, the host immune system will immediately interact with the material by inducing an inflammatory response that involves cells such as macrophages, T-cells and B-cells. Macrophages play a key role in this initial response, become activated upon receiving stimuli from their environment, and can acquire a phenotype ranging from pro-inflammatory (M1) macrophages to anti-inflammatory (M2). The aim of this study was to assess whether the behaviour of human monocyte-derived macrophages activated by biomaterials can be modulated using the compounds rapamycin, dexamethasone, celecoxib or pravastatin. These compounds are members of commonly used medication groups and are in literature described to have effects on inflammation [1-4].

METHODS: Monocytes were isolated by $CD14^+$ selection from 9 buffy coats (47±15Y). The monocytes were pooled and seeded by rotation in a tube rotator at 20 rpm on polyethylene terephthalate (PET; 1.34 g/cm^3) and polypropylene monofilament (PP; 0.9 g/cm^3) yarns. $0.85 \cdot 10^6$ monocytes were subjected to each yarn. The materials including adhering monocytes were then cultured in X-VIVO medium in 48-well plates for 3 days to allow for differentiation into macrophages. After this, 1 ng/mL rapamycin, 10^{-8} M dexamethasone, 0.1 µM celecoxib or 0.5 µM pravastatin were added to the biomaterials and cultured for an additional 3 days. DMSO was included as vehicle control for rapamycin and dexamethasone. After culture, the medium was harvested for IL-6 and CCL18 protein quantification by ELISA. The macrophages attached to the biomaterials were harvested for DNA quantification and gene expression analysis. Expression of Interleukin-1B (*IL1B*), IL6, Tumor necrosis factor-α (*TNFA*), chemokine (C-C motif) ligand 18 (*CCL18*), Mannose receptor, C type 1/CD206 (*MRC1*/*CD206*) and cluster of differentiation 163 (*CD163*) were assessed. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as housekeeper and the relative expression was determined by the $2^{-\Delta CT}$ formula. As an indication for the overall inflammatory state of the macrophages, an M1/M2-index was calculated based on the expression of genes encoding for pro-inflammatory proteins (*IL6*, *IL1B* and *TNFA*) and the anti-inflammatory proteins (*CCL18*, *IL1RA*, *CD206* and *CD163*). The experiments were repeated three times with biological triplicates using pooled monocytes from 3 buffy coats per experiment.

RESULTS: Dexamethasone reduced the overall inflammatory state of macrophages that were cultured on both PET and PP as expressed by a lower M1/M2-index and decreased CCL18 protein production. Pravastatin tended to have an anti-inflammatory effect on the macrophages cultured on PP as indicated by a lower M1/M2-index. Rapamycin decreased CCL18 production of macrophages cultured on PET and celecoxib increased IL-6 production to some extent.

DISCUSSION & CONCLUSIONS: These data indicate that the behaviour of macrophages can be modulated by dexamethasone after adhering to PET or PP. The modulatory capacity of the compounds might depend on the biomaterial that activates the macrophages since pravastatin tended to modulate macrophages adhering to PP and not adhering to PET, while rapamycin seemed to suppress the anti-inflammatory state of macrophages that were cultured on PET. Combined, this provides insights into the possibility of using a compound in combination with a specific biomaterial to direct macrophage behaviour and thereby possibly avoiding unwanted effects after implantation of a biomaterial.

Effects of varying degrees of surface strain anisotropies in combination with fluid flow shear stresses on endothelial cells

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INTRODUCTION: Cyclic strain and fluid flow are well known to affect cell behaviour. It is also known that isotropic and anisotropic strain can affect cells differently [1]. While in-vivo cells experience varying degrees of anisotropy (d.o.a.), in-vitro anisotropic strain studies have mostly focused on uniaxial strains. In order to create a better understanding of cellular behaviour under physiological strain conditions, the response of cells to strains with varying d.o.a. should be investigated.

In this study, we determined the effects of varying d.o.a., in combination with fluid flow shear stresses, on human umbilical vein endothelial cells (HUVECs) using a newly developed device. Studies like this can be used to determine and optimize the mechanical stimulation needed to elicit physiological cellular responses, and are beneficial for applications such as tissue engineering and organs-on-chip systems.

METHODS: The device is a modification of our previously developed device to apply surface strains in combination with flow induced shear stresses to cells [2]. It has 100 units producing various anisotropic strains. This is achieved by stretching a polydimethylsiloxane (PDMS) membrane over circular pillars into surrounding ellipse trenches. The dimensions of the ellipse determine the d.o.a., which is defined as the ratio of maximum to minimum principal surface strains. The presence of fluid flow channels at varying angels to the ellipses allows for the determination of combined effects of anisotropic strains and flow induced shear stresses.

HUVECs were seeded on the device and allowed to attach for 18 hours. The cells were then mechanically stimulated (maximum principal strain up to 10% at 1 Hz, sine strain profile). Simultaneously, cells were subjected to fluid flow shear stresses of ~5 dyne/cm^2. The cells were fixed after 24 hours and stained with Alexa 488 Phalloidin (actin stain) and DAPI (nuclear stain) and imaged again. Images were analyzed using CellProfiler to detect the effects of the mechanical stimuli on the cells.

RESULTS: The models and empirical measurements showed that strains with varying d.o.a. could be generated on the device. Maximum principal strains up to ~20% could be achieved.

HUVECs were found to become elongated and align along the minimum principal strain direction when only strain was applied. An increase in d.o.a. resulted in increased cell alignment. Cells aligned along the flow direction when only flow was applied. When flow and strain were combined, alignment was predominantly in the direction of the flow, but an offset towards the minimum principal strain direction was detected compared to flow alone (see fig.1).

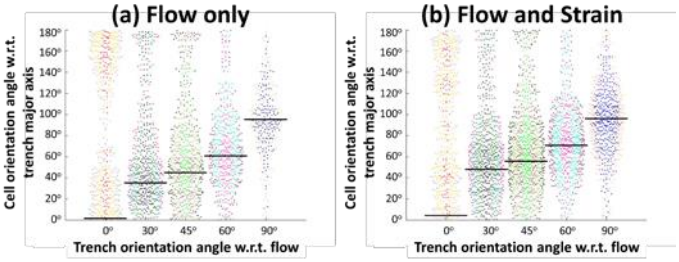

Fig. 1: Plots showing the orientation angles of individual cells with respect to the trench major axis. Panel A shows cells exposed to flow alone, while panel B shows a combined exposure to flow and strain.

DISCUSSION & CONCLUSIONS: HUVECs respond to various d.o.a. With an increasing d.o.a., the cells show an increasing alignment response. The variations in response of cells highlight the need to study the effects of strains of varying d.o.a. on cells. Our device permits such experiments with an increased throughput, which makes it an important tool to better understand these mechanobiological principles.

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Human liver organotypic membrane bioreactors

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INTRODUCTION: The development of bioartificial devices that are able to favour the liver reconstruction and to modulate cell behaviour is an important challenge in liver tissue engineering. As strategy we realized liver organotypic systems by using membrane bioreactors [1-2]. The organotypic systems consist of human hepatocytes in co-culture with non-parenchymal cells and/or mesenchymal stem cells.

METHODS: Oxygen-permeable membrane bioreactor and hollow fiber membrane bioreactor have been used for the development of liver organotypic systems. Co-culture of primary human hepatocytes with endothelial cells and mesenchymal stem cells have been performed in an oxygen permeable membrane bioreactor in which cells are seeded between flat-sheet gas-permeable membranes, which ensure the diffusion of O_2 and CO_2 and simultaneously provide a support for cell adhesion. A different system has been realized by using hollow fiber membrane bioreactor in which human hepatocytes were co-cultured together with sinusoidal endothelial liver primary cells and stellate liver cells. This bioreactor consists of two bundles of hollow fiber membranes that provide cells nutrients and metabolites and remove catabolites from cell compartment mimicking in this way the in vivo arterious and venous blood vessels. The metabolic performance of liver organotypic were evaluated in terms of urea synthesis, albumin production and diazepam biotransformation functions.

RESULTS: The morphological analysis shows the formation of a tissue-like structures ranging in size from 250 µm to over 1 mm (Fig.1) in organotypic systems. The immunohistochemical studies confirmed the tissue-like arrangement of parenchymal and non-parenchymal liver cell populations in the organotypic culture. Hepatocyte aggregates contained tube-like structures that stained positive for the endothelial cell marker CD31, and stellate cells as detected by the staining for desmin (Fig. 2). The analysis of liver-specific functions showed significantly higher albumin and urea synthesis rates throughout the experiment in the case of organotypic culture in comparison to the homotypic ones.

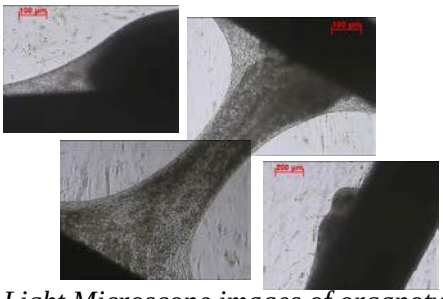

Fig. 1: Light Microscope images of organotypic culture in batch bioreactor.

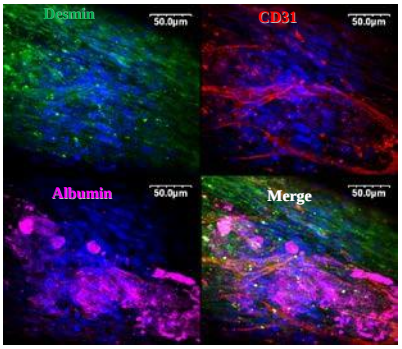

Fig.2 Confocal laser micrographs of human hepatocytes, sinusoidal and stellate cells in organotypic HF membrane bioreactor.

DISCUSSION & CONCLUSIONS: Organotypic membrane co-culture systems by using primary human hepatocytes in co-culture with non-parenchymal cells (stellate and sinusoidal endothelial cells) and/or mesenchymal stem cells were developed in membrane bioreactors to ensure an adequate mass transfer of nutrients and metabolites. The formation of large tissue-like cell associations within the bioreactors indicates active reorganization of the cells in the culture system. Structures observed in the bioreactors resembled those found in human liver tissue, which indicates that a physiological cell reorganization process occurs in the 3D bioreactor perfusion systems.

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BIOART/Kidney-Liver symposium

Repair of Ischemically Damaged Renal Allografts During Ex Vivo Warm Perfusion

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INTRODUCTION: The best course to solving the world's chronic shortage of transplantable kidney allografts will be the development of technologies that can be used to repair ischemically damaged kidneys. The vast majority of recovered deceased donor kidney allografts come from the traumatic injury fatality that is declared dead by brain criteria. The result is a kidney allograft that is undamaged or minimally damaged. Kidneys with >30-minutes of warm ischemic damage are never transplanted because the damage that occurs upon cardiac arrest has been an insurmountable obstacle to recovering life-sustaining normal renal function. A goal of regenerative medicine is to successfully tissue engineer the damaged cells in a 3-trillion cell kidney allograft to restore the integrated heterogeneous cell population that is capable of providing normal renal function. The ability to achieve this goal will be dependent upon the ability to adequately support metabolic and corresponding synthetic functions in an ischemically damaged kidney during an extended period of ex vivo near-normothermic perfusion.

METHODS: The requisite need for ex vivo near-normothermic perfusion platforms that support oxidative metabolism of sufficient magnitude for new synthesis that is requisite for cellular reparative processes will be reviewed. The ex vivo organ perfusion technologies that have been developed will be presented along with the corresponding potential to completely repair ischemically damaged kidneys during extended periods of warm perfusion. Particular focus will be placed on the need to have perfusion periods of long enough duration to support reparative processes in conjunction with the requirements to accomplish specific reparative pathways for adequate organ regeneration.

RESULTS: Successful ex vivo warm perfusion of warm ischemically damaged human kidney allografts has been achieved for periods of several days. During the ex vivo perfusion oxidative metabolism can be successfully resuscitated. The resuscitated oxidative metabolism restores the intracellular ADP/ATP ratio providing the cellular energetics requisite to supporting the synthetics

functions that are the basis of renal regeneration. Sufficient renal regeneration results in restored cytoskeletal integrity during a 48-hour period of warm ex vivo perfusion if oxidative metabolism and synthetic functions are adequately resuscitated.

Cytoskeletal Evaluation by ZO-1 Staining

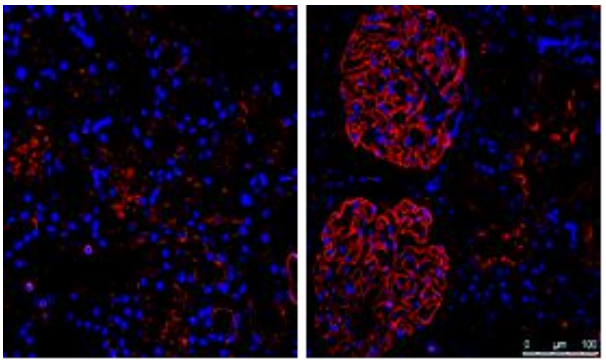

Fig. 1: A. Ischemically damaged kidney with diffuse cytoplasmic staining (left). B. Kidney after 48hrs of EMS perfusion with localized ZO-1 staining to cellular junctions. (right).

DISCUSSION & CONCLUSIONS: In summary warm perfusion of human renal allografts results in resuscitated oxidative metabolism of sufficient magnitude to result in restored intracellular ATP pools, recovered synthetics functions and the observed renal regeneration. The observed renal regeneration that is mediated by the ex vivo warm perfusion requires a period of 48 hours. The ability to overcome a severe ischemic insult of as much as two hours represents a framework where the majority of fatalities from traumatic injuries in the major metropolitan areas would be a large untapped pool of organ allografts that could lead to a paradigm shift in the field of transplantation.

DISCLOSURE: The author has nothing to disclose.

Immunologic basis for a novel organ-specific therapy: immunocloaking

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INTRODUCTION: We have previously reported on a novel organ-specific immunomodifying therapy referred to as immunocloaking (IC) that provides protection from early allograft rejection without the need for immunosuppressive drugs [1]. The IC membrane is comprised of extracellular matrix proteins and is applied during ex vivo warm perfusion where it binds to the luminal surfaces of the vasculature within kidneys. The IC membrane functions by interrupting the interface between the donor vascular endothelium (VEC) and recipient immune cells; without adversely affecting renal function. The result is a newly engineered non-thrombogenic/non-immunogenic apical surface. We now report the underlying protective mechanisms involved with IC.

METHODS: The IC membrane was applied to confluent human VEC. The immunologic testing included: effect of IC on antigen presentation, T cell activation and diapedsis using standard methodology with flow cytometry, Luminex screening, proliferation assays and chemotactic-driven diapedesis. The testing used responding mononuclear cells (MNC) stimulated by confluent allogeneic VEC without IC (positive control) and the same VEC that were IC (test). Transendothelial migration involved VEC grown on filters in Transwell™ plates. MNC were added to the upper chambers and the chemoattractant SDF-1, a potent lymphocyte chemotactic factor, was added to the lower chambers to form chemokine gradients.

RESULTS: The immunocloaking membrane prevents antigen presentation, early T cell activation (Figure 1) as well as T cell proliferation. Transmigration: In negative controls without SDF-1 few MNC migrated through the VEC layers. In positive controls >65% of the cells migrated through the VEC into the chambers containing the SDF-1. When VEC monolayers were IC, the MNC migration into the chambers with the SDF-1 was inhibited by a mean of 86.7% (Figure 2).

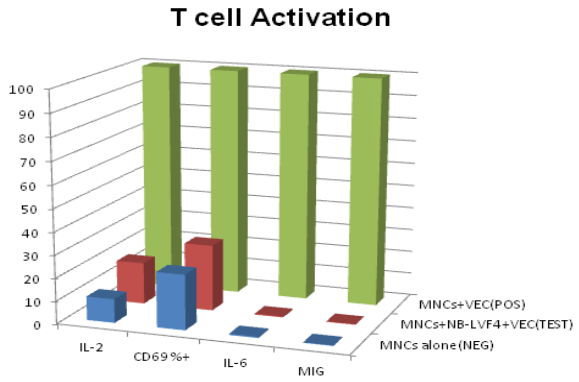

Fig. 1: Allo-immune assays were performed with or without the immunocloaking membrane. Results were analyzed on the Luminex and by Flow cytometry.

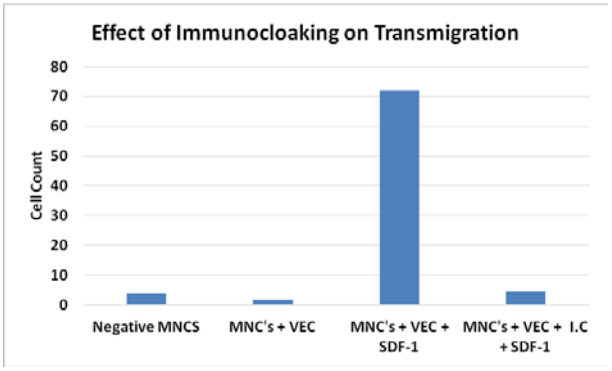

Fig. 2: Chemotactic migration of MNC through Transwell with SDF-1 (10 ug/mL). Final counts were performed using a Necexelom Cellometer.

DISCUSSION & CONCLUSIONS: We previously reported that IC prevents renal allograft rejection for a mean of 30 days without systemic immunosuppression; although the mechanisms involved were unknown. We now show that IC temporarily prevents alloresponses. Significantly, IC prevents chemokine-stimulated transmigration. Eliminating the need for nephrotoxic drugs during the early posttransplant period could help to ameliorate the severity of delayed graft function.

DISCLOSURE: The authors have nothing to disclose.

Controlled differentiation of mesenchymal stem cells towards distinctive chondrogenic phenotypes for tailored articular cartilage tissue engineering

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INTRODUCTION: Articular cartilage is the connective tissue present at the end of long bones allowing for frictionless movement upon skeletal articulation. Cartilage is a multi-zonal material in which chondrocytes assume different phenotypic states depending on the zone they are, i.e., persistent, proliferative and hypertrophic chondrocytes. These distinct chondrocyte sub-types are responsible for the deposition of extracellular matrix (ECM) that is specific to each of the cartilage zones, providing the tissue with unique mechanical properties. However, upon injury or degeneration, cartilage is unable to self-heal and surgical intervention is usually required. Current cartilage regeneration and tissue engineering efforts have so far only resulted in the deposition of an immature and isotropic neocartilage that lacks adequate differentiation status of the cells and insufficient mechanical properties to withstand the loads applied to the tissue.

METHODS: We hypothesized that mimicking the biochemical environment of cells in the different zones of cartilage will drive the differentiation of MSCs towards the distinct chondrocytic phenotypes found in native tissue, guiding the deposition of a specialized ECM. We identified a library of molecules responsible of cell-cell and cell-matrix interactions that are involved in the differentiation of MSCs towards chondrogenic and osteogenic lineages during development.

We performed a systematic study of the differentiation of MSCs towards the aforementioned phenotypes by presenting different peptide sequences and ratios representative of E- and N-cadherin (cell-cell interactions), collagen I and IV and, decorin and heparin sulphate cell recognition sites (cell-matrix) (Figure 1).

RESULTS: Peptide presentation was achieved in a controlled manner via peptide-modified ß-cyclodextrin molecules that were conjugated to surfaces coated with a phase separating (Polystyrene-co-Polyethyleneoxide)-Adamantyl block copolymer.[1] The phenotype of MSCs cultured on surfaces presenting different peptide sequence combinations and ratios were analysed by morphometry, polymerase chain reaction (PCR) and inmunohistological experiments. Our results indicate that fine tuning of the phenotype of MSCs differentiated towards persistent, proliferative and hypertrophic chondrogenic lineages is indeed possible and dependent on the presented biochemical environment.

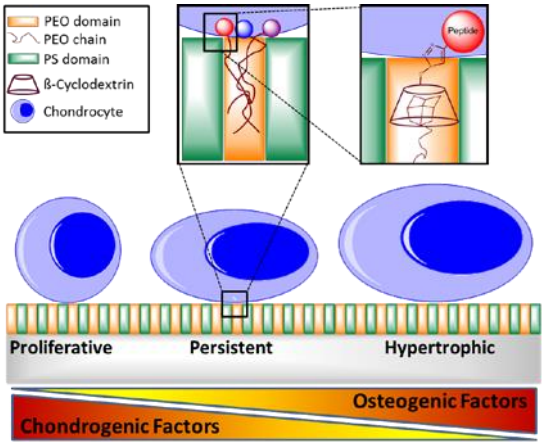

Fig. 1: Schematic illustration of the phase separating block copolymer and the differentiation of MSCs into the distinct chondrogenic lineages found in articular cartilage in function of the presented peptide sequences.

DISCUSSION & CONCLUSIONS: Our findings detail a well-defined chemistry that can be exploited in three-dimensional spatially-controlled biomaterials to engineer anisotropic and mature hyaline cartilage with properties that are reminiscent of the native tissue.

ACKNOWLEDGEMENTS: This work was supported by the Swiss National Science Foundation (Early Postdoc.Mobility. P2FRP2_158734) and the Australian Research Council Discovery Grants Scheme (DP140104217).

Directed cell growth in multi-zonal tissue engineering scaffolds for cartilage regeneration

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INTRODUCTION: Articular cartilage is the connective tissue responsible for frictionless movement between bones, supporting the loads applied during movement. Upon damage of the tissue due to injury or wear and tear, cartilage cannot efficiently regenerate and, usually requires surgical intervention. Articular cartilage was predicted to be the first successfully engineered tissue but, it turned out to be difficult to reproduce the complex structure and biomechanical properties of the native tissue. Here, we report the fabrication and use of multi-zonal nanocomposite scaffolds that mimic in a spatially controlled manner the architecture, chemical cues, and mechanical properties of mature hyaline cartilage.[1]

METHODS: The new scaffold was based on poly(D,L-lactide), which was selected on account of its biocompatibility, mechanical properties, and on-set of degradation. Chemical cues that mimic the extracellular matrix (ECM) and potentially tune the cell morphology were imparted by the addition of cellulose nanocrystals (CNCs). These nanoparticles were chosen due to their ability to support cell growth, the chemical similarity to glycosaminoglycans (GAGs) and the ease of surface modifications. Sulphated -CNCs (S-CNCs) were chosen on account of their chemical similarity to sulphated- GAGs whereas phosphated- CNCs (P-CNCs) were chosen based on the expectation that they would promote the formation of hydroxyapatite (Figure 1) and provides an anchoring point to subchondral bone. The fabrication of the scaffold was done from independent porous layers that were prepared by different thermally induced phase separation methods and fused together in a final step affording scaffolds with virtually no interface. Thus, the multi-zonal scaffolds featured a superficial tubular layer oriented parallel to the surface, a middle isotropic layer, and a deep tubular layer oriented perpendicular to the surface. The mechanical and biochemical properties of the scaffolds were analysed before and after culture of human foetal chondrocytes for 2-4 weeks.

RESULTS: These scaffolds were able to guide the morphology, orientation, and phenotypic state of cultured chondrocytes in a spatially controlled manner, as revealed by histological images and gene expression measurements, respectively. The formation of cartilaginous tissue was evidenced by histology and by the increase in compressive mechanical properties of the tissue-scaffold construct that reached values of aggregate and Young's modulus on the range of the natural analogue. Moreover, the multi-layered scaffolds were able to selectively promote the formation of hydroxyapatite, as shown by electron microscopy and energy dispersive x-ray measurements.

Fig. 1: *Schematic representation of the architecture of native hyaline cartilage (left) and the multi-zonal scaffold (right).Reprinted from Camarero-Espinosa, S. et al. Biomaterials 74, 42-52 ©2016.*

DISCUSSION & CONCLUSIONS: Our results suggest that such biomimetic multi-zonal scaffolds can be used to grow neocartilage with characteristics that are similar to those of mature tissue and we expect that the architecture introduced here will serve as a blueprint for future tissue engineering efforts.

ACKNOWLEDGEMENTS: This work was supported by the Swiss National Science Foundation (National Research Programme 64, Project #406440_131264/1) and the Adolphe Merkle Foundation.

Liver microtissues in hollow-fiber membrane systems

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INTRODUCTION: Liver failure, due to various etiologies, is a life-threatening medical condition leading to over 220,000 deaths per year in Europe [1]. The only proven therapy is orthotopic liver transplantation; however, due to the limited number of donors, it is crucial to consider alternative therapies. Bioartificial liver (BAL) devices present themselves as a promising alternative that can act as a temporary support for liver failure patients. The BAL device should be able to maintain hepatocytes functional for long periods; since the liver-specific functions of primary hepatocytes, such as albumin secretion or drug-metabolizing activity, are rapidly down-regulated during *in vitro* cultures, limiting their use in BAL devices.

METHODS: Primary human hepatocyte spheroids were realised utilizing an agarose mold with 400-µm wide wells. Hepatocyte spheroids were seeded in a perfused crossed hollow-fiber membrane (HFM) bioreactor made of alternating polyethersulphone (PES) HFMs. Spheroids were also seeded on PES HFMs in batch system to compare between the static and perfused conditions.

Liver-specific functions were assessed in terms of diazepam metabolism, urea and albumin synthesis. Confocal laser scanning microscope (CLSM) and scanning electron microscope (SEM) analysis of the spheroids was carried out to study the interaction of cells within the spheroids as well as the interaction of spheroids with the HFMs. The cultures were maintained for more than 3 weeks.

RESULTS: Hepatocyte spheroids fused over time forming human liver microtissues around the hollow-fiber membrane as observed by light microscope. CLSM image (Fig. 1) confirms the fusion of multiple spheroids. The formation of tight cell-cell interactions, as well as the formation of bile canaliculi was observed in CLSM images, thus confirming the maintenance of polarity of hepatocytes which is important for their survival. SEM image (Fig. 2) shows the weak interaction of the spheroids with the PES HFMs that allow spheroids to be attached but prevents them from spreading and losing their 3D structures. Liver-specific functions were maintained in the perfused bioreactor throughout the whole period of the culture at levels higher than those in static conditions.

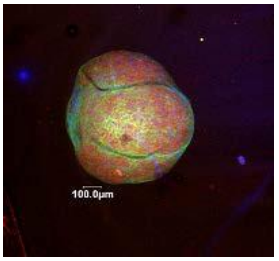

Fig. 1: CLSM image of fusing human hepatocyte spheroids cultured on crossed PES/PES hollow-fiber membranes.

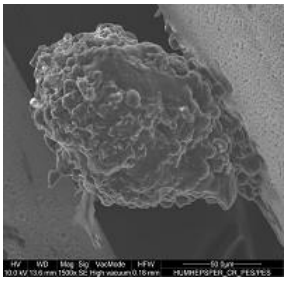

Fig. 2: SEM image showing the interaction of human hepatocyte spheroids with the PES hollow-fiber membranes. Magnification 1500X

DISCUSSION & CONCLUSIONS: A hollow-fiber membrane bioreactor was realised that allows the long term maintenance of functional human hepatocytes through the formation of liver microtissues via fusion of hepatocyte spheroids, paving the way for the development of a BAL device.

ACKNOWLEDGEMENTS: This work has been funded by a grant from the European Commission through the BIOART project within the framework of Marie Curie Initial Training Network, Contract No. FP7-PEOPLE-2012-ITN-316690. The authors declare that they have no conflict of interest.

BIOART/KIDNEY-LIVER

Effect of heterodimeric BMP2/7 conjugated to a fibrin-hyaluronan hydrogel on organ cultured intervertebral disc

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INTRODUCTION: Intervertebral disc (IVD) degeneration is a major cause of low back pain, a key factor of disability and enormous healthcare cost worldwide. Biological treatment with growth factors has evolved as potential therapy for IVD regeneration. Bone morphogenetic protein 2 (BMP2) and BMP7 have shown promise in this regard. In this study, we evaluated the effect of a BMP2/7 heterodimer releasing biomimetic fibrin-hyaluronan conjugate hydrogel (FBG-HA) [1] for IVD regeneration *in vitro* and in organ culture.

METHODS: *In vitro study:* Bovine nucleus pulposus (NP) cells were cultured at a density of $4x10^6$ cells/mL in beads of FBG-HA hydrogel containing 0, 1000 or 5000 ng/mL of covalently bound BMP2/7 [2] (n=6). Glycosaminoglycan (GAG), DNA content and gene expression of the samples were measured after 7 and 14 days. *Organ culture study:* Discoid polyurethane scaffolds (PUS) were manufactured with a hydrogel based core with swelling capacity in between two electrospun nanofiber envelope sheets [3]. Bovine caudal IVDs with endplates were nucleotomized by incision through the endplate and refilled with either (1) PUS, (2) PUS surrounded by 50-80 µL FBG-HA or (3) PUS surrounded by 50-80 µL FBG-HA containing 5000 ng/mL BMP2/7 (n=8). Empty discs served as controls. IVDs were cultured in an organ culture bioreactor under dynamic load (0-0.1 MPa, 0.1 Hz; 3 h/day) for 14 days. Disc height was recorded after load and recovery. After 14 days, gene expression, GAG and collagen content were assessed; proteoglycan synthesis was analyzed by S-35 incorporation rate. Histology was evaluated by Safranin O/Fast Green staining. Fourier transform infrared (FTIR) microscope imaging was performed on sagittal sections. ANOVA was used to determine statistical significance between groups.

RESULTS: *In vitro study*: On day 7, there was a trend of higher aggrecan expression in hydrogels with 5000 ng/mL compared to without (p=0.065) and 1000 ng/mL (p=0.067) BMP2/7. GAG/DNA ratio was significantly increased in cell-seeded hydrogel containing 1000 or 5000 ng/mL BMP2/7. On day 14, there was a trend of higher collagen II expression in hydrogels with 5000 ng/mL compared to without (p=0.085) and 1000 ng/mL (p=0.053) BMP2/7. *Organ culture study:* After dynamic load, all implant groups maintained their disc height, while it dropped by 7% in empty controls ($p<0.001$ *vs.* implant). When FBG-HA hydrogel containing BMP2/7 was implanted, the gene expression of aggrecan in the remaining NP increased 4.5-fold. The expression of collagen I and alkaline phosphatase was not affected by BMP2/7 treatment, indicating no adverse fibrotic or osteogenic effects. In the remaining NP in contact with the biomaterial, the GAG/DNA ratio increased by 46% when BMP2/7 was applied. Histologically the envelope of the PUS was observed as a ring in direct contact with the remaining NP, indicating favorable integration. FTIR analysis revealed localized differences in proteoglycan, collagen I and II to tissue ratios.

DISCUSSION & CONCLUSIONS: This study aims to develop a minimally invasive treatment strategy for NP regeneration by restoration of the IVD with implanted biomaterial and simultaneous NP regeneration with biological factors. Results indicate that the PUS is able to immediately restore the disc height after partial nucleotomy. The biomimetic FBG-HA hydrogel with BMP2/7 may support biological NP repair by stimulation of anabolic genes and restoration of new matrix, promoting integration of the biomaterial implant.

ACKNOWLEDGEMENT: This study was funded by the European Commission under the FP7-NMP project NPmimetic (246351).

3D printed PCL/ ß-TCP prosthesis for reconstruction of mandibular defects

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INTRODUCTION: The reconstruction of defects in face are very complicated because of its anatomical complexity and cosmetically importance. We developed novel artificial prosthesis using CAD assisted 3D printing. **METHODS:** A 10mm X 8mm sized defect was made on rabbit's right side mandible. The defect's CAD-CAM images were taken from three dimensional CT images of whole mandible (Fig. 1). Using these images double layered scaffold which was adjusted to the defect's shape were printed with mixture of polycaprolactone (PCL) and ß-tricalcium phosphate (ß-TCP) in equal proportion[1]. Scaffolds were designed to have double-layered structure (dense outer layer for fixation plate and porous inner layer for reconstruction of defect site). Eight rabbits were used for animal study; 2 animals for negative controls, 3 animals for scaffold-only graft (SC group), and 3 animals for scaffold soaking in peripheral blood (PB group). Scaffolds were fixed into the defect site with screw fixation. Evaluation of mechanical property and histology performed at 12 weeks after implantation.

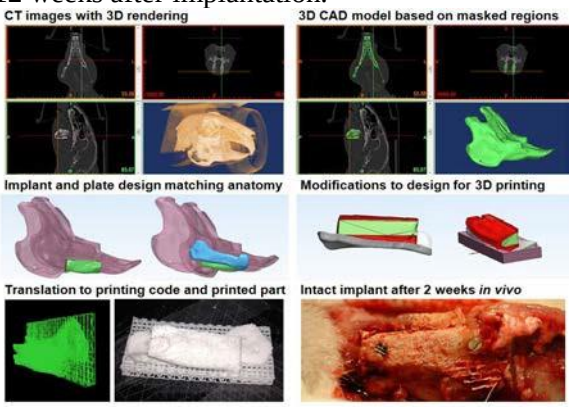

Fig. 1: Scheme of the reconstruction of bony defects with 3D printed PCL- ß-TCP scaffold

RESULTS: Significant regeneration of bone was noted in PB and SC group (Fig. 2). The pores of scaffold were filled with regenerated bone tissue. Compact cortex bone was also covered over the scaffolds. Its morphology was similar with normal lamella bone. There were no significant difference between PB and SC group (Fig.2).

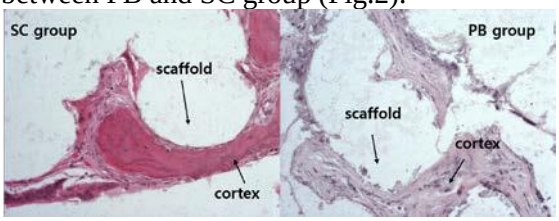

Fig. 2: Lamellar cortex bone was regenerated. The pores of the scaffold were filled with bone tissue (H&E staining, X 20)

Mechanical properties of implanted scaffolds showed significant differences between two groups. A mean compressive modulus were 1214 MPa (normal group), 28.15MPa (SC group) and 99.00MPa (PB group), respectively. Mean maximal strength were 115.08MPa (normal bone), 42.02MPa (SC group) and 88.35MPa (PB group) (Fig. 3). PB group showed more compatible mechanical properties than that of SC group.

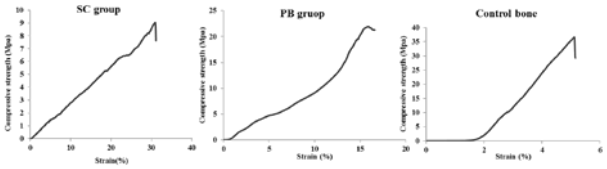

Fig. 3: Results of mechanical property test.

DISCUSSION & CONCLUSIONS: 3D printed PCL and ß-TCP scaffolds has proper mechanical property and biocompatibility on bone regeneration.

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

Gene transfer strategies to promote chondrogenesis and cartilage regeneration

Gun-Il Im

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Various transgene and gene transfer methods have been used to enhance chondrogenic differentiation and hypertrophy in adult stem cells and to reduce dedifferentiation in culture expanded articular chondrocytes. The current challenge for the clinical applications of gene modified cells is ensuring the safety of gene therapy while guaranteeing the effectiveness, namely, therapeutic levels of translated factors. Viral gene delivery methods have been mainstays in reported literatures. Enhanced safety features recently have shown great potential for clinical application of such technologies. On the other hand, efficiency has been greatly improved in nonviral delivery, which also includes the use of various scaffold materials. Considering that cartilage defect is nonlethal disease unlike genetic disease such as enzyme deficiency, the safety issue take precedence over efficiency issue. In this regard, nonviral delivery method is likely to be prevailing mode of clinical application for enhancing chondrogenesis, provided that the efficiency of nonviral gene transfer closely catch up with those of viral gene transfer. It is also not determined how long the transgene should be expressed to prevent unnecessary events. Future investigations are warranted to provide answers to these questions and finely tune the transfer technologies.

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

Stem cell culture on thermoresponsive nanobrush surface for continuous harvest

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INTRODUCTION: Stem cell culture is typically based on batch-type culture, which is laborious and expensive. Here, we propose a continuous harvest method of stem cells cultured on thermoresponsive nanobrush surfaces [1]. In this method, stem cells are partially detached from the nanobrush surface by reducing the temperature of the culture medium below the critical solution temperature needed for thermoresponsive. The detached stem cells are harvested by exchange into fresh culture medium. Following this, the remaining cells are continuously cultured by expansion in fresh culture medium at 37 °C.

METHODS: The thermoresponsive nanobrush surfaces were prepared by coating block copolymers containing polystyrene (for hydrophobic anchoring onto culture dishes) with three types of polymers: (a) polyacrylic acid with cell-binding oligopeptides, (b) thermoresponsive poly-N-isopropylacrylamide, and (c) hydrophilic poly(ethyleneglycol)methacrylate. Human adipose-derived stem cells (hADSCs) and human embryonic stem cells (hESCs) were cultured on the thermoresponsive nanobrush surfaces.

RESULTS: We developed a partial detachment stem cell culture process using a thermoresponsive nanobrush surface for continuous stem cell harvest (Fig. 1). During the cell detachment process, 50-90% of the stem cells are detached from the thermoresponsive nanobrush surface. Detachment is achieved by lowering the temperature to 4-20 °C after the cells have become confluent (first cycle). The few cells that remain attached start to re-expand after fresh culture medium is added and they are placed in a dish at 37 °C. The cells regain confluence after a few days in culture, and the process is repeated (Fig. 1). Stem cells can be continuously harvested during each culture medium replacement from the same dishes. The optimal coating durations and compositions for these copolymers to facilitate adequate attachment and detachment of stem cells were determined. hADSCs and hESCs were continuously harvested for 5 and 3 cycles, respectively, via the partial detachment of cells from the surfaces.

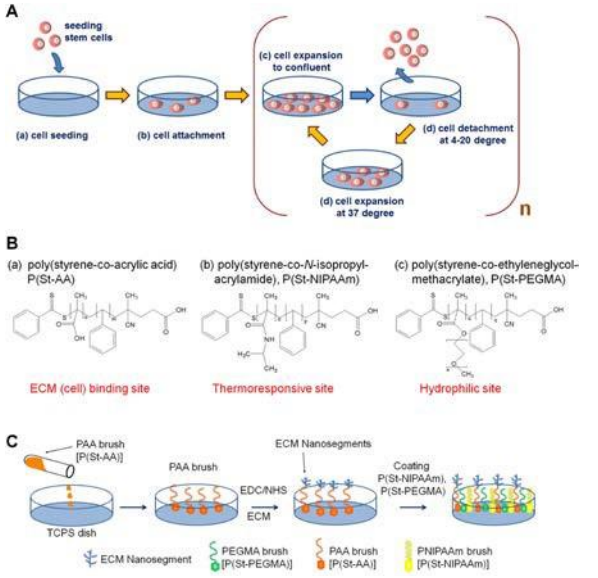

Fig. 1: Preparation of a thermoresponsive nanobrush surface. (A) Concept of continuous stem cell harvest. (B) Chemical structures of P[St-AA], P[St-NIPAAm]), and P[St-PEGMA]. (C) Preparation of a thermoresponsive nanobrush surface by coating a dish with P[St-AA] grafted with oligoVN, P[St-NIPAAm], and P[St-PEGMA].

DISCUSSION & CONCLUSIONS: We successfully demonstrated that culture on a thermoresponsive nanobrush surface enabled hADSCs and hESCs to be continuously harvested for five and three cycles, respectively, when using a partial detachment protocol. Such continuous harvest of stem cells should downsize the equipment requirements for stem cell culture and simplify the culture process. These improvements should decrease the costs of therapies using hESCs and hiPSC. As cost is the current bottleneck for clinical therapies using hESCs and hiPSCs, the methodology presented herein should expand treatment options.

ACKNOWLEDGEMENTS: Deanship of Scientific Research at King Saud University is acknowledged for its funding of Research Group Project No RG-1435-065.

shoot the target: hyaluronic acid based nano-therapies for cancer treatment

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INTRODUCTION: Innumerable cases of cancer are diagnosed every day, being responsible for more than 68% of deaths worldwide. Despite the increasing efficacy of treatments and important advances in understanding the molecular basis of the disease, chemotherapy resistance mechanisms still remain poorly understood.

The vast majority of cancer therapeutics screens are performed using simplistic *in vitro* models that fail at recreating physiological conditions e.g. multicellular, fluid-dynamic, 3D. Development of complex and dynamic *in vitro* tumour microenvironment (TME) is necessary in the optimisation of drug delivery systems.

CD44, a cell surface glycoprotein, is one of the biomarkers most commonly associated with malignant and chemo-resistant tumours [1]. Since the early 1990s, CD44 has been recognised as: 1) the major receptor of hyaluronic acid (HA) in cell membranes [2] and 2) one of the major players in tumour/metastasis genesis and growth [3]. As a result, HA-based therapies have been developed to target CD44 and treat cancer.

We here introduce an *in vitro* TME connecting three of the main cell types (cancer cells, macrophages, and fibroblasts) as a tool for tracking the fate of targeted HA-based nano-therapies. This more effective drug screening *in vitro* aims at: 1) reducing the costs (and time) of chemotherapy design and testing, 2) assessing tumour-targeting and treatment efficacy, and 3) speeding-up the pre-clinical *in vivo* development stage.

METHODS: Tumour-associated macrophages (THP-1-M), fibroblasts (HDF) and CD44-expressing cancer cells (PANC-1, HCT-116, HT-29, AsPc-1) were cultured both in static and dynamic condition. Cell viability, cytokines production, and matrix remodelling were measured in both cell culture conditions. HA-based nano-particles (NPs) were prepared as described by Almalik *et al.* [4]. In this study, chitosan (35 kDa), HA (300 kDa) and a fluorescently-labelled siRNA sequence (siGLO control, Dharmacon, UK) were used to prepare NP (~200 nm hydrodynamic diameter). Specifically, siRNA was used as reporter for both the quantification of targeted delivery, and the functional readout.

RESULTS: CD44 expression was characterised for several cancer cell lines, HDF and THP-1-M (Fig.1a), and a TME was selected. The targeted delivery of NP in the tumour population was monitored in both static (Fig.1b) and dynamic conditions by LSCM; while the targeted-delivery quantified using FCM.

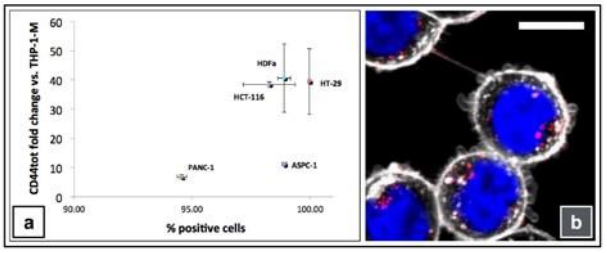

Fig. 1: a) Flow Cytometry: CD44tot in TME cells; b) Confocal microscope: NP uptake in HT-29 (2h, 0.125 mg/mL NP). Scale Bar: 10μm.

DISCUSSION & CONCLUSIONS: Targeted therapies for the selective transport of drugs to CD44-expressing tumour cells are presented herein. A more physiologically relevant *in vitro* TME was exposed to HA-based NP, showing the targeted delivery and efficacy under different culture conditions. Nevertheless, the design of more complex *in vitro* models will provide a means to understand the TME and its evolution during drug treatments, helping in the design of more accurate therapies for aggressive solid tumours.

ACKNOWLEDGEMENTS: Mr. Rios de la Rosa acknowledges EPSRC for a PhD studentship as part of the North-West Nanoscience (NoWNano) Doctoral Training Centre, EPSRC grant EP/G03737X/1. The Bioimaging Facility microscopes used in this study were purchased with grants from BBSRC, Wellcome and the University of Manchester Strategic Fund.

Purification of ruminant mammary stem cell population and uses thereof for production of transgenic proteins *in vivo*

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INTRODUCTION: while strong evidence has been gathered on adult mammary stem cells in mice and humans, only circumstantial evidence has been presented about their counterparts in the ruminant species. Given the economical interest revolving around dairy species and the histological similarities between the human and bovine mammary gland, the hypothesis of the existence of a bovine mammary stem cell population is certainly intriguing [1]. Given the high milk yield that can be obtained from a dairy breed like the Holstein-Frisian, the opportunity to use the mammary gland as a high efficiency bioreactor for the production of heterologous proteins has been quite appealing in recent years [2]. Thus weisolated a cell population enriched in stem cells which are able to (re)generate polarized and functional structures *in vivo* when transplanted in an animal model. We focused on engineering these cells for transgene production in milk since they represent a preferential target for *in vitro* manipulation.

METHODS:

Primary epithelial mammary cells or induced-pluripotent mammary stem cells (IPCs) [3] were used as source of mammary stem cells. Colony forming cell assay (CFC) and xenograft in NOD/SCID mice were used as models for testing functional properties in vitro and in vivo. Immunofluorescent and flow cytometry analyses were performed to characterized the regenerated structures.

RESULTS:

We report that in addition to a $CD49f^+$ phenotype, stem cells are $ALDH^-$ and p-$cadherin^-$. Furthermore we give evidence that stem cells come from the basal layer of the lumen ($K14^+$ and $K18^-$). These cells can be maintained in culture for up to 35 days but no expansion was observed. IPCs can be a continuous source of mammary stem cells but commitment to a mammary phenotype is hard to achieve.. Progesterone and estradiol administration induces a partial differentiation towards the mammary phenotype.

The complete regeneration of functional alveolar tissue in vivo was obtained only through the use of epithelial purified cells, therefore we obtained production of exogenous proteins by transfecting primary cells.

DISCUSSION & CONCLUSIONS: We were able to demonstrate that bovine mammary tissue contains lineage-restricted progenitors with in vitro clonogenic activity as well as more primitive uncommitted cells that regenerate bilayered multilineage milk-producing mammary structures when transplanted in immunodeficient mice. We reported the ability of isolated ruminant mammary cells to regenerate mammary tissue following their transplantation into a histocompatible recipient and also a proof concept that it is possible to engineer these cells to produce human beta casein, a major component of human milk [1].We also propose that the cells responsible to secrete endogenous and exogenous proteins in the lumen of alveolar-like structures retain peculiar features: they lack aldehyde dehydrogenase activity and p-cadherin expression while expressing high levels of CD49f. We propose that basal myoepithelial cells contain cells with stemness and that only luminal progenitors in the mammary gland are $ALDH^+$. A great milestone to achieve is to purify a large amount of these cells for further manipulation. Long term culture of primary mammary cells and new IPCs derived from mammary epithelial cells are far to be used for field applications.

ACKNOWLEDGEMENTS: This work was supported by University of Turin grant 2015.

In vitro assessment of the efficiency of poly(ester-urethane) scaffolds for the repair of bone defects

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INTRODUCTION: Elastomeric scaffolds may provide promising bone graft substitutes since they could fit easily in the bone defect and suppress shear forces at the bone-biomaterial interface leading to enhanced cells proliferation and bone regeneration promotion [1,2]. A new generation of porous biodegradable scaffolds based on poly(ester-urethane) (PCLU) was recently developed in our laboratory through a polyHIPE method [3]. With the aim to use these scaffolds in bone tissue engineering, the present study focuses on preliminary *in vitro* assays to assess the efficiency of PCLU scaffolds in terms of supporting the proliferation and differentiation of human mesenchymal stem cells (hMSCs).

METHODS: Porous cross-linked poly(ε-caprolactone urethane) (PLCU) scaffolds were prepared through a polyHIPE method as described previously [3]. Scaffolds were sterilized by autoclave prior hMSCs seeding. hMSCs-seeded PCLU scaffolds were maintained in osteogenic culture medium for up to 30 days to achieve cell infiltration, proliferation and differentiation. Environmental Scanning Electron Microscopy (ESEM) and DAPI staining were employed to visualise the hMSCs within the PCLU scaffolds. Alizarin red staining was carried out to assess hMSCs differentiation. The degradation of the PCLU scaffolds was also investigated up to 5 months in the culture medium through the determination of mass loss, water absorption measurements and FTIR analyses. The variation of the scaffold morphology was monitored by ESEM.

RESULTS: No scaffold degradation or morphological change was noticed during 5 months of incubation in the culture medium. The *in vitro* study demonstrated that hMSCs were able to adhere and proliferate within the PCLU scaffold. After 7 days of culture, hMSCs were elongated and well spread onto pore walls of the porous structure. After 30 days of incubation, pore walls were widely covered with a cell layer exhibiting a 3D spatial distribution layout (figure 1). Finally, the differentiation of hMSCs was put in evidence through alizarin red staining. Indeed, mineralization occurred after 14 days of incubation.

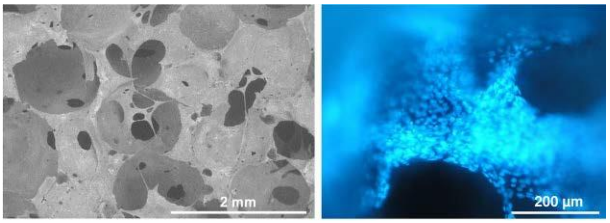

Fig. 1: Images of PCLU scaffold seeded with hMSCs after 30 days of incubation: ESEM micrograph (left), DAPI staining (right).

DISCUSSION & CONCLUSIONS: In a previous study, it was demonstrated that PCLU scaffolds did not elicit a cytotoxic response towards hMSCs and could be suitable biomaterials for supporting the growth of hMSCs [3]. The present work has shown that PCLU scaffolds are stable 3D structures that provide a suitable environment for hMSCs proliferation in 3D spatial arrangement. Moreover, PCLU scaffolds are adequate for the hMSCs differentiation into an osteoblastic phenotype lineage. Long-term *in vitro* and *in vivo* investigations are underway to assess the efficiency of PCLU scaffolds as graft substitute for the repair of bone defects.

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Tissue-engineered fibrin-based heart valve with bio-inspired textile reinforcement

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INTRODUCTION: Tissue-engineering has the potential to produce heart valves with growing and remodeling capabilities, able to last for a life-time without the need for reoperations. However, the mechanical properties of tissue-engineered heart valves (TEHVs) still need to be improved to allow their implantation in the systemic circulation [1]. The aim of this study was to develop a tissue-engineered textile-based valve for the aortic position by using a bio-inspired composite textile scaffold.

METHODS: The textile composite leaflet scaffold consisted of i) single multifilament fibres arranged similarly to the collagen bundles in the native aortic leaflet, fixed by ii) a thin electrospun (e-spun) layer directly deposited on the pattern. For each valve, three textile-reinforced leaflets were positioned into a 3D system for the fabrication of semilunar heart valves by injection moulding. Subsequently the components to form a fibrin gel containing vascular cells were injected into the mould and left to polymerize for 45 minutes. Once demolded, the valves (n=3) were cultivated into a custom-made bioreactor system for dynamic stimulation. After 21 days the valves' hydrodynamic performance was assessed under physiological aortic flow and pressure conditions according to ISO 5840-2. Mechanical properties were evaluated by burst strength measurements and tensile tests. Tissue composition was verified by histological and immunohistochemical stainings.

RESULTS: The Young´s modulus of the e-spun construct (2.1 ± 1.4 MPa) was significantly increased by the addition of single multifilament fibres (7.4 ± 1.0 MPa). Valves exposed to physiological aortic conditions demonstrated excellent functionality, with a mean gradient pressure of 10.7 ± 0.7 mmHg, regurgitation fraction of 4.0 ± 1.0 % and effective orifice area (EOA) of 1.4 ± 0.1 cm^2 (valve diameter of 1.8 cm). The neotissue burst at 1020 ± 100 mmHg. Immunohistochemistry revealed a pronounced deposition of collagens I and III and chondroitin sulphate.

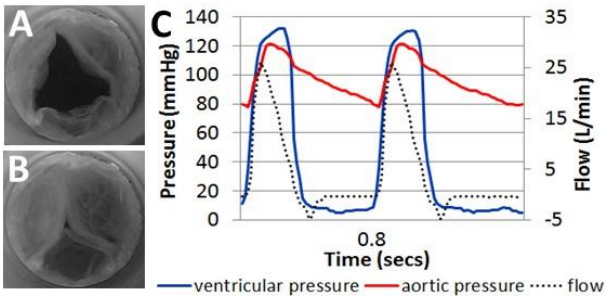

Fig. 1: Valves were tested in a flow mock-circulatory system. Frames showing the open (A) and closed (B) positions of the valve and representative pressures-flow curve (C).

DISCUSSION & CONCLUSIONS: TEHVs with anisotropic leaflet mechanical properties were developed. The obtained Young`s modulus of the textile composite scaffold was comparable to the native aortic leaflet [2]. The mean pressure gradient was comparable to the valves available on the market[3] and the EOA and regurgitation fraction were within the limits indicated by the ISO for a valve replacement of 19 mm (i.e. EOA ≥ 0.85 cm^2 and regurgitation ≤ 15%). This study shows the potential of biomimetic textile-reinforcement towards the fabrication of TEHVs to be implanted in the systemic circulation.

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).

Graphene Oxide promotes embryonic stem cell differentiation to haematopoietic lineage

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INTRODUCTION: Pluripotent stem cells represent a promising source of differentiated tissue-specific stem and multipotent progenitor cells for regenerative medicine and drug testing. The realisation of this potential relies on the establishment of robust and reproducible protocols of differentiation. Several reports have highlighted the importance of biomaterials in assisting directed differentiation. Graphene oxide (GO) is a novel material that has attracted increasing interest in the field of biomedicine.

METHODS: GO was prepared according to a modified Hummers method [1] followed by exfoliation, and characterised by Raman and XPS. Glass cover-slips were coated with GO by spin-coating and characterised by AFM and SEM.

Murine embryonic stem cells (ESCs) were differentiated to haemangioblasts as described previously [2]. Man-5 human ESCs were maintained on γ-irradiated MEF feeders in hESC basal medium as described previously [3].

Cell culture and differentiation was monitored by flow cytometry, hematopoiesis CFU-C assay, quantitative RT-PCR analysis, cell cycle and apoptosis analysis.

RESULTS: We demonstrate that GO coated substrates significantly enhance the differentiation of mouse ESCs to both primitive and definitive haematopoietic cells. GO does not enhance haematopoietic lineage specification through an increase in cell proliferation or survival. Instead, GO increases the generation of both primitive and definitive haematopoietic cells from haemangioblasts. As a result, GO promotes the transition of haemangioblasts to haemogenic endothelium and thereby positively influence both primitive as well as definitive haematopoiesis. Importantly, GO also improves, in addition to murine, human ES cell differentiation to blood cells.

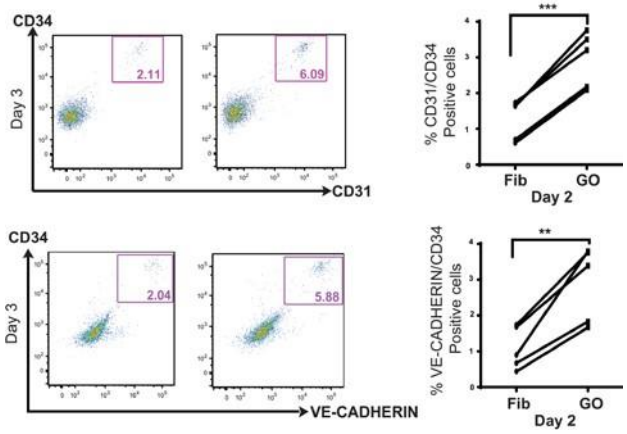

*Fig. 1: Role of GO on human haemogenic endothelial cell fate. (left) FACS analyses of human haemangioblast cultures at day3 grown on either Fibronectin or GO. Percentages of CD31/CD34 or VE-CADHERIN/CD34 positive cells in haemangioblast cultures grown on either Fib or GO at day2 (N=3). Asterisks indicate significant differences (Paired t-test. ***p<0.001, **p<0.01)*

DISCUSSION & CONCLUSIONS: Taken together, our study reveals a positive role for GO in haematopoietic differentiation and suggests that further functionalization of GO could represent a valid strategy for the generation of large numbers of functional blood cells. Producing these cells would accelerate haematopoietic drug toxicity testing and treatment of patients with blood disorders or malignancies.

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Graphene oxide scaffolds for neuronal and glial cell cultures to promote peripheral nerve regeneration

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INTRODUCTION: Peripheral nerve injuries represent a major health related problem as there are no pharmaceutical therapies available: the treatment of choice is the microsurgical repair and whenever an end-to-end suturing is not possible, the gold standard is the autologous nerve graft which results in loss of sensation at the donor site and full recover is not achieved. Therefore there is urgent need to find therapeutic alternatives to autologous nerve graft [1].

A new experimental approach to solve the lack of treatment of peripheral nerve injury is the incorporation of stem cells into biomaterial scaffolds in efforts to improve peripheral nerve regeneration. Our aim was to determine if graphene based substrates are suitable materials for this purpose. Four different groups of substrates were fabricated: graphene oxide (GO), reduced graphene oxide (rGO), IKVAV-functionalized graphene oxide (GO-IKVAV) and bare glass as control.

METHODS: AFM, XPS, FT-IR and Raman spectroscopy was used to show complete coverage of the graphene flakes on the substrates, confirmed the covalent binding between GO and IKVAV peptide, and verified the maintenance of the structural integrity after the functionalization process.

To assess the biocompatibility of the fabricated substrates, human adipose-derived stem cells differentiated toward neuroglial lineages were cultured on the graphene substrates. The expression of neurotrophic markers was evaluated by Real-Time quantitative PCR. Rat peripheral neurons were cultured on the graphene substrates and neuronal attachment and neurite outgrowth were evaluated by immunocytochemistry.

RESULTS: Real time quantitative PCR, showed significant multi-fold increase in the expression of BDNF (***P<0.001 on rGO and ****P<0.0001 on GO) and nestin (****P<0.0001 on rGO and ***P<0.001 on GO), together with other neurotrophic markers. Proliferation of human adipose-derived stem cells on the graphene substrates was comparable to the bare glass substrate. Furthermore, functionalisation of graphene coated substrates with the IKVAV peptide enhanced neuronal attachments and facilitated axonal outgrowth of rat peripheral neurons, as shown by immunocytochemical analyses.

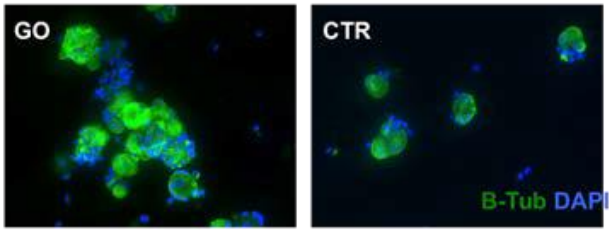

Fig. 1: Immunocytochemical analyses of peripheral neurons grown on GO and Glass control (CTR) substrates.

DISCUSSION & CONCLUSIONS: Altogether this evidence strongly positions graphene-based substrates as promising tools towards the improvement of stem cell differentiation and to enhance nerve regeneration.

Graphene-based substrates can offer many advantages compared to other materials: for instance graphene oxide due to its functional groups can be ideal to biochemically stimulate neurite outgrowth while reduced graphene oxide due to its electrical conductivity can be suitable to electrically stimulate neurite outgrowth and electrically-guided cellular alignment.

ACKNOWLEDGEMENTS: The research was supported by the Engineering and Physical Sciences Research Council (EPSRC/UK), National Institute for Health Research, the British Society for Surgery of the Hand and the Academy of Medical Sciences.

The development of a 3D tracheobronchial co-culture model with a bilayered collagen-hyaluronate scaffold: An innovative platform for respiratory tissue engineering

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INTRODUCTION: Epithelial dysfunction plays an important role in the pathophysiology of incurable chronic respiratory diseases. The development of physiologically-relevant respiratory epithelial models can improve disease modelling and the translation of novel inhalable therapies to the clinic [1]. This study sought to engineer a novel 3D tracheobronchial co-culture model with a bilayered collagen-hyaluronate (CHyA-B) scaffold. This CHyA-B scaffold was characterised and feasibility to support the growth and differentiation of a bronchial epithelial cell line assessed. Thereafter, a co-culture model of bronchial epithelial cells and lung fibroblasts was established and validated by analysis of biomarkers and epithelial barrier strength.

METHODS: CHyA-B scaffold was fabricated by a specialised freeze-drying process [2]. The top-layer was seeded with Calu-3 bronchial epithelial cells and the sublayer with Wi38 lung fibroblasts for 14-21 days of culture. MUC5AC (mucus), ZO-1 (barrier) and FOXJ1 (cilia) were examined as markers of epithelial differentiation. Barrier strength was quantified by transepithelial electrical resistance (TEER) and paracellular transport.

RESULTS: The CHyA-B scaffold was successfully manufactured and consisted of a dense top-layer 20µm in thickness fused to an interconnected porous sub-layer with pores of 80µm in diameter. Mechanical testing found a compressive modulus of 1.9kPa and demonstrated sufficient adhesive strength between the layers. Analysis of MUC5AC and FOXJ1 revealed greater expression levels on CHyA-B compared to standard tracheobronchial models (Fig. 1a, 1b). This translated to increased mucin secretion and ciliation (Fig. 1c, 1d). ZO-1 was also expressed by cells on CHyA-B. Co-cultured Wi38 fibroblasts migrated into the CHyA-B sublayer, reflecting *in vivo* bronchial tissue structure (Fig. 1e). The resultant cell-cell crosstalk in a 3D environment reduced the TEER to a more physiologically-relevant level, from 902Ωcm^2 to 694Ωcm^2 (Fig. 1f).

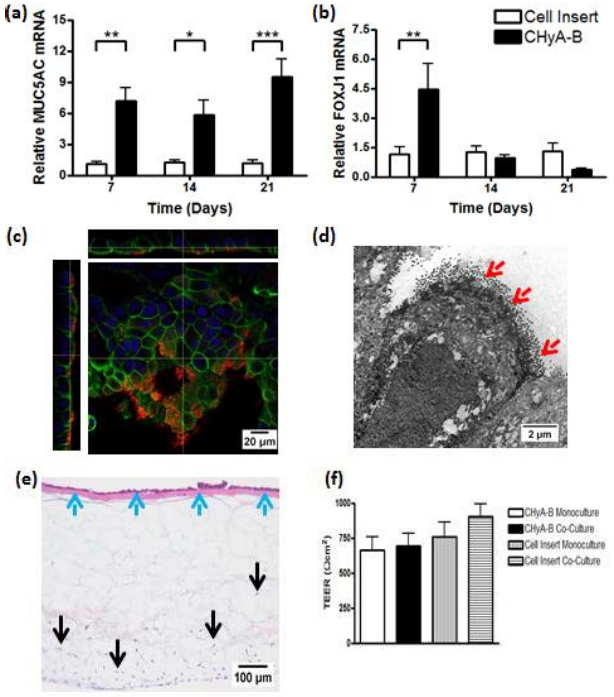

Fig. 1: (a, b) Relative MUC5AC and FOXJ1 mRNA expression by Calu-3 cells. (c) MUC5AC glycoprotein (red) secretion from Calu-3 cells. (d) TEM of Calu-3 cell ciliation on CHyA-B (red arrows). (e) H&E image of a Calu-3 monolayer (blue arrows) and fibroblasts (black arrows) in co-culture on CHyA-B. (f) Average TEER values of Calu-3 epithelial cell barriers (>day 10).

DISCUSSION & CONCLUSIONS: CHyA-B scaffolds supported the growth and differentiation of a bronchial epithelial cell line. A 3D co-culture model was developed that was reflective of *in vivo* bronchial architecture and a functional epithelium. Overall, CHyA-B scaffolds are an innovative 3D platform for the development of an *in vitro* tracheobronchial co-culture for respiratory drug development and disease modelling applications.

ACKNOWLEDGEMENTS: Funded by the PRTLI Cycle 5 & the ERDF. No conflicts of interest to disclose exist.

Bio-inspired tissue-engineered heart valve for mitral replacement with evidence of elastogenesis in vitro

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INTRODUCTION: Tissue-engineered heart valves (TEHVs) are promising candidates to outperform the available prostheses. However the in-vitro fabrication of heart valves which i) can function in the systemic circulation and ii) have elastin as extracellular matrix component is still a major challenge [1]. In this study we developed a TEHV for the mitral position able to withstand the physiological hemodynamic conditions and showing considerable elastogenesis in vitro.

METHODS: The tissue-engineered valve recapitulates the anatomical components of native mitral valves i.e. annulus, asymmetric leaflets and chordae tendineae and is fabricated by using fibrin gel embedding vascular cells isolated from human umbilical cord veins. After 25 days of dynamic cultivation, the valves' functionality was evaluated by hydrodynamic testing according to ISO standards. Tissue analysis included histology, immunohistochemistry, transmission electron microscopy, two-photon scanning microscopy, gene expression analysis and biochemical assays for collagen, sulphated glycosaminoglycans (sGAG) and elastin quantification. Elastogenesis was further evaluated with a time course by evaluation of valves after 7, 14, 21 and 25 days of cultivation (n=3 per time point).

RESULTS: The valves exhibited excellent hydrodynamic performance with an effective orifice area (EOA) of 2.2 ± 0.2 cm^2 (valve diameter of 2.6 cm), regurgitation fraction of 4.5 ± 1.5 % and mean gradient pressure of 2.4 ± 1.2 mmHg. Tissue samples burst at 916 ± 137 mmHg. Biochemical assays demonstrated presence of considerable amounts of collagen, sGAG and elastin. Elastogenesis was detected one week after initiation of dynamic cultivation with a steady increase of the elastin amount in the following cultivation period. Fibrillin-1, a molecule involved in elastogenesis, was detected throughout the whole cultivation period.

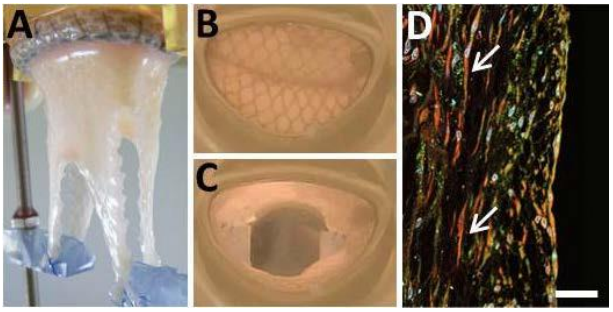

Fig. 1: TEHV after cultivation (A), closed (B) and open (C) positions of the valve inside the mock-circulatory system and two-photon microscope picture (D) showing presence of elastin (in red) as indicated by the white arrows. Scale bar: 50 µm.

DISCUSSION & CONCLUSIONS: TEHVs exhibited excellent hydrodynamic performance with regurgitation and EOA values within the limits established by the ISO 5840-2 for a replacement of 25 mm (i.e. regurgitation ≤ 15% and EOA ≥ 1.2 cm^2). The absence of mature elastin in cardiovascular tissue-engineered constructs might limit its long-term functionality in vivo [2]. We were able to detect elastogenesis in the valves, which is commonly not seen in TEHVs in vitro. These results demonstrate the feasibility of fabricating a fully functional, viable prosthesis to be implanted in the mitral position.

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).

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

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INTRODUCTION: Stromal Vascular Fraction (SVF) cells, freshly isolated from adipose tissue, are an abundant and easily accessible source of mesenchymal/endothelial progenitors. Previous studies from our group demonstrated the combined osteogenic and vasculogenic properties of adipose-derived cells. We aimed at investigating safety and feasibility of the clinical implementation of SVF cells for bone fracture augmentation in elderly individuals. The contribution of implanted cells to bone formation and vascularization was also investigated in a nude rat model of segmental fracture healing.

METHODS: Autologous human SVF-cells, intra-operatively isolated by the use of an automatic Celution system (Cytori, CA, USA), were loaded onto ceramic granules within a fibrin gel and implanted into 8 patients (age: 69 ± 7 years old; BMI: 27 ± 5) with standard open reduction and internal fixation (ORIF) following low-energy proximal humeral fractures. Safety was defined by absence of risks linked to the treatment. Feasibility was defined as the possibility to manufacture the autologous graft following good clinical practice (GCP) regulations and implant it, with control and traceability of the manufacturing process and by defining quality control markers to assess the graft. The repair tissue was biopsied upon plate revision or removal and analyzed. Similar constructs were implanted in a segmental femoral defect in immune-compromised rat after locking-plate osteosynthesis (RatFix, RISystem, Switzerland), with cell-free grafts as control.

RESULTS: The intra-operative cell isolation from abdominal liposuction (volume: 272 ± 63 ml), yielded 121.4 ± 72 million SVF cells. The manufacturing of the graft was feasible, as no relevant procedural deviations from the protocol were noted, and reproducible. The total duration of surgery (212 ± 26 minutes) was prolonged as compared to a standard ORIF (60-90 minutes) without bone grafting, due to liposuction (≈ 30 min) and cell isolation (≈ 110 min). The procedure was safe, without adverse reactions within a 12 month follow-up. Moreover, the duration of hospitalization (11 ± 3 days) and course of rehabilitation were in the same range as the ones observed in patients treated by ORIF alone. Microtomography and histology of the repair tissue demonstrated formation of ossicles (fig 1.), disconnected and morphologically distinct from osteoconducted bone, suggesting osteogenicity of implanted SVF cells. In the rat segmental defect model, only SVF cell-treated fractures did not fail mechanically and displayed vascularized bone with osteocytes and vascular structures of human origin.

Fig. 1: Hematoxylin/eosin staining of a section from a biopsy of graft implanted for 6 weeks in a humeral fracture in human. Compact red staining shows bone tissue inside a pore of the ceramic with embedded osteocytes

DISCUSSION & CONCLUSIONS: This study indicates that SVF cells, without expansion or exogenous priming but within a fracture-microenvironment, can de novo generate bone tissue. Clinical effectiveness of the proposed mode of action should now be tested in controlled trials with larger patient cohorts.

ACKNOWLEDGEMENTS: AO Foundation (Start-up grants S-12-08S / S-09-112S) and the Swiss National Science Foundation (Project Grant No. 310030-156291) partially supported this study.

Injectable immobilization of hydrophobic molecules in hyaluronan hydrogels triggered by calcium ions

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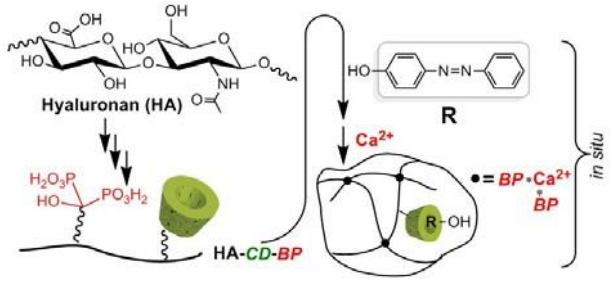
INTRODUCTION: Encapsulation of hydrophobic drugs in hydrogels is inefficient due to lack of adequate interactions with hydrophilic matrix. On the other hand, localization of relevant drugs on bone surface is advantageous for treatment of bone-related disorders and bone regeneration.[1] In the present work, we designed a hydrogel-based drug delivery system with high loading capacity and bone adhesive properties.

METHODS: HA-CD-BP was dissolved in distilled water at 4% concentration and 4-hydroxyazobenzene was added to each solution at [drug]/[CD] = 2:1 molar ratio. After complexation for 24 hours, the insoluble part was removed by centrifugation. The supernatant (200 μL) was mixed with 20 μL of 2.8M $CaCl_2$ solution to yield a hydrogel. The same amount of the model drug was similarly encapsulated in HA-BP hydrogel.

RESULTS: Hyaluronic acid (HA), a major natural component of extracellular matrix, was modified in three steps with bisphosphonate (BP) and β-cyclodextrin (CD) groups providing HA-CD-BP derivative (Scheme 1). High affinity of BPs to hydroxyapatite component of bone and the ability of CDs to form host-guest complexes with hydrophobic molecules were on the basis of rational design of our osteotropic drug delivery system. As compared to previously described direct attachment of CD to BP [2], multiple attachment of these two types of ligands to HA backbone provided possibility to immobilize the polymer in the form of a hydrogel.[3] Sol-to-gel transition can be triggered by simple addition of calcium ions to the solution of HA-CD-BP.



Scheme 1: Hyaluronic acid dually modified with bisphosphonate (BP) and β-cyclodextrin ligands binds to a hydrophobic gest. The formed complex is jellified upon addition of calcium ions.

Next, we used 4-hydroxyazobenzene as a model hydrophobic drug for complexation with HA-CD-BP. The subsequent addition of $CaCl_2$ solution allowed us *in situ* encapsulation the hydrophobic cargo in the formed hydrogel (Scheme 1). A reference hydrogel lacking CD groups was also prepared from analogous HA-BP derivative. First, we observed that 4-hydroxyazobenzene loading capacity of HA-CD-BP hydrogel was 2.2 times higher than that for HA-BP hydrogel. Sufficient retention (50%) of the model hydrophobic drug in HA-CD-BP hydrogel was also observed after some burst release during the first 5 hours.

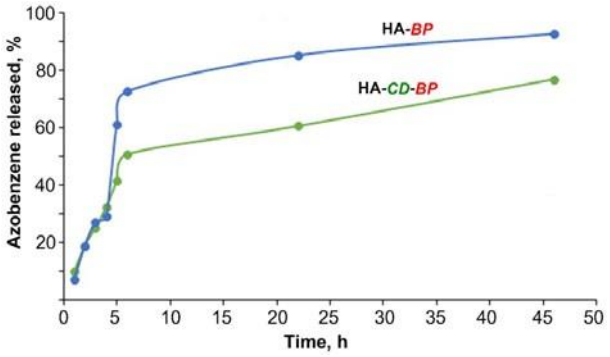


Fig. 1: Cumulative release of 4-hydroxy-azo benzene from HA-BP and HA-CD-BP hydrogels.

DISCUSSION & CONCLUSIONS: Comparative drug loading and release experiments demonstrated ability of new HA derivative with covalently attached CD and BP groups for effective immobilization of a model drug. Adhesion of the drug depot to bone is ensured by BP groups of the material.

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In vitro and in vivo characterization of a photocrosslinkable hyaluronan hydrogel for skeletal muscle repair

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INTRODUCTION: Skeletal muscle can self-repair through the activation of resident muscle satellite cells as a consequence of injury. However, when volumetric muscle losses (VMLs) occur due to trauma or surgical resection, lesions are so extensive that the regenerative potential of the muscle is overwhelmed. Current approaches to treat VMLs include autologous tissue transfer and skeletal muscle tissue engineering [1], but both approaches have been hampered by several drawbacks. Within this framework, hydrogels are becoming an increasingly attractive choice in the fields of regenerative medicine, wound care and tissue engineering as scaffolds, due to their high water content, good biocompatibility and for their ability to mimic the environment of the extracellular matrix.
In this work, 200 kDa hyaluronic acid (HA) was modified with coumarin moieties and photopolymerized into networks using a near-UV irradiation [2]. The resulting hydrogels have been tested *in vitro* for biocompatibility and *in vivo* in a mouse model of muscle loss.

METHODS: Two hydrogel prototypes with different percentage (p) of derivatization (p30 and p40) and concentration (30 and 20 mg/mL) were selected for the study. To determine their mechanical properties, the hydrogels have been tested by means of mechanical compression and Atomic Force Microscopy (AFM); furthermore, the FRAP assay has been used to evaluate the hydrogels permeability.
Primary murine myogenic precursors (MPC) have been used for biocompatibility assays. Cells were encapsulated within the hydrogels and tested for their metabolic activity (Alamar Blue®). Cellular proliferation (EdU) and immunofluorescence were carried out to determine the ability of encapsulated cells to maintain the expression of two muscle markers: MyoD and desmin. *In vitro* tests were performed at 48 hours.
In vivo tests have been performed through the partial ablation of tibialis anterior (TA) and the hydrogels have been photopolymerized within the created pocket. Mice were sacrificed after 2 and 6 weeks, and histology (Hematoxilin & Eosin and Sirius red) has been performed to assess hydrogels degradation and muscle regeneration.

RESULTS: Based on mechanical compression data, the hydrogels showed bulk elastic moduli of 42±1 and 23±2 kPa for p30 and p40, respectively. AFM data showed surface elastic moduli of 213±5 and 173±9 kPa for p30 and p40, respectively. Data obtained from FRAP assay exhibited a diffusion cut–off for molecules with MW higher than 250 kDa. Biocompatibility tests revealed that MPC cells encapsulated within the hydrogels showed lower metabolic activity values and a lower percentage of proliferating cells (25±3 for p30 and 35±5 for p40) compared to standard culture conditions. Interestingly, MPC cells retained the expression of key myogenic markers: MyoD (p30: 72±1%, p40: 60±1%) and desmin (p30: 99±1%, p40: 94±1%) compared to the controls (86±2% for MyoD and 99±1% for desmin). Once the hydrogels were injected and photopolymerized within the muscle pocket, they showed good repair after 6 weeks. No significant inhibition of muscle regeneration has been observed, also after removing an important amount of tissue (≈50%).

DISCUSSION & CONCLUSIONS: Hydrogels for skeletal muscle repair have been developed and characterized. They showed good mechanical properties and permeation to nutrients and small soluble factors. Biocompatibility tests showed that hydrogels were able to sustain cellular proliferation and maintain myogenicity of MPC cells after 48 hours in culture. *In vivo* experiments highlighted the low immunogenicity of the hydrogels. Muscle repair is a balance between new tissue formation and dissolution of engineered scaffolds: HA hydrogels disclosed a good degradation profile (residence time: 2 weeks).

Clinically-compliant cartilage bioprinting

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INTRODUCTION: Cartilage tissue has several characteristics which make it amenable to bioprinting technology. Firstly, cartilage derives its function largely from its structural architecture and mechanical properties, both which can be easily reproduced with medical imaging data and 3D bioprinting. Secondly, chondrocytes receive the majority of signals through the extracellular matrix, with cell-cell contacts being relatively uncommon. New generations of bioinks which incorporate sophisticated methods to present biological signals are yielding bioprinted tissue analogues with close resemblance to native tissue. Thirdly, given that cartilage is an avascular, hypoxic tissue, the transplantation of printed cartilage grafts is anticipated to be more straightforward compared to tissues where rapid connection to the host vasculature is required. Given these advantages, what is holding back translation of bioprinting into the clinics? In this presentation, we discuss the evolution of a clinically compliant bioink and the steps which can be taken to improve function and assure safety and stability of bioprinted grafts.

METHODS: Generic bioinks composed of gellan (3%) and alginate (2%)[1] were mixed to give a base cartilage bioink. Methods to enhance the overall performance of the printed graft include addition of matrix signals, optimization of the extrusion nozzle, internal design of the graft and post-extrusion crosslinking conditions (Table 1). Quantitative measurements of the graft's mechanical properties were evaluated according to ISO standards and compared to bulk properties[2].

RESULTS: Our results show that biochemical alteration of the bioink can result in highly improved function of the cells as determined by their ability to spread and proliferate. In addition, nozzle geometry is critical and can be highly detrimental to the cell viability, even in the presence of a highly functional bioink[3]. For extrusion of very large and thick structures, the inclusion of macroporosity is critical to maintain highly viable cells throughout the structure. The printing of cartilage grafts therefore involves optimization of multiple parameters.

Table 1. Modification of the bioprinting process.

Process	Modification	Effect
Bioink formulation	Addition of ECM particles	Increased matrix deposition
Extrusion	Nozzle Geometry	Increased Viability
3D model	Addition of macroporosity	Better core viability
Crosslinking	Cation Concentration	Stiffer constructs

DISCUSSION & CONCLUSIONS: Bioprinting's great promise is to deliver tissue replacement grafts which are personalized in terms of geometry, material properties and the patient's own cells. As a new technology, bioprinting also raises concerns from a regulatory point of view as to its safety and reliability. In the context of a clinically-compliant bioprinting formulation, we explore the space to improve the entire printing process and to standardize the evaluation of the printed materials.

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3D tissue models: microvascularization and osteogenesis in PEG hydrogels

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INTRODUCTION: In vitro engineered tissues which recapitulate functional and morphological properties of osteogenic tissue would be desirable to study bone regeneration under fully controlled conditions. To enable the study of cells on a blank slate synthetic hydrogels such as poly(ethylene glycol) (PEG) can be used. Here, we combine a 3D PEG system with human mesenchymal stem cells (MSCs) and human umbilical vein endothelial cells (HUVECs) toward the establishment of an artificial vascularized bone tissue-like environment. In doing so, we make use of the dual role of MSCs, which is their ability to give rise to osteogenic cells and their ability to support endothelial cells forming microvascular structures.

METHODS: TG PEG hydrogels are formed by the cross-linking of 8-PEG-MMPsensitive-Lys and 8-PEG-Gln precursors via FXIIIa transglutamination [l]. The cell adhesion peptide Lys-RGD is cross-linked into the gel at the same time. HUVECs and MSCs are 3D encapsulated in TG-PEG hydrogels during the gelation process at 1:1 ratio. Hydrogels were cultured in medium containing bone morphogenetic protein-2 (BMP-2) and/or fibroblast growth factor-2 (FGF-2) or without growth factors. After 14 days of culture hydrogels were analyzed for microvascularization by means of immunostaining or subcutaneously implanted in immunocompromised mice. To evaluate the osteogenic differentiation of the constructs, hydrogels were analyzed for alkaline phosphatase activity and cells were isolated and analyzed by qRT-PCR for their osteogenic differentiation.

RESULTS: In this synthetic hydrogels BMP-2 or FGF-2 promotes the formation of microvascular networks (an example of microvascular structures is depicted in Fig.1). Moreover, the resulting microvascular networks are functional in vivo as they inosculate in the host vasculature when implanted subcutaneously in immunocompromised mice. However, while osteogenic differentiation is obtained with BMP-2, the treatment with FGF-2 suppressed osteogenic differentiation.

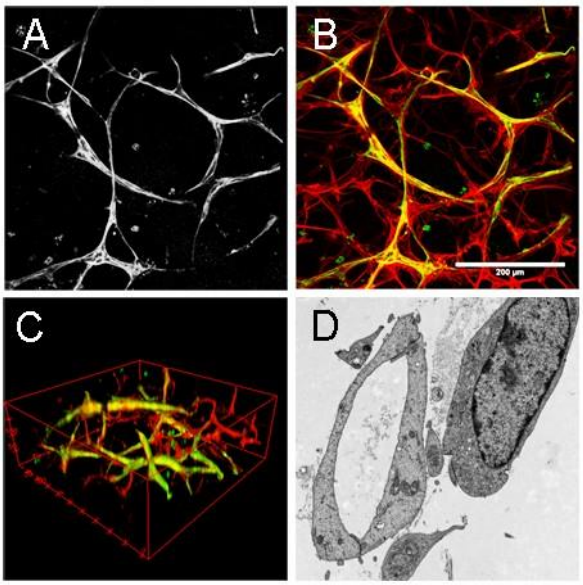

Fig. 1: Microvascularization in PEG hydrogels after 14 days of in vitro culture revealed by A) immunofluorescence of CD31 staining for HUVECs; B) CD31 (green) and F-Actin staining for all cells (red), scale bar 200 µm; C) 3D reconstruction of B). D): Lumen formation of microvessels supported by perivascular cells visualized by TEM microscopy.

DISCUSSION & CONCLUSIONS: We have established a microvascularized 3D tissue model, in which MSCs support HUVECs and can be simultaneously osteogenic differentiated or prevented from osteogenic differentiation. Thus we recapitulate some of the features of native bone tissue in a synthetic microenvironment by combining vasculogenic and osteogenic processes in PEG hydrogels.

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 Biofabrication of soft tissue: Material challenges

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INTRODUCTION:

Three dimensional bioprinting is being applied to tissue engineering and regenerative medicine as a manufacturing tool to produce 3D tissues and organs suitable for transplantation. Difficulties with printing cell-laden natural materials such as collagen as well as concerns over immunogenic responses to these materials are well documented. In our present work we synthesised easily printable, stimuli responsive polymers specifically tailored for housing cells to serve as wound dressings for wound healing.

METHODS: The copolymer was synthesized by the copolymerizing of PEGMEMA, MEO_2MA and PEGDA via an in situ deactivation-enhanced atom transfer radical polymerization approach as previously described [1]. ^{1}H NMR was carried out on a 300 MHz Bruker NMR with Mnova processing software. To make the polymer more semi-solid than viscous, 5% porcine gelatin (sigma, UK) was added to the polymer solution and stored at 4°C for 20 minutes until a clear gel of polymer was formed. Specialised pressure syringes were filled with this polymer combination and printed using the RegenHu 3DDiscovery printer at constant pressure, strand diameter and print-head speed. The PMP is crosslinked after printing using UV light (350nm) for 2 minutes.

RESULTS: The polymer (termed PMP) molecular weight and polydispersity index were determined using Gel permeation chromatography (M_w=10,800 Da, PDI=1.32).

Investigation into the effect of UV light on cell viability found that 2 minutes was ideal to maintain around 90% of cells viable without affecting the crosslinking efficiency of the polymer. This crosslinking efficiency created a strong rubbery like hydrogel that supports cell growth and proliferation.

The polymer can also be easily printed when mixed with 5% gelatine and cells at 4°C. Preliminary data show high cell viability post printing too (results not shown here).

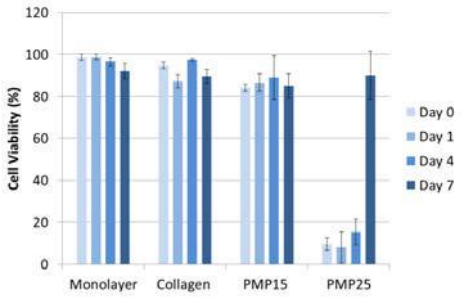

Fig. 1: Cell viability of 3T3 Fibroblasts in the PMP hydrogel before printing. (15% polymer:PMP15, 25% polymer: PMP25)

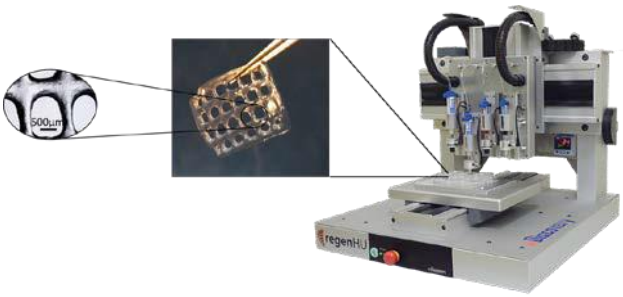

Fig. 2: 3D printed PMP hydrogel after photocuring and removing gelatine showing precise and accurate printing of 5 layers of the polymer.

DISCUSSION & CONCLUSIONS: With the aid of 3D printing technology, the photocurable property of the synthesised polymer means that strong and flexible hydrogels, faithful to the precise shape of the injury, can be made to replicate many of the tissues including human skin. Moreover, cells can survive and proliferate within the gel (results not shown here) for more than seven days a testimony for the future application of such polymers in tissue engineering.

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 PCOFUND-GA-2012-600181.

Magnetic hybrid scaffolds for bone tissue regeneration

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INTRODUCTION: Magnetic scaffolds have recently attracted significant attention in tissue engineering, due to the prospect of improving bone formation by acting as a "fixed station" able to accumulate/release soluble mediators such as growth factors in the defect area under the influence of an external magnetic field [1]. Here, we assessed the in vivo biocompatibility and osteoconductive potential of different hybrid magnetic scaffolds, i.e. collagen/HA, macroporous HA and PCL-based scaffolds containing a ferromagnetic phase, both in presence and absence of an external permanent magnet, in rabbit femoral condyle model.

METHODS: Collagen/HA hybrid magnetic scaffolds were prepared following two different protocols. The first method was based on the concurrent nucleation of biomimetic hydroxyapatite and ferromagnetic nanoparticles on self-assembling type I collagen fibrils [2]. The second method was based on the infiltration of collagen/HA scaffolds with a ferro-fluid solution leaving ferromagnetic nanoparticles entrapped in the construct [3]. Non-magnetic commercial biomimetic scaffold obtained by biologically inspired nucleation of biomimetic hydroxyapatite on self-assembling type I collagen fibres (RegenOss, Fin-Ceramica SpA, Italy) were tested as control. Magnetic macroporous HA scaffolds were obtained by mixing hydroxyapatite and magnetite nanopowders with a foaming agent, casting in water draining moulds and finally drying in air at room temperature. Magnetic PCL scaffolds were produced by embedding FeHA nanoparticles into a PCL matrix. Superparamagnetic FeHA nanoparticles were formerly prepared by adding a source of Fe^{2+} and Fe^{3+} ions to a suspension of calcium hydroxide. For all the investigated scaffolds, bone regeneration has been evaluated at 4 and 12 weeks from the implantation of the scaffolds in the femoral condyle of rabbit by means of histological, histomorphometric and nano-mechanical analyses.

RESULTS: In all the cases, the osteoconductive properties of scaffolds were well preserved despite the presence of a magnetic component. Interestingly, when using bio-resorbable collagen/HA magnetic scaffolds, the reorganization of the magnetized collagen fibres under the effect of the static magnetic field generated by the permanent magnet produced a highly-peculiar bone pattern, with highly-interconnected trabeculae orthogonally oriented with respect to the magnetic field lines. In contrast, only partial defect healing is achieved within the not magnetic control groups.

DISCUSSION & CONCLUSIONS: Our results suggest the possibility to improve implant fixation and control bone morphology of regenerated bone by synergically combining static magnetic fields and magnetized biomaterials. Notably, the magnetic properties of hybrid biocompatible scaffolds could be exploited to guide important cytokines/growth factors for bone healing, such as vascular endothelial growth factor (VEGF), previously bounded to magnetic nanoparticles (MNP-VEGF), directly to the site of scaffold implantation.

ACKNOWLEDGEMENTS: This study was supported by the European Project "Magnetic Scaffolds for in vivo Tissue Engineering" (NMP3-LA-2008-214686).

Silica nano-carrier as a sustained delivery system of GDF5 for intervertebral disc regenerative medicine

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INTRODUCTION: Intervertebral disc (IVD) degeneration is one of the major causes of low back pain. The sustained delivery of therapeutic factors able to promote IVD regenerative processes in situ is contemplated and biomaterials-based delivery systems are widely studied. Among all biomaterials, mesoporous silicas are of interest with respect to their properties and lack of cytotoxicity[1]. In this context, we studied mesoporous silica nanofibers (MSNFs)[2] as a drug delivery system. The loading and release capacity of MSNFs and the protein-silica interactions were explored using a model protein (lysozyme). MNSFs were then characterized as a bioactive carrier for Growth Differentiation Factor 5 (GDF5), a nucleopulpogenic factor[3].

METHODS: Lysozyme was incubated with MSNFs (50x500nm) in different conditions of pH, time and concentrations. Analyses of protein-silica interactions were performed with transmission electron microscopy (TEM), attenuated total reflectance infrared (ATR/IR) and zeta potential (ZP). Release was performed in PBS, pH 7.2 at 37°C for 20 days. Lysozyme concentration and biological activity were measured with BCA method and enzymatic assay, respectively.GDF5 was incubated with MSNFs at pH 7 for 48h at concentrations from 1-4µg/mL. Release was performed in PBS, pH 7.2 at 37°C for 48h. GDF5 concentration and bioactivity were measured with ELISA and by testing the Smad 1/5/8 pathway activation in human adipose stromal cell (hASC) by western blot, respectively. Nucleopulpogenic commitment of hASC by released GDF5 was finally assessed using RT qPCR (aggrecan, type II collagen, OVOS2, PAX1, CD24) and immunohistology (alcian blue, type II collagen, aggrecan, OVOS2, PAX1, CD24).

RESULTS: Lysozyme was successfully adsorbed on MSNFs and the highest amount of adsorbed lysozyme was obtained at pH 10, concentration of 200 mg/mL and 48h of incubation. Different lysozyme morphologies were observed by TEM. The ATR/IR study, which compared amides, Si-O-Si, Si-OH and water bonds between lysozyme, MSNFs and MSNFs/lysozyme, combined with ZP results, suggest that protein-silica interactions must be mostly driven by hydrogen bonds involving the amide II of the protein.

Release experiments showed a burst within the 1st hour followed by a slower release rate, leading to a sustained release for up to 20 days. The biological activity of the released protein was maintained at all time points. We also demonstrated that GDF5 could be adsorbed and released onto MSNFs. GDF5 bioactivity, evaluated by Smad 1/5/8 phosphorylation, was evidenced for higher GDF5-loaded concentrations onto MSNFs and cells commitment was assessed.

DISCUSSION & CONCLUSIONS: MSNFs are promising nano-carriers for therapeutic factors delivery. Moreover, protein-silica interactions were extensively described and revealed hydrogen bond-mediated interactions. Further experiments will focus on the association of GDF5-loaded MSNFs with hASC with an adapted scaffold for in vivo proof of concept.

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Evaluating the effect of EGF diffusion on the migration of placental cells using a 3D printed, bioengineered placenta model

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INTRODUCTION: The current paucity of clinically relevant experimental models for preeclampsia (PE), a leading cause in maternal and perinatal morbidity, impedes any effort to identify treatment options [1]. The migration of trophoblasts is suspected to play a critical role in the development of PE [2]. We hypothesized that a bioengineered placenta model (BPM) that captures the maternal decidua's ECM composition and mechanics as well as its growth factor-secreting vascular network, can be bioprinted to measure the effects of epidermal growth factor (EGF) on the migration rates of placental cells and to develop novel treatments for PE. Specifically, our objectives are 1) To identify the appropriate loading level of EGF and other design parameters of the BPM and 2) To bioprint the BPM and evaluate the effect of EGF diffusion on the migratory response of two placental cell types (trophoblasts and hMSCs).

METHODS: Migration of trophoblast on fibronectin (FN)-coated glass was quantified through time-lapse microscopy. Diffusion of EGF in gelatin methacrylate (GelMA) was investigated by time-lapse fluorescent microscopy of labeled EGF and modeled by diffusion equations. BPM were bioprinted using extrusion-based bioprinter. For the migration experiments, placental cells were bioprinted along the periphery of the cylindrical BPM and an EGF source was printed in the center (*Fig 1, top right*). The distances between the edge of the bioengineered placenta model and the migration front (*Fig 1, red arrows*) were measured and averaged over time to obtain the migration rates. Live/Dead stain was utilized to confirm viability of printed cells. A p-value less than 0.05 indicate significant difference between samples.

RESULTS: Our results demonstrate that the trophoblast migration rate increases (72.7±0.5–155.8±20.9μm/day) as EGF concentration increases (10-100nM) on FN-coated glass in 2D. Diffusion coefficient of EGF in GelMA was determined to be 2.5×10^{-8} cm^2/s and the EGF concentration profiles were calculated numerically. Based on these findings, we bioprinted BPM and found dosage-dependent 3D migratory responses of two placental cell populations towards EGF (0-13 μM): trophoblasts (-3.2±9.4–16.8±11.5μm/day) and hMSCs (132.6±44.8–191.0±50.41μm/day) with excellent viabilities. Next, we utilize our bioprinter to evaluate the effect of EGF source geometry on placental cell migration rates, as geometry of EGF source will alter the concentration profile of $_2$ EGF that induces migration of placental cells .

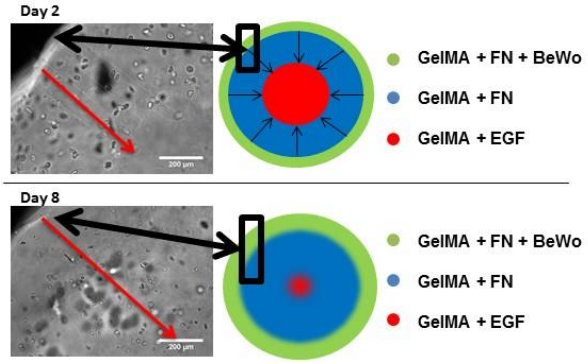

Fig. 1: Migration Results from the Bioengineered Placenta Model (Scale bars: 200 μm).

DISCUSSION & CONCLUSIONS: Our results suggest that EGF is a potential therapeutic agent for preeclampsia by directly promoting trophoblast migration and indirectly by recruiting hMSCs to the site of trophoblast invasion, as hMSCs alleviate symptoms of PE [2]. To the best of our knowledge, BPM is the first 3D *in vitro* model that captures the stiffness of decidua and contains a controlled gradient of chemokines to induce trophoblast and hMSC migration. Such clinically critical biophysical information as tissue elasticity and chemotactic gradient has not been previously incorporated to build a functional tissue model for disease testing and treatment development.

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Construction of a three dimensional in vitro embryo implantation research model using alginate macro porous scaffold

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INTRODUCTION: Implantation failure remains an unsolved obstacle in reproductive medicine and is a major cause of infertility in otherwise healthy women. Indeed, only about 20% of embryos transferred to the uterus, following in vitro fertilization (IVF), lead to the birth of a healthy infant. Due to obvious ethical restrictions there is an unmet need to establish an in vitro model that mimics the events in the uterine wall during the implantation process. The available two-dimensional models do not fully represent the event taking place at implantation.

METHODS: Alginate scaffolds were prepared by a freeze-dry technique [1]. Endometrial cell lines, RL95-2 or HEC-1A, displaying receptive and non-receptive endometrial properties, respectively, were seeded into alginate scaffolds and cultivated in four different media: (a) 3 weeks in Estrogen containing medium, (b) Progesterone containing or (c) hormone-free media, or (d) one week priming of Estrogen, following by two weeks of Progesterone containing medium. E-cadherin mRNA expression levels were evaluated by qPCR. E-cadherin protein expression in cell was evaluated by specific immuno-staining. In order to evaluate cell constructs receptivity to trophoblast, JAR spheroids were seeded on top of 3 week-old cell constructs, and incubated for 24 h. Attachment of JAR spheroids to RL95-2 culture was evaluated by H&E staining.

RESULTS: Cultivation under 3D conditions within macro-porous alginate scaffolds enabled long-term cultivation of the cells for at least 4 weeks, as indicated from MTT analysis. E-cadherin mRNA expression levels, evaluated by qPCR, were shown to be hormone-dependent in RL95-2 cell constructs (Fig.1A), in contrast to HEC-1A cells, where no hormonal effect was evident (Fig.1B). In 2-weeks old RL95-2 constructs, Estrogen treatment significantly increased E-cadherin mRNA expression, compared to other hormone treatments (Fig.1A, $p<0.05$, Bonferroni's post-test). E-cadherin immuno-staining of cell constructs revealed pronounced protein expression on cell membranes of RL95-2 cell constructs, compared to HEC-1A. Most importantly, JAR spheroids attachment to 3 weeks old RL95-2 culture was confirmed by H&E staining. JAR attachment was not evident in HEC-1A constructs.

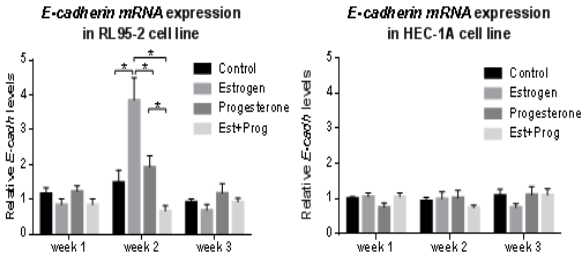

*Fig. 1: MRNA E-cadherin expression levels in RL95-2 cell constructs (A), vs. HEC-1A cell constructs (B), evaluated by qPCR. Expression levels were normalized to control group (hormone-free medium), week 1. *- $p<0.05$.*

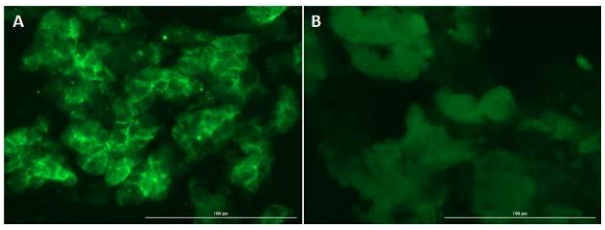

Fig. 2: Immunostaining for E-cadherin expression in RL95-2 cell constructs (A), compared to HEC-1A cell constructs (B).

DISCUSSION & CONCLUSIONS: Our 3D culture models within macro-porous alginate scaffolds enabled long-term culture of viable endometrial cells. These cultures may serve as a research model for studying the regulatory mechanism governing implantation process and evaluation of potential novel therapeutic strategy for regulating implantation defects and restoring the ability to implant embryos in patients with repeated implantation failure (RIF).

ACKNOWLEDGEMENTS: This study was funded by Azrieli College of Engineering (DS).

Off-the shelf scaffolds: the future of urethroplasty

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INTRODUCTION: Actual surgical procedures using existing autologous tissues for the treatment of congenital malformations or injuries of the urethra are associated with post-operative stenosis and fistula. Tissue engineered collagen tubes could be a promising alternative for this reconstructive surgery to overcome current post-operative complications. In our previous work, we could demonstrate the spontaneous regrowth of urothelium and ingrowth of smooth muscle cells after implantation of high-density collagen gel tubes[1] in a rabbit model. Other groups claim that autologous cell therapies are the path of the future[2]. However, such solutions are associated with high costs, regulatory issues and added patient discomfort. If an off-the shelf solution could show a good regenerative potential, it would have a great chance of broad translation into clinics.

METHODS: We have developed two manufacturing procedures resulting in two acellular collagen tubes with enhanced mechanical properties, allowing better handling of the graft as compared to our previous collagen gel tubes[1]. The tubes were used as urethral grafts in New Zealand white rabbits and sutured between the native prostatic and the very distal urethra following subtotal excision of more than 80% of the total urethral length. No catheter was placed postoperatively. This procedure was applied in 40 male rabbits (20 rabbits/scaffold) in two different transplantation centers. At 1, 3, 6, and 9 months the animals were macroscopically evaluated and contrast voiding cysto-urethrography was performed. After sacrifice histological examination was done.

RESULTS:

This multi-centric study revealed from the contrast voiding cysto-urethrography that one of the produced acellular collagen tube had a 40% failure rate, while the other had a 10% failure rate,

steadily decreasing during the learning curve (Table 1). Furthermore, spontaneous urothelial coverage of the grafts and time-dependent smooth muscle cell migration could be observed in all grafts.

Table 1. Results from contrast voiding cysto-urethrography from 20 rabbits per scaffold

	No Complication	Fistula	Stenosis
Scaffold 1	60%	20%	20%
Scaffold 2	90%	10%	0%

DISCUSSION & CONCLUSIONS: Results obtained in this rabbit study show the promise of off-the shelf tubular collagen scaffolds from a surgical and regenerative perspective. Therefore, we will invest into further optimization and regulatory compliance of such scaffolds to turn them into a clinical product for future urethroplasty surgeries.

ACKNOWLEDGEMENTS: This research was supported by two CTI projects (project-14577.1PFLS-LS, 16627.1PFLS-LS)

Human adult venous endothelial cells form vessel networks within engineered human muscle grafts

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INTRODUCTION: Current treatment options for the loss of large masses of muscle tissue are limited and one promising solution is the use of engineered muscle grafts. Since the skeletal muscle tissue is a thick tissue with a high oxygen demand, vascularization is a fundamental concern. We hypothesized that this vascularization can be achieved by populating the engineered muscle with adult venous endothelial cells (EC) which will form organized vessel networks while cultured in the lab and integrate with the host blood vessels upon implantation.

METHODS: Adult primary venous endothelial cells (adult EC) and adult smooth muscle cells (SMC) were isolated from lower-extremity vein segment as previously described [1]. Adult EC were transduced with ZsGreen fluorescent protein lentivirus.

Porous sponges composed of 50% poly l-lactic acid (PLLA) and 50% polylactic glycolic acid (PLGA) were fabricated utilizing a particle leaching technique to achieve pore sizes of 212−600μm and 93% porosity [2].

Different multicellular cultures were seeded on the scaffolds and cultured for 14 days. Then, the scaffolds were fixated and stained with rabbit anti desmin primary antibody. Lastly, vessel network development was analysed and quantified using the AngioTool software (AngioTool®).

Tri culture scaffold composed of adult EC-ZsGreen, adult SMC and human myoblasts were grown 14 days *in vitro* and then implanted to fill a defect that was created in the abdominal wall of nude mice.

RESULTS*:* Different cell combinations were seeded on PLLA/PLGA scaffolds in order to create various co and tri cultures and to compare adult and young endothelial cells and support cells. Our results demonstrated the formation of vessel network within all the groups tested. Data analysis reviled that scaffolds seeded with adult venous EC were significantly better than the same groups seeded with human umbilical vein EC. *In vivo* studies reviled that the engineered muscle tissue was viable and anastomosed with the host vasculature 9 days post implantation.

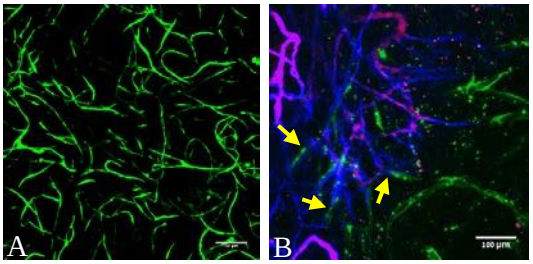

Fig. 1: Confocal Z stack images of (A) vessel network (green) formed in vitro within human myoblasts, (B) Anastomosis in vivo between adult human EC (green) and the host vasculature (blue). Functional blood vessel was visualized using dextran injection (red).

DISCUSSION & CONCLUSIONS: Here we show that adult venous EC are capable of creating vessel like structures *in vitro* and to anastomose with the host vasculature *in vivo*. We also show that these adult EC vessels can form within an engineered human muscle tissue composed of aligned human myoblasts. These results are very promising and can lead to the construction of optimal engineered autologous muscle grafts that can be used in the clinic.

ACKNOWLEGDEMENTS: We would like to thank the European Research Council: ERC Grant Agreement no. 281501 – ENGVASC for funding this project.

Influences of extracellular matrices on myofibroblast differentiation in pelvic organ prolapse

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INTRODUCTION: Pelvic relaxation is a weakness in the supporting structures of the pelvic floor that can result in pelvic organ prolapse (POP). POP affects the quality of life of many women world-wide and remains a great therapeutic challenge, with no optimal treatment currently available.

Typically, the bladder is kept in place by a connective tissue layer in the anterior vaginal wall, in which fibroblasts are responsible for tissue maintenance and extracellular matrix remodelling. Studies from us and others have shown that POP affects the tissue composition, tissue mechanical properties and cell behaviour. How the changes in the extracellular matrix affects cell behaviour and vice versa, is unknown. Decellularized tissues (DT) can provide a more physiological microenvironment for the cells than standard tissue culture plates, enabling cell-matrix interaction studies in a disease-specific manner. In this study we evaluated DT-matrices derived from vaginal tissues as cell culture systems to study cell-matrix interactions in pelvic organ prolapse. We focus on fibroblast to myofibroblast differentiation, a key process in normal and pathological soft tissue repair and regeneration,. This may provide evidence that the altered tissue microenvironment plays a role in the maintenance of POP.

METHODS: vaginal tissues from women with and without POP were decellularized using the Triton x-100/sodium-deoxycholate method[1]. DT-matrices of 6 mm diameter and 50 µm thick from the mucosal and the muscularis layers from prolapsed (DT-POP) and non-prolapsed (DT-Control) tissues were prepared. The surface stiffness of the DT-matrices was measured with a micro-indentor (PIUMA, Optics11). Fibroblasts harvested from women with and without POP were seeded on the DT-Control and DT-POP matrices and cultured for 48 hours. The viability of the cells was determined using the LIVE/DEAD Viability/ Cytotoxicity assay and total DNA was determined using the CyQuant kit (Molecular probes). Fibroblast to myofibroblast differentiation was investigated by western immunoblotting using alpha smooth muscle actin (α-SMA) as a marker. Three independent experiments were performed in duplicate, and statistical analysis was performed using unpaired t-test or one-sample t-test.

RESULTS: Fibroblasts survived and migrated into all DT-matrices. Cells from women without POP showed better attachment to the DT-matrices than cells harvested from prolapsed tissues (POP cells), regardless of matrix origin. The surface micro-stiffness of the muscularis layer of the DT-Controls was at least 2x higher than the mucosal layers. POP cells seeded on the muscularis layer of DT-Controls showed twice the α-SMA content compared to the same cells seeded on the mucosal layer. These results show that the stiffer muscularis layer of the DT-Control matrices induced myofibroblast differentiation of POP cells, but not of control fibroblasts. Fibroblast to myofibroblast differentiation of POP cells was higher in the DT-POP matrices; POP cells seeded on the DT-POP matrices had 4x more α-SMA content compared to the same cells seeded on DT-Controls. Together our results suggest that myofibroblast differentiation is stronger in cells from prolapsed tissues and also more pronounced on DT extracellular matrices from POP patients.

DISCUSSION & CONCLUSIONS: Decellularized vaginal wall matrices can be useful cell culture systems in which to study cell-matrix interactions in pelvic organ prolapse. Fibroblast to myofibroblast differentiation was higher in cells from prolapsed tissues and dependent on the extracellular matrix encountered as indicated by α-SMA. These findings support the idea that tissue repair and regeneration processes are altered in women with POP, and should be taken into account, when using tissue engineering approaches to prolapse.

Combinatorial gene therapy can accelerate regeneration: development of non-viral gene-activated scaffolds for orthopedic tissue engineering

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INTRODUCTION: Gene-activated scaffolds facilitate localized gene delivery to defect sites which can result in enhanced bone or cartilage regeneration. However, identifying an adequate vector for cellular transfection is a key challenge. Chitosan, a natural cationic polymer derived from the exoskeleton of crustaceans, is biocompatible, biodegradable and a formulation optimised in our lab has been shown to have a transfection efficiency of 45% in mesenchymal stem cells (MSCs) without causing cytotoxicity [1]. The aim of this study was to incorporate chitosan nanoparticles, carrying either osteoinductive or chondroinductive genes, into collagen scaffolds, previously optimized for bone or cartilage repair [2, 3], thus creating gene-activated scaffold systems which can accelerate MSC-mediated bone and/or cartilage tissue formation.

METHODS: Three scaffolds were assessed, composed of 1) collagen, 2) collagen hyaluronic acid (CHyA) and 3) collagen hydroxyapatite (CHA). Chitosan nanoparticles were used to deliver plasmids encoding bone morphogenetic protein-2 (BMP-2), BMP-7 and a multi-cistronic plasmid encoding both BMP-2 and 7 as well as vascular endothelial growth factor (VEGF) were assessed for bone repair while transforming growth factor-β3 (TGF-β3), the Sox trio of genes (Sox 5, 6 and 9) and histone deacetylase-4 (HDAC4) were delivered to enhance chondrogenesis. Transfection efficiency was measured by ELISA and qRT-PCR. MSC osteogenesis was assessed using histology and calcium deposition was quantified. Chondrogenesis was determined by quantifying sGAG deposition as well as histology.

RESULTS: Chitosan nanoparticles were capable of efficiently delivering therapeutic genes to MSCs and causing an up-regulation of the encoded protein expression. Following a rigorous screening process, a lead gene combination of pBMP-2 and pVEGF were identified, together significantly accelerated calcium deposition by MSCs. When incorporated into a CHA scaffold, the gene-activated scaffold induced MSCs to produce approximately 2000 µg of calcium per scaffold, significantly higher ($p<0.001$) than gene-free scaffold controls (Figure 1A). The combination of pSox Trio delivered using chitosan nanoparticles on a CHyA scaffold induced MSCs to produce approximately 70 µg of sGAG per scaffold after 28 days in culture. By day 28, this was significantly higher than gene-free scaffold controls ($p<0.001$) (Figure 1B). Furthermore, collagen type X expression was not evident on the scaffolds after 28 days in culture, indicating that the MSCs are forming stable cartilage and not undergoing endochondral ossification.

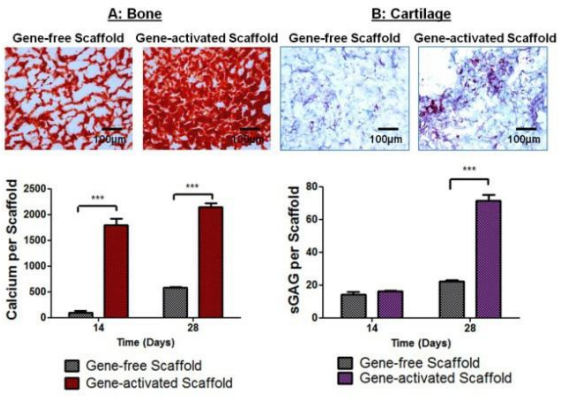

*Figure 1: Gene-activated scaffolds for bone (A) and cartilage (B) repair significantly accelerates MSC-mediated tissue formation. Data plotted represents mean ± SD. *** $p<0.001$.*

DISCUSSION & CONCLUSIONS: This study demonstrated that combinatorial delivery of osteoinductive or chondroinductive genes within collagen-based scaffolds can accelerate MSC differentiation towards bone or cartilage-like tissue respectively. Both cell-free gene-activated scaffold systems are currently being tested *in vivo* with a view to generating 'off-the-shelf' products for repair of bone and cartilage defects.

ACKNOWLEDGEMENTS: SFI Research Frontiers Programme (Grant No. 11/RFP/ENM/3063) and ERC (FP7/2007-2013) ERC grant agreement No. 239685

Improvement in contrast and in biological features of calcium phosphate cement

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INTRODUCTION: Calcium phosphate cement (CPC) has been postulated to be a suitable material for dental pulp therapy [1]. However, similar radiopacity to the natural dentin, makes the detection of CPC challenging especially when using conventional X-ray techniques. In this study, the CPC contrast was enhanced by using a dedicated dual contrast agent (DCA) for magnetic resonance imaging (MRI) and computer tomography (CT) visualization [2]. Furthermore an enhancement in new dentin formation was pursued by using bone morphogenic protein-2 (BMP-2) as promoter of the odontoblastogenesis [3].

METHODS: CPC composition was a weight/weight (wt/wt) mix of 59.1% alpha-tricalcium phoshate (α-TCP), 1.5% carboxyimethylcelulose (CMC) and 39.4% of cryo-grinded poly-(DL-Lactic-co-Glycolic acid) particles. To this mixture, customized DCA microparticles (1.5 µm) were added with a final concentration of 5% (wt/wt). Briefly, DCA consisted of superparamagnetic iron oxide (SPIO) nanoparticles coated with colloidal gold with a SPIO:gold ratio equal to 3:1 volume/volume (v/v). The core-shell SPIO/gold structured particles were then embedded in silica matrix and coated with hydroxyapatite. BMP-2 was then added directly through resuspension in the setting solution.

The enhancement in MRI and CT contrast of the CPC/DCA composition was firstly assessed in human and goat teeth *ex vivo*. Afterwards CPC/DCA/BMP-2 was used for a direct pulp treatment *in vivo* in goat incisors. The material degradation and the new dentin formation were checked at 5 weeks post-surgery using MRI and µCT.

RESULTS: The analysis of the gray value distribution of the implanted CPC with and without contrast agents showed a rise in intensity when the DCA was added. This increase in gray value resulted in an enhanced contrast between the CPC/DCA and the dentin phase (Figure 1A). MRI scans showed, as expected, a blooming effect exactly where the CPC/DCA was implanted, proving the presence of the implanted material (Figure 1B).
Five weeks after the surgery, the enhancement of both MRI and CT signals was reduced but still present. Furthermore we speculated that the combination with BMP-2 would results in an higher dentin deposition compared with the control group (i.e. without BMP-2).

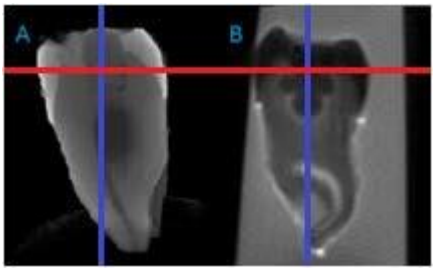

*Fig 1. Human molars. Respectively, in **A** the two-dimensional reconstruction of µCT files and in **B** the MRI ^{1}H phantom. The intersection between the red and the blue line indicates the implanted CPC material.*

DISCUSSION & CONCLUSIONS: The achieved enhanced signal allows more accurate longitudinal detection, while adding a grow factor improved the healing process. The combination of CPC with a DCA and BMP-2 seems to be an innovative strategy for pulp treatment warranting further clinical translation.

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

Engineered *in vitro* model to assess the neuroprotective effect of mesenchymal stem cell secretome on SH-SY5Y neuroblastoma cells exposed to 6-hydroxydopamine

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INTRODUCTION: Because of the biological complexity of the mammalian brain, conventional 2D culture systems fail to provide topographical cues, enable cell differentiation into specific phenotype and fully reproduce *in vivo* cell behavior [1]. Mimicking the 3D physical microenvironment is thus crucial to study both neuropathological features and novel therapeutic approaches. In this work, we optimized a miniaturized optically accessible bioreactor [2] to develop a reliable and effective *in vitro* model to study the neuroprotective effect of mesenchymal stem cell (MSC) secretome on damaged SH-SY5Y neuroblastoma cells.

METHODS: We seeded SH-SY5Y cells and MSCs on 3D poly(styrene) scaffolds (6x3x0.4 mm, 3D Biotek, Fig.1) using a 3D multifunctional shaker. To induce a stable oxidative damage, SH-SY5Y cells were incubated with different concentrations of 6-hydroxydopamine (6-OHDA) in static or shaken conditions. We then evaluated cell metabolic activity by Alamar Blue® assay, at subsequent time points, under construct perfusion. To evaluate the conditioning effect of MSC secretome we inserted the scaffolds in separate but hydraulically connected culture chambers of the bioreactor, so that MSC secretome could flow through one SH-SY5Y cell-seeded scaffold. We also tested cell protein extracts by Western blotting searching for a biochemical mediator of the protective effect of MSC secretome. We used both one-way and two-way ANOVA to assess statistical significance.

RESULTS: The Alamar Blue® assay adapted to perfused culture, resulted a reliable method to assess cell metabolic activity avoiding the need to remove the constructs from the bioreactor for the assay. In shaken conditions, SH-SY5Y cells achieved a stable oxidative damage with 125 µM 6-OHDA, more controllably than in static conditions. MSC secretome significantly reduced neuronal damage by up to 30% in terms of cell viability at 8 days of culture. We are currently searching for a molecular basis of this neuroprotection effect (Hsp70/SIRT3).

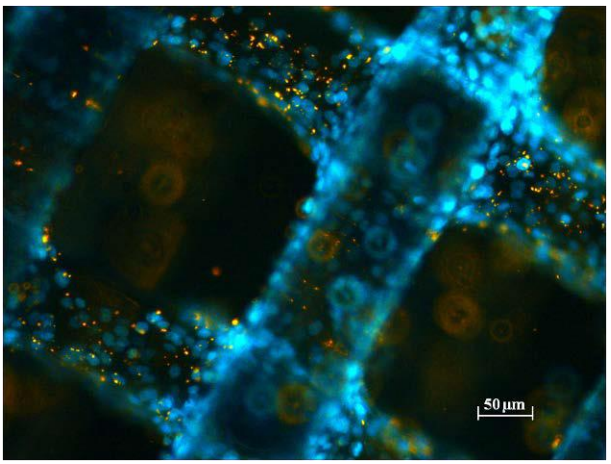

Fig. 1: Cell-seeded 3D poly(styrene) scaffold imaged in live under perfusion in the bioreactor. Cells are double-labelled with DAPI (nucleus) and quantum dots (cytoplasm).

DISCUSSION & CONCLUSIONS: Coherently with what observed in static *in vitro* models and *in vivo* [3-4], our advanced *in vitro* model was able to reproduce the neuroprotective effect of MSC secretome on damaged neuronal cells. Thanks to the perfusion and optical accessibility of the bioreactor, we were also able to follow the effect of MSC conditioning non-destructively and for a longer culture period, compared to standard 2D culture.

ACKNOWLEDGEMENTS: We acknowledge ERC grant No. 646990-NICHOID.

DISCLOSURES: The authors have nothing to disclose.

Modulation of the metastatic niche in peritoneal metastasis of ovarian cancer via mesothelial-to-mesenchymal transition

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INTRODUCTION: Cancers that arise in the peritoneal cavity (colorectal, gastric, ovarian) can disseminate via trans-coelomic route and develop peritoneal metastases. These metastases are often accompanied by the accumulation of ascitic fluid, containing suspended cells and a high concentration of cytokines and chemokines. Ovarian cancer (OvCa) is the most prominent in this type of dissemination, is generally diagnosed at advanced stages and has a high mortality rate. During peritoneal metastasis, cancer cells detach from the primary tumour, disseminate through the peritoneal fluid and attach to the mesothelial cells (MCs) that line the peritoneal cavity [1]. Then, they invade the submesothelial compact zone where carcinoma-associated fibroblasts (CAFs) accumulate. CAFs are an important population in the tumour microenvironment, derive from different sources, and participate in several stages of tumour progression [2]. One of these sources is the MCs, which convert into CAFs through a mesothelial-to-mesenchymal transition (MMT) induced by cancer cells via the TGF-β pathway [3,4].

METHODS: MCs were isolated from the ascitic fluid (AFMCs) of ovarian cancer patients and characterised by immunofluorescence (Figure 1), qRT-PCR and RNA-seq. Swiss nu/nu mice were used for peritoneal dissemination and subcutaneous xenograft models using the OvCa cell line SKOV-3-luciferase. Tumour growth was monitorised by *in vivo* bioluminiscence imaging. We pre-conditioned the peritoneum of mice with conditioned media from cancer cells, adenoviral particles encoding active TGF-β1, or lentiviral particles containing the shRNA sequence targeting Smad3. Biopsies of peritoneal implants of ovarian cancer patients were analysed by immunohistochemistry.

RESULTS: We show that AFMCs are undergoing a MMT, convert into CAFs and favour tumour growth in a subcutaneous xenograft model. This MMT is taking place in a TGF-β Smad-dependent way. In patient biopsies, we observed that pSmad3 has a nuclear localisation in MC-derived CAFs but, surprisingly, a cytoplasmic one in ovarian cancer cells. We confirmed this differential localisation *in vitro* upon TGF-β stimulation. Finally, we observed that peritoneal metastasis is significantly reduced when the TGF-β pathway is targeted, by blocking its receptor I, or by knocking down Smad3.

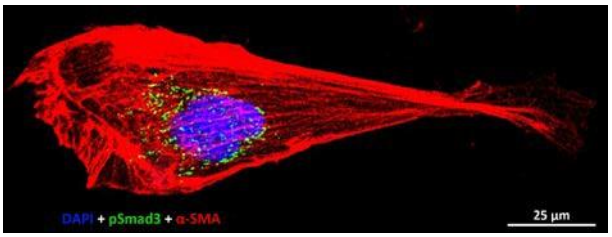

Fig. 1: Immunofluorescence of AFMC, showing a MMT has taken place: expression of the CAF marker α-SMA, and nuclear localisation of pSmad3, downstream effector of TGF-β.

DISCUSSION & CONCLUSIONS: The MMT taking place in AFMCs converts them into CAFs and promotes tumour progression. In the development of peritoneal metastases, the TGF-β Smad-dependent pathway plays a key role; however, ovarian cancer cells have this pathway truncated. This suggests ovarian cancer cells rely on MC-derived CAFs for creating a suitable metastatic niche. We suggest that modulation of the MMT could constitute an alternative target in the treatment of peritoneal metastases.

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.

Biomimetic hepatic scaffolds cannot be fabricated using human livers as a design template

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INTRODUCTION: The native liver extra-cellular matrix (ECM) is often considered as the perfect scaffold for hepatic tissue engineering. Hence, it is of interest to characterise the principal mechano-structural and physicochemical properties of decellularised liver in order to derive quantitative design criteria to fabricate or print ECM-mimetic scaffolds using novel materials. In this regard, we applied our recent decellularisation method [1] – which reproducibly conserves intra-lobular micro-structure and protein content in porcine liver – to hepatic tissue samples collected from five different human donors (H1-H5). The decellularisation outcome was evaluated histologically using Haematoxylin-Eosin (H&E) and Alcian blue-PAS (AB/PAS) stained sections, then quantitative image analyses were performed and the results compared to those obtained for liver samples harvested from one year old healthy pigs.

METHODS: Normal human hepatic tissue was obtained from 5 patients undergoing liver resection. The study was approved by the local Ethical Committee. Human liver discs were decellularised using a protocol based on Triton X- for 3 days, as described in ref [1]. Samples were fixed and embedded in paraffin. Stained sections were imaged with a 25x objective, obtaining a set of n=15 images per sample/treatment/staining for subsequent quantitative histological analyses. Images were analysed with HisTOOlogy, an open-source tool we developed to identify and quantify biological structures from histological sections [2]. All H&E stained sections were analysed to determine the eosin covered area (ECA) and the nuclear count. Mean pixel intensity (MPI) in AB/PAS stained sections was analysed in CYMK colour space, assuming that cyan is directly related to the AB dye and magenta to PAS.

RESULTS: The nuclear count obtained from histological analyses of untreated, decellularised human and porcine samples are reported in Figure 1. The data show that the use of non-ionic detergents is not sufficient to remove cells from 4 out of the human 5 livers. Similarly, the ECA as well as PAS and AB content differed from sample to sample. We also applied a more aggressive ionic decellularisation method replacing 1% Triton X-100 with 0.1% SDS on all samples but the results were still unsatisfactory and highly variable.

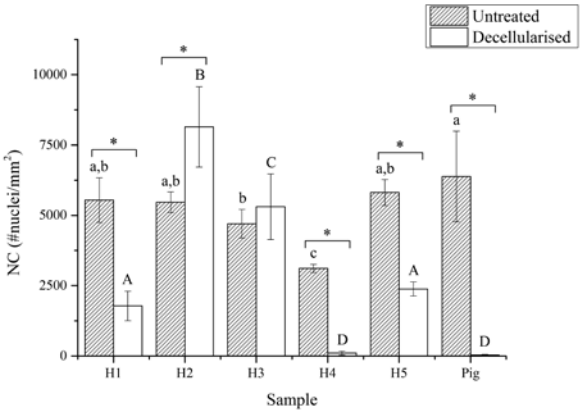

Fig. 1: Number of cell nuclei obtained from quantitative histological analyses. Different letters indicate significant differences between groups (one-way ANOVA, $p < 0.05$): lowercase letters are referred to ANOVA results of untreated samples, while capital letters denote ANOVA results of decellularised samples.

DISCUSSION & CONCLUSIONS. This study was undertaken to derive a set of quantitative design parameters for the fabrication of biomimetic liver ECM scaffolds using decellularised human livers as an ideal template. Our results show that neither mild nor aggressive detergents are capable of removing all cells from all human donor samples and that there is a high degree of donor to donor variability, reflected in different decellularisation outcomes for each donor. Thus decellularised human hepatic tissue cannot be used to derive quantitative parameters for the fabrication of scaffolds which mimic the architecture of healthy human livers.

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Hyaluronic acid increases chondrogenesis in AMSCs-Chondrocytes co-culture systems

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INTRODUCTION: Cartilage tissue features poor self-healing capacity and it is avascular by nature. Its regeneration represents a unique clinical challenge for which current treatments are unsuccessful. Cell-based cartilage repair represent a very attractive alternative. Particularly, co-culture systems of mesenchymal stem cells (MSCs) and chondrocytes have the advantage of lowering the amount of chondrocytes needed and avoiding their dedifferentiation during expansion [1]. In addition, combining MSCs and chondrocytes have been demonstrated to improve chondrogenesis [2] as well as to reduce hypertrophy and tissue mineralization [3]. On the other hand, a relevant aspect to consider is the design of an adequate 3D matrix in order to induce tissue regeneration via specific cell stimulation.
This study intends to apply the advantages of mixed cell culture to create a system for more efficient cartilage formation. The aim is to mimic the environment in the native cartilage as a better stimulation for both cell types may increase differentiation, proliferation and production of extracellular cartilage components.

METHODS: Human articular chondrocytes (hAC) and human adipose-derived mesenchymal stem cells (hAMSCs) were cultured together in different concentrations (100% hAMSCs; 75% hAMSCs/ 25% hACs; 50% hAMSCs/50% hACs; 25% hAMSCs/75% hACs, 100% hACs) in a hydrogel consisting of type-I collagen and hyaluronic acid (HA). The percentage of HA in the composition of the gel was varied in the range 0-5%. Total cell seeding per construct was $1x10^5$ and culture duration was up to 35 days. No growth factors were used. Cell viability was evaluated by MTT and LDH activity. Cell proliferation was measured by PicoGreen assay. Glycosaminoglycans (GAGs) were quantified by binding to 1,9-dimethyl-methylene blue (Blyscan assay). Histological evaluation (Alcian blue and Safranin O) and immunohistochemistry (IHC) for Collagens II, III and X was performed. Sox-9, Aggrecan, and Collagens II, III, and X were evaluated by qPCR.

RESULTS: Cell viability tests showed no detrimental effect on the co-culture groups after 7 days. On the contrary, the cell viability of all co-culture groups was significantly higher when compared to the 100% hACs. Cell proliferation was increased in all co-culture groups and increased constantly with the time of culture. The Collagen/HA construct resulted in better cell viability and proliferation independent of the HA concentration used. Significantly higher amounts of GAGs in the Collagen/HA constructs were present compared to collagen alone. The co-culture group 25%hAMSCs/75%hACs showed the highest GAGs production (fig.1). This superiority on cartilage forming capacity of the co-culture in vitro was also confirmed by IHC and gene expression. This was also observed for the gels containing collagen/HA composite.

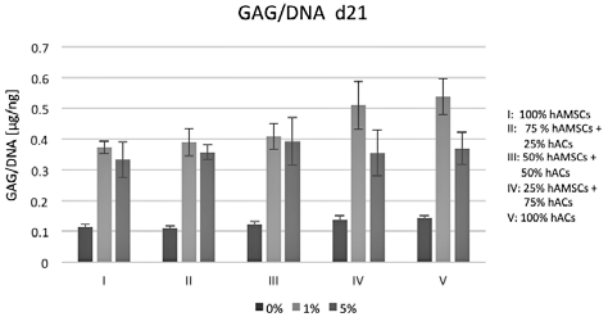

Fig. 1: GAGs production after 21 days of culture.

DISCUSSION & CONCLUSION: Our study shows that chondrogenesis can be improved by selecting an adequate 3D matrix selection, but also by cell-based approaches like co-culture systems. The positive effect of the HA content was demonstrated on the elevated GAGs production of hAMSCs alone seeded on this matrix. On the other hand, our study demonstrated that hACs could be partially replaced by hAMSCs without influencing extracellular matrix production. It was shown that a adding a small % of hAMSCs was able to even increase the chondrogenic potential. Moreover, the co-culture system highly favored the chondrogenesis process to occur in vitro. In order to make our study particularly relevant for future clinical applications, we have isolated both ACs and AMSCs from human donors (n=6). This is an advantage compared to other reports in which one or both cell types used for the co-culture are from animal origin.

Biodegradable scaffolds for soft tissue engineering based on reactive isocyanate-containing polylactone prepolymers

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INTRODUCTION: The incidence of soft tissue loss or damage makes efficient reconstructive therapies a challenge in medicine. In a scaffold-based approach, biomaterials matching the complex properties of soft tissues are still a matter of ongoing research. We report on reactive, isocyanate-containing polylactone (ICP) prepolymers which can be processed either by chain elongation resulting in linear poly(ester-urethane)s (lPUs) or by crosslinking leading to *in situ* foamable scaffolds. The application potential of the materials in soft tissue reconstruction was evaluated by testing mechanical properties, biodegradation, and biocompatibility.

METHODS: Linear and star-shaped poly(L-lactide-co-ε-caprolactone)s were prepared by ring-opening polymerization using 1,8-octandiol and meso-erythritol as starters [1]. Providing the reactive ICPs, polymers were treated with L-lysine diisocyanate ethyl ester. Chain elongation of ICP forming lPUs was performed by reaction with PEG 400 [2]. Crosslinking was carried out by mixing ICP with DMSO/H_2O and 1,4-diazabicyclo[2.2.2]octane and hardening overnight. Polymers were characterized by conventional techniques (FT-IR, NMR, GPC, titration for isocyanate quantification). The mechanical properties were determined using a texture analyzer TA-XT2i. Morphology of foams was studied by SEM and pore sizes were determined using a gas pycnometer. *In vitro* degradation was studied gravimetrically in Sorenson buffer at 37°C for defined time intervals. *In vitro* cytocompatibility was tested using WST-1® and live/dead assays [1]. Electrospun fleeces were prepared from lPU as previously described [2]. *In vivo* biocompatibility was tested in a murine subcutaneous model for up to 4 weeks and foreign body reaction was assessed histologically in HE-stained slides.

RESULTS: Chain elongation of ICPs with PEG 400 resulted in lPU with molecular weights (M_W) up to 100 kDa. Determination of the mechanical properties of lPU films provided values of 8 MPa for the tensile strength and 0.1 GPa for the Young's modulus which is considerably lower as measured for a conventional poly(L-lactide-co-D,L-lactide) (70/30, Resomer LR 708, PLA, Evonik) confirming the higher elasticity of lPU. The degradation of lPU was found to be only slightly diminished compared to PLA reference. Both WST-1® and live/dead assay performed for 7 days revealed a good *in vitro* cytocompatibility. lPUs can be further processed by various scaffold manufacturing processes. Exemplarily, micro-structured fleeces with a fiber diameter of about 1 µm were produced by electrospinning.

Crosslinking of ICP gave inter-connectively porous foams (porosity: 89%) with pore sizes of 200 to 600 µm. Foams showed a tensile strength of 0.3 MPa (Young's modulus: 0.4 MPa) and a compressive strength of 0.03 MPa. After storing the foams in Sorensen buffer for 36 weeks, an overall weight loss of 50 wt% was detected. WST-1® assay revealed a release of toxic substances during the first 24 h as indicated by a decrease in cellular dehydrogenase activity (CDA) of incubated 3T3 cells. On the following days, no reduction of CDA was found. On formed foams the cells adhered, and proliferated as shown by live/dead-staining. A low-grade mononuclear inflammatory reaction was observed *in vivo*.

DISCUSSION & CONCLUSIONS: Reactive ICPs are versatile prepolymers to receive either non-crosslinked lPUs processible to eletrospun fleeces or crosslinked *in situ* foamable polymeric network scaffolds. Both types of polymer materials show a gradual biodegradation and mechanical properties adaptable to soft tissue requirements. These properties along with the good biocompatibility render the new polymers promising scaffold candidates in soft tissue reconstruction.

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Tailoring bone growth in highly orientated macroporous scaffolds by pore size modification

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INTRODUCTION: Biomaterials are designed to provide the best possible microenvironment to guide cell behavior towards tissue repair and endogenous regeneration. We have previously shown *in vitro* that migration and tissue formation inside scaffold pores is influenced by the pore size. Unclear remained which pore size would best support endochondral bone regeneration. Here, the influence of scaffold pore size on the healing outcome was investigated *in vivo* in a critical sized bone defect in rats.

METHODS: Four different prototypes with variation of the pore size were produced via directional freezing in a controlled temperature gradient and subsequent freeze drying. All prototypes were produced from a 1.5% aqueous collagen dispersion (wt/wt) and featured the same highly aligned channel-like pore architecture. Pore sizes were 62±15µm (prototype S_{60}), 89±15µm (prototype S_{80}), 105±25µm (prototype S_{100}) and 120±21µm (prototype S_{120}).

For *in vivo* evaluation, a 5 mm critical sized femoral defect was created in Sprague Dawley rats stabilized with an external fixator. Prototype S_{60}, S_{80}, S_{100} and S_{120} were implanted into the defect with a group size of n=7-8 per prototype. *In vivo* micro CT (µ-CT) was performed at 2, 4, and 6 weeks and in vitro µ-CT at 6 weeks post implantation to quantify newly formed bone. All specimen were analyzed histologically with Movat's pentachrome staining at 6 weeks post implantation to evaluate tissue composition in the bone defect.

RESULTS: In µ-CT analyses, animals showed a significantly higher amount of bone volume (BV) in the prototypes S_{100} compared to S_{60} at 4 weeks post osteotomy. At 6 weeks post osteotomy, the BV of S_{100} was significantly increased to the BV of both S_{60} and S_{120}. At the same time the ratio between bone and total callus volume (BV/TV) was significantly higher in S_{100} compared to S_{60} and S_{120} both 4 and 6 weeks post osteotomy.

Overall, S_{100} showed the highest BV of all groups with decreasing values towards smaller and larger pore sizes. Histology revealed a higher incidence and higher amounts of cartilage in S_{100} compared to S_{80} indicating an ongoing osteochondral ossification process. In contrast, animals without cartilage showed a closure of the bone marrow cavity and no signs of ongoing bone formation. Interestingly, cartilage was found exclusively within the scaffold filling the critical sized defect verifying the active role of the scaffold in the induction of endochondral ossification.

DISCUSSION & CONCLUSIONS:

In our study, the pore size of 100 µm (S_{100}) was most efficient in inducing endochondral bone growth and led to a higher volume of newly formed bone than smaller or larger pore sizes. It was reported previously that pore sizes of 90-120 µm are favorable for the formation of cartilage compared to larger pores since they limit vessel ingrowth and create a hypoxic environment *in vivo* [1]. However, in our study vessel density was not the decisive factor for endochondral bone growth and could not explain the optimal pore size at 100 µm. Rather we found indications that the influence of the scaffold's pore architecture on extracellular matrix patterning is the critical parameter for the occurrence of cartilage. This finding is remarkable since biomaterial-based approaches without the use of growth factors or cells have so far failed to induce endochondral ossification for improved bone regeneration.

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3D Printing of a biomimetic environment for central nervous tissue formation in vitro

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INTRODUCTION: Morphogens gradients (e.g. retinoic acid or sonic hedgehog) control the formation of neural tube during central nervous system (CNS) development which may provide a model to differentiate pluripotent stem cells to CNS tissues. Replication of those gradients in a suitable biomaterial which favours neuronal differentiation may promote the formation of a region-specific CNS tissue, *in vitro* [1]. 3D printing technology offers a manufacturing tool to fabricate a complex biomimetic environment for tissue engineering application [2]. The aim of this work is to create a biomimetic gradient hydrogel tube using 3D printing, in addition to investigate the effect of the effect of hydrogel matrix on the neuronal differentiation of mouse embryonic stem cells.

METHODS: An extrusion-based multi-head 3D printer (Regen HU, Switzerland) was used for 3D printing a hollow tubular construct composed of polycaprolactone (PCL, MWT 45 kDa) and semi-cross linked alginate hydrogel (1.5%). A gradient of fluorescein isothiocyanate conjugated bovine albumin (FITC-BSA) was created within the alginate hydrogel structure by controlling the ratio of FITC-BSA – alginate/ alginate layers as a function of tube distance. The formation of gradient was determined by measuring the fluorescent intensities along the tube after sectioning, in addition to fluorescence imaging.

Differentiation of mouse embryonic stem cells (mESCs) into neurons by retinoic acid was investigated in embryoid body –encapsulated 3D hydrogels. Alginate (1.5%) and methacrylated gelatine (10%) were used in 3D hydrogel differentiation experiments. Immunostaining was used to characterize the loss of pluri-potency and neuronal differentiation, respectively.

RESULTS: 3D printing offers a precise control over the formation of gradient and PCL/alginate tubular structure (Fig. 1). Fig. (2) Shows βIII tubulin positive neurons in both alginate and methacrylated gelatine. However, cells in methacrylated gelatine showed clear cell migration and axonal outgrowth.

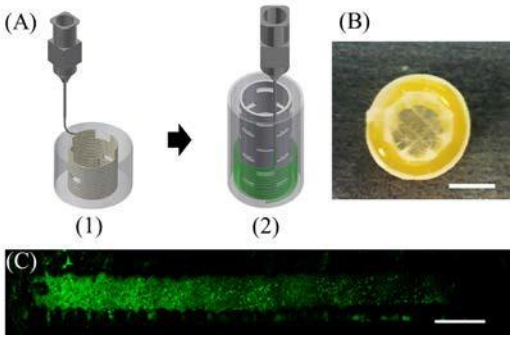

Fig.1: 3D printing of the hollow PCL tubular structure (A-1) and alginate hydrogel between them (A-2), top view of the final structure, scale bar (5mm) (B) a fluorescence image of the tube cross-section showing the gradient of albumin in alginate, scale bar (2mm)(C).

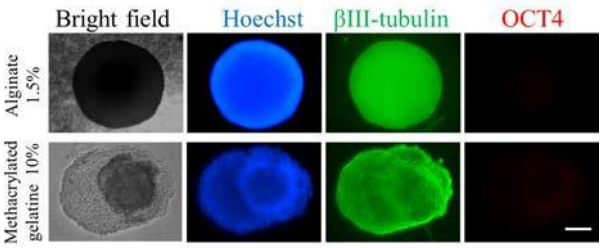

Fig.2: Immunostaining of the differentiated embryoid bodies (day 8). Scale bar (100µm).

DISCUSSION & CONCLUSIONS: 3D printing of a controlled molecular gradient in a hydrogel tubular construct with using neuronal growth permissive hydrogels will allow us to replicate the developmental process of neural tube. Ultimately, the more precise control over neural differentiation of stem cells in 3D hydrogel can reveal potential spinal cord regenerative strategies.

ACKNOWLEDGEMENTS: We thank the Higher Committee for Education Development in Iraq for sponsoring Omar Hamid's PhD.

Platelet derivatives combined to PGA-HA scaffold support cartilage repair and maintain hyaline cartilage phenotype by Chondromodulin-I induction

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INTRODUCTION: Platelets are known to play an important role in homeostasis and in the process of tissue healing. The use of platelet derivatives in regenerative approaches in cartilage repair is becoming more common (1). Here, we showed the synergic actions of platelet-rich plasma (PRP) and PGA-HA scaffold on cartilage regeneration and the capability to maintain hyaline cartilage phenotype by chondromodulin (Chm) induction.

METHODS: Primary human articular chondrocytes (HAC) from bioptic specimens were cultured in vitro in presence of platelet derivatives (PL) to induce HAC proliferation maintaining chondrogenic potency (2). We performed an in vivo study in which HAC, previously expanded, were seeded in PGA-HA scaffolds in presence or not of platelet- rich plasma (PRP) and implanted in nude mice to mimic cartilage neo-tissue formation. Histological and immunohistochemical analyses were performed to characterize the cartilage tissue formed.

RESULTS:

As previously described human articular chondrocytes are extremely sensitive to PL treatment (2). When 5% of platelet derivatives (PL) were added to the chondrocytes, we observed a drastic increase of cellular proliferation. After 1 week of cell expansion, HAC were combined with PGA-HA scaffolds and implanted in mice. The analyses after 40 days of the recovered constructs (HAC+ PGA-HA) implanted, revealed a complete reabsorption of PGA-Ha scaffolds and newly hyaline cartilage formation was detected. Moreover, we observed the stability of ectopic cartilage formed; after 2 months in vivo, all the sample showed a strong expression of GAG and COL II, no COL X was observed, while the antiangiogenic factor Chm-1 was high expressed (Fig.1).

We further investigated the influence of Chm-1 in vitro, HAC were cultured in presence of 10% FCS or 5% Lyset (PL) and we observed a drastic increased of Chm-1 protein and mRNA expression in presence of platelet derivatives (Fig.2).

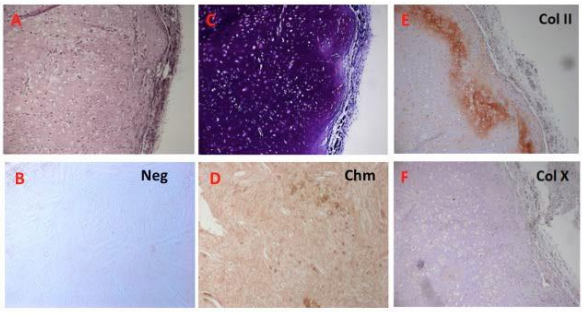

Fig. 1 Histological analysis of neo-cartilage formed in vivo A) H&E C) Toluidine Blue E) COLII, D) Chm, F) COL X expression, B) Negative control

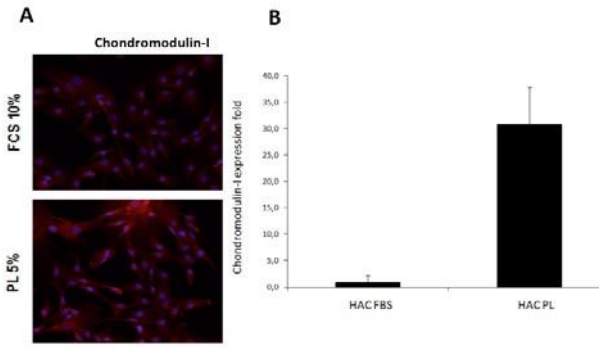

Fig. 2: Chm-1 in vitro expression: a) protein and b) mRNA induction of Chm in presence of 5% PL

DISCUSSION & CONCLUSIONS:

The combination of articular chondrocytes, platelet derivatives and PGA-HA scaffold can synergistically promote cartilage formation. However, the ectopic cartilage regeneration and homeostasis is maintained by the overexpression of Chondromodulin mediated by platelet derivatives treatment. This study provides a novel model based on tissue engineering strategy for future clinic cartilage therapy.

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Improved cell seeding results in improved mineralization

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INTRODUCTION: Cell seeding onto a 3D scaffold is the first step for engineering a bone tissue-like structure. An efficient and homogenous method to seed cells is needed to provide a reproducible amount of attached cells with a uniform distribution. Even though dynamic seeding methods have been reported to be superior to static ones because of their more homogeneous cell distribution using kinetic forces [1], many laboratories still apply cells by pipetting them on top of their scaffolds. With this study, we present an improved cell seeding strategy that results in an improved amount of attached cells and significantly affects the amount and distribution of mineralized extracellular matrix (ECM) deposited by human mesenchymal stem cells (hMSCs) on porous silk fibroin (SF) scaffolds.

METHODS: Scaffolds (height: 3 mm; diameter: 5 mm) were manufactured as reported previously [2]. Static seeding involved pipetting a cell suspension (1×10^6 cells/20 µL) onto pre-wetted scaffolds and incubation for 90 min at 37°C. In the dynamic seeding process, scaffolds were incubated with a cell suspension (1×10^6 cells/4 mL) in 50-mL tubes placed on an orbital shaker for either 2 h, 4 h or 6 h in an incubator at 37°C. Cell number was determined after seeding by DNA quantification. Cell distribution was assessed by H&E staining on histological sections. For culture, the constructs were transferred into a bioreactor with osteogenic cell culture medium. After 3 weeks, microcomputed tomography (µCT) imaging was used to visualize and quantify mineralized ECM.

RESULTS: DNA quantification showed significantly higher cell numbers using the dynamic seeding approach for 4 h and 6 h (Tab. 1).

Table 1: DNA and mineralized ECM quantification of cell-seeded scaffolds

Seeding Method	Static	Dynamic 4 h	Dynamic 6 h
DNA [ng]	23±3	44±5	58±4
ECM/DNA [mm³/µg]	0,2±0,2	21,3±4,7	32,4±14,4

Dynamic seeding resulted in a more homogenous cell distribution throughout the bulk of the scaffold and increased ECM mineralization (Tab. 1). In general, the mineralized ECM was more homogenously distributed both vertically and horizontally as compared to the static seeding approach (Fig. 1).

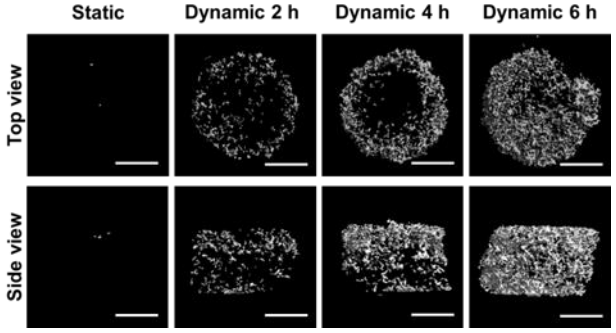

Fig. 1: µCT images of mineralized ECM produced after 3 weeks by hMSCs seeded on SF scaffolds with a static or dynamic seeding method using an orbital shaker for 2 h, 4 h or 6 h. Scale bar: 2 mm.

DISCUSSION & CONCLUSIONS: In this study, we demonstrated that the use of a dynamic seeding method using an orbital shaker leads to significantly higher numbers of cells inside a SF scaffold, which in turn results in increased bone-like tissue formation. Our results not only imply that the initial cell seeding density significantly affects tissue formation but also indicate that homogenous cell-seeding using a dynamic method supports uniform tissue development. The application of this simple seeding technique can go beyond bone tissue engineering and be used for seeding similar porous scaffolds with hMSCs.

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Highly branched poly(β-amino ester)s as new gene delivery vectors

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INTRODUCTION: Over the last decade, linear poly (β-amino ester)s (LPAEs) have emerged as a new class of gene vectors[1]. However, their linear structure has severely limited the further improvement of their performance [1,2]. Herein, a novel type of dendritic polymer, highly branched poly (β-amino ester)s (HPAEs, Fig. 1), was developed as a new class of gene vector.

METHODS: S4, TMPTA and BE, were copolymerized to generate base polymers, MPA was then used to endcap the base polymers to formulate HPAEs (Fig. 1).

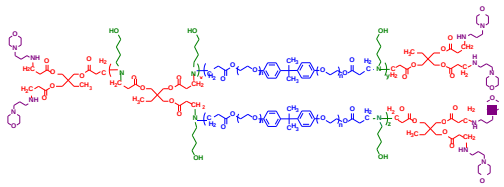

Fig. 1: HPAEs are developed as gene delivery vectors via a one-pot "A2+B3+C2" Michael addition approach.

RESULTS: GPC results show that HPAEs have molecular weight around 10 kDa with Mark-Houwind value below 0.5. NMR confirms the copolymerization of S4, TMPTA and BE. Gene transfection show that in HKC8, COS7, NHK, SHSY-5Y, rADSC and Neu7, HPAE can mediate over 80% GFP expression (Fig. 2). Utilizing a RDEB knockout mouse model, HPAEs can carry pcDNA3.1COL7A1 to restore the expression of Collagen VII (C7) after single or triple injection (Fig. 3).

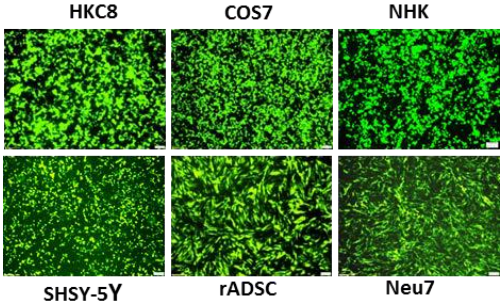

Fig. 2: Representative fluorescence images of cells transfected by HPAE-4 at the w/w ratio of 15:1.

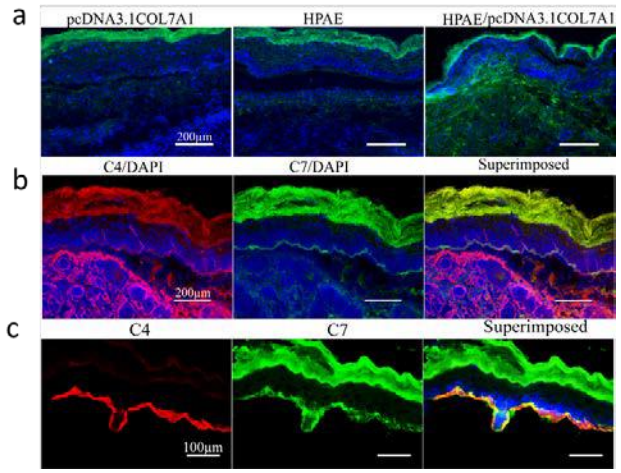

Fig. 3: (a) Intradermal administration of HPAE carrying the pcDNA3.1COL7A1 shows large regions of human C7 restoration in the RDEB (-/-) mouse; (b) human C7 was restored in the basement membrane zone (BMZ); (c) The ventral area of RDEB (-/-) mouse shows large regions of human C7 restoration.

DISCUSSION & CONCLUSIONS: One of the main bottlenecks for clinical gene therapy is the lack of safe and efficient gene delivery vectors. Given the superiority of highly branched polymer in gene transfection efficiency compared to their linear counterparts, we updated the LPAEs to HPAES. GPC and NMR results demonstrated that the "A2+B3+C2" polymerization strategy is highly reliable and flexible for HPAE synthesis. The high performance in gene transfection over multiple cell lines and the restoration of C7 expression in RDEB knockout mouse model imply the great promise of HPAEs in gene therapy in clinic.

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Vascularized skin-on-a-chip: strategies combining skin equivalents with vasculature in a multi-organ-chip platform

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INTRODUCTION: Current *in vitro* skin models are mostly based on static culture systems emulating human epidermis or a combination of epidermis and dermis in so-called full thickness skin equivalents. As human skin is a complex tissue composed of various cell types, these systems are inadequate for studying the complex human interactions required to fully replace animal models in drug development[1]. In particular, the presence of a vasculature is a prerequisite for sufficient supplementation of the skin and it's appendices with required nutrients and oxygen. Our multi-organ-chip (MOC) platform uses a miniaturized circulatory network with an integrated micropump to provide pulsatile circulation of microliter-volume of medium to the tissues in a similar way to blood[2]. Here, we combine skin equivalents with vasculature in the two-organ variant (2OC) aiming to model a microvasculature driven cutaneous homeostasis of our skin equivalents.

METHODS: The 2OC's circuit contains only 600 µl of medium. The built-in micropump provides a constant pulsatile flow and ensures oxygen and nutrient supply. No external reservoirs need to be attached that would otherwise dilute the enriched medium. Human dermal microvascular endothelial cells (HDMECs), isolated from human foreskin, were injected into the microfluidic channel system. After even cell distribution inside the circuit, the device was incubated at 37 °C under static conditions for 3 h to allow the cells to attach to the channel walls. A continuous pulsatile flow was applied that provided an optimal shear environment. After 3 days of mono-cultivation, the skin equivalent was added for another 7 days. Skin equivalents were build up prior to the MOC experiment under static conditions. Full thickness skin equivalents (ftSE) were constructed by growing keratinocytes on Matriderm® punches colonized with skin fibroblasts.

RESULTS: Mediated by the shear stress the HDMECs demonstrated physiological-like elongation and orientation with the direction of flow. The cells remained viable over the co-cultivation period. FtSE developed in vivo-like epidermal architecture with regular differentiation and marker expression. The skin equivalents cultivated in the perfused 2OC system together with the endothelial cells showed remarkable consistency and vitality. Expression of specific basal membrane and extracellular matrix proteins (Collagen IV, Laminin 5, Collagen I) and defined mesenchymal and epithelial markers (Vimentin, CK10/15) remained stable after 7 days of MOC co-culture.

DISCUSSION & CONCLUSIONS: The results clearly demonstrate the capability of the MOC system as a useful tool for long-term co-culture of skin equivalents and endothelial cells, keeping most of their structures undamaged. Additionally, the on-chip micro-pump was able to provide a suitable shear stress environment for the endothelial cells.

In vitro testing of substances using the MOC, whether applied topically or into the medium, might be performed with significantly prolonged test periods, enhanced validity and online endpoint analysis compared to static cultures.

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Cardiomyocytes Differentiated From Adipose Derived Mesenchymal Stem Cells and Cell Platforms for Heart Tissue Regeneration

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INTRODUCTION: The incidence of heart failure across the world is one of the most important challenges that remained to be solved. Currently many strategies for repairing damaged heart are under investigation [l]. In this study, we established modified method to decellularized the heart and obtained an in vitro cardiac cell platforms to seed differentiated adipose derived mesenchymal stem cells (ADMSCs) for heart tissue failure therapy.

METHODS: The rat gonadal adipose tissue was isolated in the first part of the study. 5-Aza was used as a cardiomyogenic differentiation factor. Fluorescent staining with specific molecules were used for characterization following the cell differentiation. RT-PCR analysis was performed to investigate the expression of cardiac specific genes. ADMSCs which were expected to differentiate to the cardiomyocytes were labeled with BrdU by incubation for 24 hours in culture medium. Heart samples were obtained from perfused rats and cut into pieces for applying modified decellularization protocol. After that, all samples stained by H&E and Masson's Trichrome. For seeding labeled cells, lyophilization method was applied to all decellularized heart pieces except control hearts. Then cells were incubated with the decellularized cardiac cell platforms for 48 hours. The proliferation and adhesion potential of cells on scaffolds was measured by MTT assay. The viability of cells on scaffolds was assessed using AO/PI fluorescent staining. Also SEM was performed. After that, all samples in three different groups were implanted to the rat retroperitoneum. All groups sacrificed at 1^{st}, 2^{nd} and 4^{th} weeks following the implantation. The collected tissues scaffolds were examined by RT-PCR.

RESULTS: Our study showed the most effective method for decellularization of hearts after analysis that described above was done. Histological evaluation revealed no remaining nuclei in all groups. MTT assay demonstrated increasing cell proliferation at the second day. SEM analysis of decellularized heart showed well preserved extracellular matrix (ECM) components for cell attachment at 48 hour after cell seeding. Protein expression of differentiated cardiomyocytes on cell platforms showed enhanced expression in ECM+Cell group at second week.

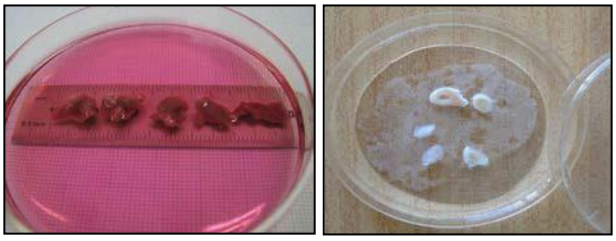

Fig. 1: Macroscopic images of control (a) and decellularized (b) heart pieces.

Table 1. Total RNA amount of implanted control ECM and ECM+Cells at different time duration (W-week).

Sample	Total RNA (ng/ul)
Control Matrixs	117.5
ECM+Cells-1W	257.7
ECM+Cells-2W	429.8
ECM+Cells-4W	321.8

DISCUSSION&CONCLUSIONS: In conclusion, mechanical interactions between cells and the ECM play important role in the healing progress. That is why our study indicated that using decellularized tissue after lyphilization, as a cell platform, can support cell seeding and adhesion, survival of cells and also maintain the cell-to-cell contacts after implantation.

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Ultrasound as a tool to direct microvessel network formation and morphology

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INTRODUCTION: Engineering microvessel networks that structurally and functionally mimic native microvasculature is critical for the fabrication and survival of a wide range of bioengineered tissues. We have developed a non-invasive ultrasound-based method to spatially pattern cells within 3D hydrogels, and have demonstrated the feasibility of translating this technology to microvascular tissue engineering [1, 2]. Here, we determine how acoustic frequency and amplitude affect initial cell patterning and resulting microvessel network morphologies.

METHODS: Human umbilical vein endothelial cells (HUVECs; $1x10^6$ c/mL) were suspended in soluble type I collagen (1 mg/mL). Solutions were exposed to ultrasound standing wave fields (USWFs) with acoustic frequencies of 0.5, 1, and 2 MHz and a range of peak acoustic pressure amplitudes. Sham-exposed samples were treated identically, but the sound was not activated. Samples were exposed for 15 min, during which time cell patterning and collagen polymerization occurred. High-frequency (38-MHz) ultrasound imaging was used to visualize initial cell patterns through the depth of 1-cm thick gels. Gels were cultured for 10 days and images of microvessel networks were obtained. Initial cell patterning and resultant microvessel morphology were quantified as a function of acoustic frequency and pressure.

RESULTS: USWFs rapidly patterned cells into planar bands. High frequency ultrasound was used to generate B-mode images of the full depths of USWF-patterned hydrogels (Figs. 1A, B). Distances between planar bands of cells and the density of cells within planar bands were quantified. Distance between planar bands of cells decreased with increasing acoustic frequency. The density of cells within each planar band increased as peak pressure amplitude increased for each frequency investigated. After 10 days, microvessel networks were present throughout the volume of USWF-exposed samples. The morphology of the resultant microvessel networks was influenced by acoustic parameters employed for the initial cell patterning (Fig. 1 C, D). Microvessel networks formed in response to patterning with 0.5-MHz USWFs formed capillary-like networks characterized by vessels less than 25 µm in diameter (Fig. 1D). In contrast, three different microvessel morphologies formed in response to 1-MHz USWF patterning. Capillary-like networks formed as a result of 0.1 MPa exposure; non-branching microvessels with diameters of 50-200 µm formed in response to 0.2 MPa exposure; and hierarchically-branching networks formed in response to 0.3 MPa exposure (Fig. 1C). Microvessel networks formed with 2-MHz USWFs were capillary-like for exposures at 0.05 MPa and branching networks at 0.1 MPa and 0.15 MPa. Sham-exposed samples underwent limited network formation.

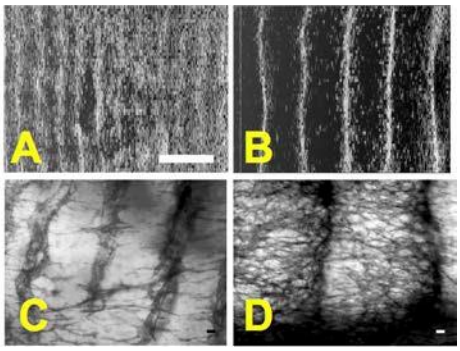

Fig. 1: USWF-induced patterning and network formation. B-mode images of cells patterned at 1 MHz (A) and 0.5 MHz (B). Scale bar = 2 mm. C & D) Microscopy images of resultant microvessel networks formed from initial patterning in A and B, respectively. Scale bar = 100 µm.

DISCUSSION & CONCLUSIONS: USWF-patterning of HUVECs leads to rapid formation of 3D microvessel networks within collagen hydrogels. The acoustic frequency and pressure amplitude of the USWF control both the initial cell patterning and the resulting microvessel network morphology. High-frequency ultrasound imaging provides a valuable tool to visualize and quantify 3D cell patterning throughout thick collagen gels.

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

Local treatment of inflammation in degenerated intervertebral disc using poly(γ-glutamic acid)-based nanocomplexes promotes extracellular matrix remodelling

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INTRODUCTION: Intervertebral disc (IVD) degeneration and associated inflammation often lead to low back pain, one of the major causes of disability worldwide. Available treatments decrease symptoms' progression, but they fail to restore native IVD properties. Poly(γ-glutamic acid) (γ-PGA) supplementation of chondrogenic medium has recently been shown to enhance chondrogenesis of mesenchymal stem/stromal cells[1]. Moreover, Chitosan (Ch) and γ-PGA nanocomplexes (NCs) with a non-steroidal anti-inflammatory drug, Diclofenac (Df), were shown to counteract a pro-inflammatory response of human activated macrophages[2]. Hence, the effect of γ-PGA/Ch and γ-PGA/Df/Ch NCs were analysed, in different models of IVD organ culture.

METHODS: γ-PGA/Ch and γ-PGA/Df/Ch NCs were prepared by co-acervation method[2]. Two IVD organ cultures were used: 1) nucleotomized bovine IVDs with cartilaginous endplate[3] and 2) IVD organ cultures of nucleus pulposus (NP) punches stimulated with needle-puncture and IL-1β[4].

RESULTS: The results showed that γ-PGA/Ch NCs can be internalized by IVD cells in organ cultures. Nucleotomized IVDs treated with γ-PGA/Ch NCs at pH 7.4 and γ-PGA by itself were able to promote glycosaminoglycans production and significant Col II synthesis. In pro-inflammatory and degenerative culture conditions, the injection of γ-PGA/Df/Ch NCs down-regulated *IL-6*, *IL-8*, *MMP1* and *MMP3*, and decrease PGE_2 production, compared with IL-1β-stimulated IVDs. Interestingly, at the same time γ-PGA/Df/Ch NCs promoted an up-regulation of matrix proteins, namely Col II and Aggrecan, both at gene and protein level. γ-PGA/Ch NCs by itself also reduced *IL-8* expression and PGE_2 production, although no significant differences were observed compared to IL-1β group. Nonetheless, γ-PGA/Ch NCs alone significantly decreased *MMP3*, but only slightly down-regulated *MMP1*. Concerning ECM proteins, γ-PGA/Ch NCs group also significantly increased Agg expression and production, but the same was not observed for Col II.

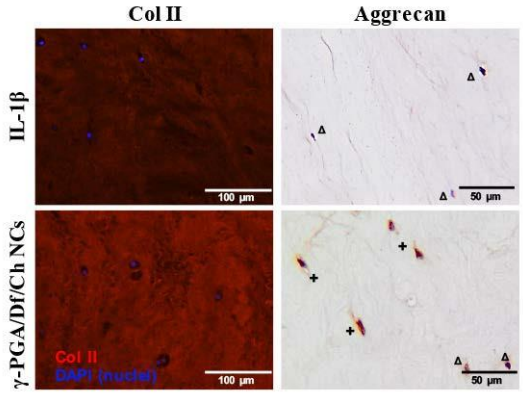

Fig. 1: Sagittal sections of disc punches, after a 14-days culture, stained for Col II and Aggrecan (negative (Δ) and positive (+) cells).

DISCUSSION & CONCLUSIONS: The results obtained in this study suggest that γ-PGA/Ch NCs can promote recovery of IVD native matrix. Furthermore, γ-PGA/Df/Ch NCs decreased pro-inflammatory markers in degenerated IVD, as well as promote ECM remodelling in IVD. The results obtained suggest the involvement of MMP3, but not MMP1. This works opens new perspectives on the development of alternative therapeutic approaches for IVD degeneration.

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Inhibition of IL-1R1/MyD88 signaling promotes mesenchymal stem cell-driven tissue regeneration

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INTRODUCTION: In-depth understanding of the role of the immune system during tissue healing could provide clues to therapeutic avenues for restoring damaged tissues, and controlling the immune regulations of tissue healing may become an attractive option in regenerative medicine. Commonly, tissue injury and the healing response lead to the release of various endogenous danger signals including toll-like receptor (TLR) and interleukin-1 receptor, type 1 (IL-1R1) ligands, which modulate the immune microenvironment. These danger signals are involved in the recruitment and the activation of immune cells engaged in host defense. In addition, TLRs and IL-1R1 have been shown to influence the repair process of several tissues. In this study, we explored the role of TLRs and IL-1R1 during bone regeneration, seeking to design regenerative strategies integrating a control of their signaling.

METHODS: We analyzed calvarial bone defect (5 mm diameter) regeneration in mice deficient for TLRs, IL-1R1, and molecules involved in their signaling such as MyD88. Bone regeneration was evaluated using μCT. As cell models, we used bone-derived mouse mesenchymal stem cells (MSCs) and primary osteoblasts. The effect of IL-1R1/MyD88 signaling on MSCs and osteoblasts was analyzed with various technics, including cell proliferation assay, migration assay, qPCR, cell-signaling arrays, and ELISA. To track MSC fate *in vivo*, MSCs were labeled with carboxyfluorescein succinimidyl ester. MSCs were transplanted with a fibrin matrix and proliferation as well as differentiation into osteoblasts were analyzed by FACS and qPCR. MSC-like cells mobilization into calvarial defects was monitored using flow cytometry using typical MSC markers. Depletion of monocytes/macrophage in mice was done with clodronate liposome. Lastly, an IL-1R1/MyD88 signaling inhibitory peptide was engineered to bind fibrin, and to be released on cell-demand.

RESULTS: We show that interleukin-1β (IL-1β) via IL-1R1/MyD88 signaling negatively regulates bone regeneration in the mouse. IL-1β is released mostly by monocytes and macrophages at the bone injury site for about 10 days. *In vitro*, we demonstrate that IL-1β inhibits MSC migration, proliferation, and differentiation into osteoblasts. We also show that proliferation and differentiation of transplanted MSCs is inhibited by IL-1R1/MyD88 signaling *in vivo*. Moreover, recruitment of MSC-like cells into bone defects of $Myd88^{-/-}$ and $Il1r1^{-/-}$ mice is twice higher compared to wt mice, indicating that IL-1R1/MyD88 signaling inhibits stem/progenitor cell mobilization. Mechanistically, we reveal that IL-1R1/MyD88 signaling impairs MSC regenerative functions by inhibiting the Akt/GSK-3β/β-catenin pathway. Lastly, we engineered a MSC delivery system based on fibrin. An IL-1R1/MyD88 inhibitory peptide was engineered to have a fibrin-binding sequence, a protease sensitive sequence, and a membrane translocation sequence. Consequently, the peptide can bind fibrin and is released by proteases following matrix remodeling. Then, the peptide translocates into cells and ultimately inhibits IL-1R1/MyD88 signaling. Using this strategy, we considerably improve MSC-based bone regeneration in a mouse calvarial defect model leading to >95% defect coverage, while MSCs delivered without inhibitor do not significantly improve bone regeneration.

DISCUSSION & CONCLUSIONS: We reveal that the innate immune response to bone injury via IL-1R1/MyD88 impairs bone regeneration and the regenerative capacity of MSCs. By using a simple cell-delivery system with an inhibitor of IL-1R1/MyD88 signaling, we significantly improve MSC-driven bone regeneration. This work highlights the crucial role of the immune response in modulating the regenerative capacity of MSCs and the importance of integrating a control of the immune system into regenerative strategies.

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Molecular mechanisms of bioactive glass induced osteogenic differentiation of human adipose stem cells

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INTRODUCTION: Bioactive glasses (BaGs) have been widely exploited in bone tissue engineering due to their inherent ability to induce osteogenic differentiation of stem cells [1]. However, the molecular level response of cells to BaGs is currently poorly understood. In the present study we hypothesized that cell attachment on the BaG surface plays a role in the BaG-induced osteogenic differentiation of human adipose stem cells (hASCs), and focal adhesion kinase (FAK) and mitogen-activated protein kinases (MAPKs) have a distinct function in the glass-induced bone formation. We were also interested to compare the effect of two different glass compositions, S53P4 and 1-06, on the cellular responses.

METHODS: To study the mechanism of BaG-induced osteogenic differentiation we cultured hASCs on BaG discs S53P4 and 1-06 (Table 1) in the presence of inhibitors for FAK, extracellular signal-regulated kinase (ERK1/2), p38 and c-Jun N-terminal kinase (JNK). The activation of FAK and MAPKs on the glasses was studied by Western blot analysis. Cell attachment on the BaGs was examined by vinculin immuno-cytochemistry as well as integrinβ1 and vinculin immunoblotting. The osteogenic differentiation was evaluated by quantitative alkaline phosphatase (qALP) activity and the expression of osteogenic marker genes *RUNX2a* and *OSTERIX*. Statistical significances were evaluated with non-parametric statistics using Mann-Whitney post hoc test.

Table 1. Compositions of the S53P4 and 1-06 bioactive glasses (wt-%).

	Na_2O	K_2O	MgO	CaO	P_2O_5	B_2O_3	SiO_2
S53P4	23.0	0.0	0.0	20.0	4.0	0.0	53.0
1-06	5.9	12.0	5.3	22.6	4.0	0.2	50.0

RESULTS: Based on our results, both BaGs stimulated hASC osteogenic differentiation but 1-06 was a stronger inducer than S53P4. Cells cultured on the BaGs had enhanced integrinβ1 and vinculin production, and focal adhesions were smaller but more dispersed when compared to the polystyrene control, as seen in Figure 1. The phosphorylation of FAK, ERK1/2 and JNK downstream target c-Jun were upregulated by both glasses. Furthermore, osteogenic differentiation, as determined by qALP activity, was significantly reduced by inhibition of FAK, ERK1/2, JNK and p38, although with p38 the extent of the decrease was smaller than with the others.

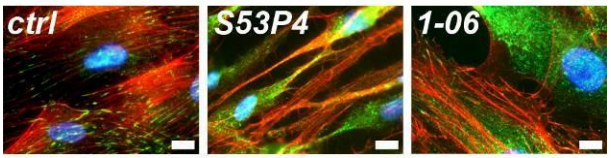

Fig. 1: Cell attachment on different surfaces. Vinculin (green) phalloidin (red) DAPI (blue) staining at 7d. ctrl=polystyrene, scale bars 10 µM.

DISCUSSION & CONCLUSIONS: FAK and MAPKs seem to be important players in osteogenic differentiation induced by S53P4 and 1-06. The glass surfaces also had a distinct effect on the cell attachment mode which may be one underlying factor explaining their osteogenesis-inducing ability. 1-06 was a stronger inducer of osteogenic differentiation which might be explained by the glass compositional differences. This is the first study analyzing the molecular level responses of hASCs on BaGs S53P4 and 1-06 and thus gives a valuable insight into the mechanism of BaG-induced osteogenic differentiation.

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DISCLOSURES: Authors declare no conflict of interest.

Preclinical intradiscal application of celecoxib loaded hydrogels in experimental dogs and canine patients with low back pain

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INTRODUCTION: Low back pain due to intervertebral disc (IVD) disease is common in both humans and dogs. Inflammatory mediators such as prostaglandin E2 (PGE_2) cause structural changes of the IVD and pain. The current conservative and surgical treatment modalities fail to achieve functional repair. Injectable formulations that enable local application and controlled release of anti-inflammatory drugs may provide an alternative to surgical treatment. *In vitro*, controlled release of celecoxib (CXB), a COX-2 inhibitor, from a thermoreversible PCLA-PEG-PCLA hydrogel and a pNIPAAM MgFe-LDH hydrogel, inhibited PGE_2 production from IVD cells stimulated by TNF-α. The aims of this study were firstly, to determine the safety and feasibility of intradiscal application in laboratory dogs with naturally occurring IVD degeneration and secondly, as a step towards translation, to treat client-owned dogs with low back pain related to IVD degeneration with CXB-loaded hydrogels.

METHODS: Biocompatibility and safety of the pNIPAAM MgFe-LDH hydrogel loaded with CXB (three dosages) were studied in laboratory beagle dogs. COX2 (immunohistochemistry) and PGE_2 levels (ELISA) of IVDs from canine patients treated for IVD disease were determined (n=150). Client-owned dogs with low back pain diagnosed with mild to moderate IVD degeneration by MRI were included in the clinical study. They were considered surgical candidates but were offered conservative management by percutaneous injection (n=10 per formulation) in the center of the IVD under fluoroscopy guidance. Follow-up consisted of clinical examination, owner questionnaires, and objective gait analysis at 6 weeks, 3 and 6 months after injection. MRI (T2- and T1-weighted images and T2-mapping) was repeated at 3 months follow up.

RESULTS: Experimental dogs. Safe intradiscal injection in lumbar IVDs was demonstrated without foreign body reaction. Safety was further confirmed by histology and gene expression profiling of relevant genes. Controlled release of CXB resulted in a non-significant maximal inhibition (~35%) of the PGE_2 content in the IVDs regardless of the CXB loading dose, without affecting GAG/DNA levels [1].

Clinical samples. PGE_2 levels were significantly higher in the IVD tissue isolated from patients compared to healthy and degenerated IVDs without clinical disease [2]. Accordingly, the % of COX-2 positive cells was significantly increased in later stages of IVD degeneration.

Canine patients. None of the dogs showed adverse reactions after intradiscal injection. Clinical improvement was achieved by reduction of the low back pain in 9/10 dogs (PCLA-PEG-PCLA hydrogel) and in 6/10 dogs (pNIPAAM MgFe-LDH hydrogel), as was shown by clinical examination, force plate analysis and owner questionnaires. Follow up MRI showed no worsening of the IVD degeneration. Three (PCLA-PEG-PCLA hydrogel) and two (pNIPAAM MgFe-LDH hydrogel) patients needed an additional intervention for their low back pain, consisting of standard-of-care surgical treatment.

DISCUSSION & CONCLUSIONS: This study showed biocompatibility and safe intradiscal application of CXB loaded and unloaded thermoreversible hydrogels in a large animal model (experimental dogs and canine patients). Ongoing studies concentrate on (a) the long term clinical follow up of these canine patients, and (b) determining the optimal loading dose of CXB for clinical efficacy. In this setup, the dog can be used as a model for the development of novel treatment modalities in both canine and human patients with chronic low back pain.

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Expression of TGF-β superfamily members and functional relevance of Smad signaling during chondrogenesis of MSC

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INTRODUCTION: Mesenchymal stromal cells (MSC) are an attractive cell source for cartilage regeneration strategies but unfortunately they undergo hypertrophy during chondrogenesis leading to the formation of transient cartilage instead of stable articular cartilage. In order to identify molecular determinants of chondrogenesis and prevent hypertrophy, aim of this study was to assess differential expression of members of the transforming growth factor β (TGF-β) - superfamily, their receptors and antagonists between MSC and human articular chondrocytes (HAC) the latter of which are resistant to hypertrophy under chondrogenic conditions.

METHODS: Chondrogenesis of human MSC and redifferentiation of HAC was induced in micromass pellet culture. Gene expression of MSC (n=5) and HAC (n=5) was compared using a transcriptome analysis on Illumina platform. Functional regulation of relevant candidate molecules was assessed in independent MSC and HAC populations by qRT-PCR. Smad signaling during chondrogenic re- and differentiation was analysed by immunohistochemistry and Western Blotting. BMP signaling in both populations was modulated by co-treatment with BMP-4/7 or an inhibitor of Smad1/5/9 signaling. Proteoglycan and DNA content, collagen type II and -X deposition, gene expression of chondrogenic and hypertrophic markers as well as alkaline phosphatase (ALP) activity were quantitatively assessed at different time points.

RESULTS: In HAC, TGFβ receptor 2 and 3 (TGFBR2/3) were up-regulated to significantly higher levels than in MSC. BMP4, expressed during HAC expansion, was suppressed while CHL2 and CHRD levels raised. In MSC, BMP4 and BMP7 were induced while TGFBR2 and TGFBR3 were down-regulated. Staining for pSmad1/5/9 in HAC demonstrated positive cells dispersed throughout the pellets at day 3 and 5, which decreased during chondrogenesis, in line with Western Blot results. Lower pSmad1/5/9 immunostaining was observed in MSC pellets. Medium supplementation with BMP-4/7 did not improve cartilaginous matrix deposition by MSC but raised ALP-activity. When Smad1/5/9 phosphorylation was blocked in MSC culture (day 14-42) COL2A1 and COL10A1 expression decreased as well as GAG and collagen type II deposition and ALP activity.

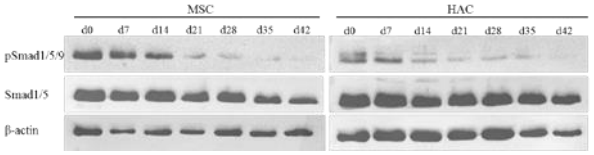

Fig. 1: Western blot analysis of pSmad1/5/9 and Smad1/5 protein levels during in vitro chondrogenesis in MSC and HAC.

DISCUSSION & CONCLUSIONS: Overall, down-regulation of BMP4, up-regulation of BMP antagonists CHRD and CHL2 and modulation of TGF-β signaling via TGFBR2/3 may be a means by which chondrocytes prevent hypertrophy. Indeed, Smad1/5/9 inhibitor strongly reduced development of MSC, inhibiting cartilaginous matrix deposition and hypertrophy suggesting no simple correlation between beneficial Smad2/3 versus unwanted Smad1/5/9 signaling during chondrogenesis.

ACKNOWLEDGEMENTS: This work was supported by funds of the German Research Foundation (DFG Ri707/7-1) and the Orthopaedic University Hospital Heidelberg.

Towards load-bearing bioceramics: mechanical reinforcement of calcium phosphate bone cements through the use of polymeric micro-fibers

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INTRODUCTION: Calcium phosphate cements (CPCs) are self-setting, injectable cements considered to be a favourable bone substitute primarily due to its excellent biocompatibility, osteoconductivity, and injectability [1]. However, their clinical applications are limited to non-load bearing sites due to their inherent brittleness and poor mechanical strength [2]. The main aim of this study was to investigate the influence of fiber type (PLLA or PVA) as well as fiber length and concentration on the mechanical properties of CPCs.

METHODS: Fiber-reinforced CPC specimens were prepared by mixing a solid phase (α-tricalcium phosphate with PLLA or PVA fibers) with a liquid phase (4 wt% aqueous solution of $NaH_2PO_4 \cdot 2H_20$) in compositions listed in Table 1. The CPC paste was cast in a silicon mold to create bars (4 x 4 x 25 mm) for three-point bending tests to be performed. The resulting stress versus strain curves were used to calculate the flexural strength, flexural modulus, and toughness (expressed herein as work-of-fracture (WOF)) of the CPC bars.

Table 1. Fiber-reinforced CPC compositions.

Fiber Type	Length (mm)	Diameter (µm)	Concentration (wt%)
PLLA	1.5, 3 and 6	11	1.25, 2.5, and 5
PVA	3 and 6	26	1.25, 2.5, and 5

RESULTS: The stress vs. strain curves (Fig. 1) demonstrate how fiber-free CPCs can only withstand a stress of ≤ 7 MPa before resulting in complete failure under minimal strain, whereas fiber-reinforced CPCs can withstand stresses upwards of 25 MPa before succumbing to a slow, gradual reduction in stress over an extended strain. This phenomenon is typical for tougher, more ductile materials. With respect to PLLA fibers, the WOF increased from 15 J/m^2 (fiber-free control) to 1.4 kJ/m^2 for CPC compositions containing 5wt% 1.5mm fiber lengths, confirming that increased fiber content resulted in dramatically increased WOF values (Fig. 2A). The same trend was observed for CPCs reinforced with PVA fibers where WOF values as high as 10 kJ/m^2 were observed (Fig. 2B). When compared to the literature, these values for PVA-reinforced CPCs rank among the best-performers [3].

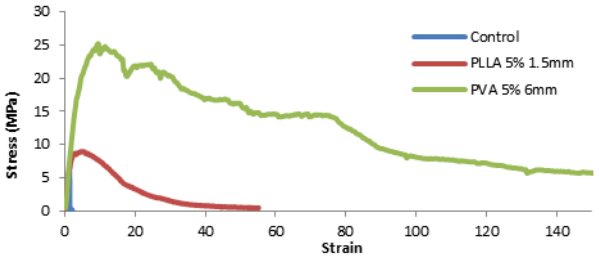

Fig. 1: Typical stress vs. Strain curves for fiber-reinforced CPCs.

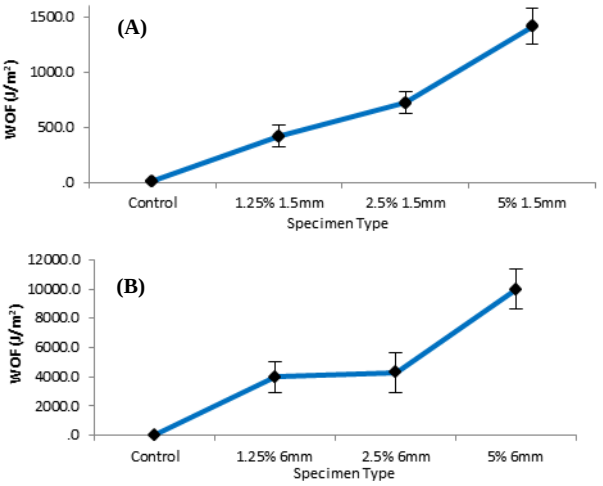

Fig. 2: Work-of-fracture for CPCs reinforced with (A) PLLA and (B) PVA fibers.

DISCUSSION & CONCLUSIONS: Incorporation of different fiber types, lengths, and concentrations all significantly influenced the mechanical properties of CPCs. The dramatic increase in the WOF was indicative of the CPC becoming tougher and less prone to catastrophic fracturing. Scanning electron microscopy revealed that incorporated fibers hindered crack propagation and bridged cracks together, thereby redistributing the applied loads throughout the entire sample and increasing their load-bearing capacity.

ACKNOWLEDGEMENTS: This study was funded through a VIDI grant (#13455) from the Dutch Technology Foundation (STW).

development of a bioartificial kidney system capable of actively removing uremic wastes

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INTRODUCTION: The development of cell based Bioartificial Kidney device (BAK) could improve existing dialysis therapies for the removal of protein-bound toxins. A key requirement for the BAK is the formation of a "living membrane" consisting of a tight renal cells monolayer with preserved functional organic ion transporters, on an artificial porous membrane. Recently, we showed the proof of concept of creating such living membrane using conditionally immortalized human renal Proximal Tubular Epithelial Cells (ciPTECs) [1] on Polyethersulfone (PES)-based flat sheet and hollow fiber membranes [2,3]. In both cases the application of a double coating of L-Dopa / collagen IV was necessary. Here, we present strategies for the upscaling of the system having several hollow fiber membranes (HFM).

METHODS: HFM were mounted in modules and double coated with L-Dopa and Collagen IV. Modules contained from 1 to 5 HFM, with surface areas ranging from 0.5 to 10 cm2. The ciPTECs were seeded on the extraluminal HFM surface or on the intraluminal side. Several coating parameters and ciPTECs seeding techniques were studied under static and dynamic culturing conditions. The properties of the new bioactive membranes were analysed, including transport of albumin. The ciPTEC morphology and monolayer quality was investigated via expression of tight junction protein Zonula Occludens-1 (ZO-1)) and permeation of inulin-FITC. The functionality of OCT2 was evaluated using a specific fluorescent substrate, ASP+, and creatinine in absence and presence of the OCT2-inhibitor cimetidine.

RESULTS: The upscaling of the L-dopa/CIV coating on the PES membranes was successful. The coating preserves membrane permeability and improves ciPTEC adhesion, both outside and inside the HFM. On the intraluminal side of HFM, high ciPTECs densities are achieved, without forming a monolayer yet. After one week of culture in static conditions, reproducible cell monolayers are formed on the extraluminal side of the HFM within modules containing 3 HFM with a surface area of 4cm2. Furthermore, L-dopa/CIV coated fiber membranes allow tight and homogeneous ciPTECs monolayer formation as can be observed from ZO-1 staining (Fig. 1) and low inulin leakage (Fig. 2). Moreover, active ASP+ uptake is observed, as well as transepithelial creatinine transport, both mediated by OCT2.

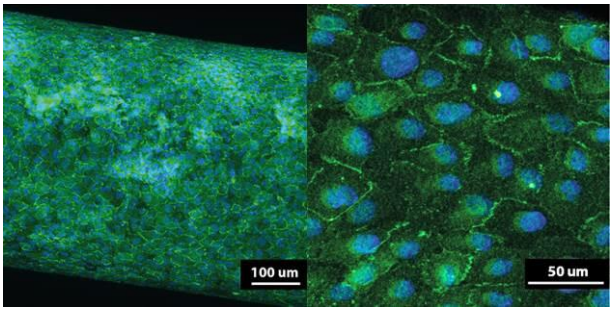

Fig. 1: Monolayer of ciPTECS on PES-based L-Dopa and collagen IV double-coated membranes within an upscaled module, after one week of static culture. Dapi (blue) and ZO-1 (green).

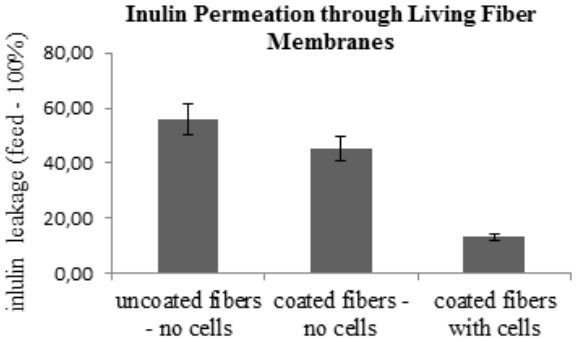

Fig. 2: Inulin permeation through PES HFM.

DISCUSSION & CONCLUSIONS: Upscaled HFM modules with a functional monolayer of renal cells were successfully developed. Future work will include characterization of protein-bound uremic toxin transport.

ACKNOWLEDGEMENTS: This work is funded by the EU Marie Curie ITN Project BIOART (grant no.316690, EU-FP7-PEOPLE-ITN-2012).

VEGF-transfected adipose-derived stromal cells enhances bone regeneration and neovascularization from bone marrow stromal cells

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INTRODUCTION: Treatment with exogenous vascular endothelial growth factor (VEGF) has also been shown to promote fracture healing and bone regeneration [1]. In this study, we devised potent angiogenic Adipose-derived stromal cells (ADSCs) by the transfection of VEGF gene using electroporation system. The aim of this study was to evaluate the potential of VEGF-transfected ADSCs ($ADSCs^{VEGF}$) to direct neovascularization and promote bone regeneration when added in small proportion to bone marrow stromal cells (BMSCs) through *in vitro* and *in vivo* models.

METHODS: 1) Construction of VEGF expressed ADSCs: The human VEGF genes were introduced to pECFP-C1 expression vectors. pECFP-C1-VEGF vector construct were transferred to ADSCs using Microporator MP-100.
2) *In vitro* coculture of $ADSCs^{VEGF}$ and BMSCs: $ADSCs^{VEGF}$ and BMSCs in various proportions were co-cultured together in osteogenic media.
3) HUVECs tube formation assay: After HUVECs were cultured on the layer of Matrigel® in down wells of transwell plate, BMSCs and/or $ADSCs^{VEGF}$ were loaded into the upper wells.
4) Gene expression profiling with oligonucleotide microarrays
5) *In vivo* implantation of ceramic/BMSCs/ $ADSCs^{VEGF}$ in critical size calvarial defect model:

RESULTS: 1) HUVEC tube formation assay: All proportion of BMSCs:$ADSCs^{VEGF}$ cocultures had 1.7 to 3.2-fold greater branch points of tube formation than those of the ADSCs control.
2) *In vitro* osteogenesis: ALP staining and activity of all mixed cocultures were significantly higher than those of BMSCs. The pattern was approximately mirrored in Alizarin Red S staining and calcium quantification. The RT-qPCR and ELISA results for osteogenic makers *OCN*, *BSP*, *COL1A1* showed a significant increase in BMSC:$ADSCs^{VEGF}$ (1:0.05) than BMSCs ($p<0.05$).
3) Gene expression profiling: Of angiogenesis-related genes, overexpression of cathepsin Z (3-fold) and downregulation of early growth response 1 (1.9-fold) were confirmed in BMSCs/$ADSCs^{VEGF}$ compared to BMSCs/ADSCs. Of osteogenesis-related genes, osteoactivin (2.3-fold) and tetranectin (6.6-fold) were upregulated in BMSCs/$ADSCs^{VEGF}$ compared to BMSCs/ADSCs.
4) *In vivo* bone regeneration: After 8 weeks, calvarial defects implanted with co-cultured BMSCs and $ADSCs^{VEGF}$ showed significantly greater regenerated bone area, volume of repair and bone mineral density compared to those implanted with BMSCs alone in the results of X-ray and micro-CT.
5) *In vivo* neovascularization: $ADSCs^{VEGF}$ alone or BMSCs/$ADSCs^{VEGF}$ at 1:0.5 induced significantly higher score in both microvessel density and staining intensity counts of CD31 than those of BMSCs alone whereas BMSCs/$ADSCs^{VEGF}$ at 1:0.05 induced significant increase in staining intensity only.

DISCUSSION & CONCLUSIONS: VEGF-transfected ADSCs added to BMSCs promoted osteogenesis *in vitro* and *in vivo* at the optimal ratio of BMSCs/$ADSCs^{VEGF}$ at 1:0.05. Coculture of BMSCs and $ADSCs^{VEGF}$ significantly enhanced the expression of CLEC3B and GPNMB compared with those of BMSCs and untransfected ADSCs. $ADSCs^{VEGF}$ added to BMSCs enhanced microvessel density and staining intensity of CD31 on the regenerated bone defect than BMSCs alone. It is concluded that $ADSCs^{VEGF}$ added in small proportion to BMSCs effectively promotes bone regeneration and neovascularization.

ACKNOWLEDGEMENTS: This research was supported by the National Research Foundation of Korea (Grant no: 2013R1A1A2062978).

Boosting *in vivo* angiogenesis by *in vitro* priming of human stromal vascular fraction cell-based engineered tissues

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INTRODUCTION: The therapeutic efficacy of angiogenic cell-based treatments for ischemic tissues relies mainly on paracrine effects and is impaired by cell death during the delivery/homing. After *in vitro* organization in engineered tissues, implanted cells show a superior *in vivo* survival [1]. Anyway, a prompt and adequate vascularization of the engineered tissues right upon implantation is also crucial [2]. For this purpose, an *in vitro* approach was here used to generate constructs with a preformed microvasculature prior to their implantation [3]. Human adipose tissue-derived stromal vascular fraction (SVF) was used for its known high vascularization potential, conferred by containing both mesenchymal and endothelial progenitor cells [4]. However, in case of mm-thick engineered tissues the static SVF culture leads to a not uniform *in vitro* pre-vascularization, especially concentrated at the periphery of the construct. This limitation could be overcome by using a bioreactor for direct perfusion. In this study, we hypothesize that the unidirectional perfusion of culture medium will not only support a uniform cell distribution in 3-mm-thick collagen sponges but also enrich the SVF for the specific endothelial/mural compartment.

METHODS: Human SVF cells were isolated from healthy donors and seeded (~10^8 cells/cm^3) on a type I collagen sponge (Ultrafoam™, Avitene) using a perfusion bioreactor for 1 day at 3 ml/min. Constructs were further cultured for additional 5 days either in perfusion at a flow rate of 3 ml/min or in static condition. Effects of perfusion and static culture were assessed in terms of SVF cell subpopulation composition, cell proliferation, protein release and *in vitro* endothelial vessel-like structure organization. Vascularization potential of the resulted engineered tissues was then assessed in a subcutaneous rat model at different time points (3, 7 and 28 days).

RESULTS: Interestingly, our findings showed that the culture method strongly influences the final SVF cell composition. Compared to static culture, perfusion significantly enriched the initial SVF population for pericytes ($CD45^-$ $CD34^-$ $CD146^+$, ~7.5-fold increase) and mature endothelial cells ($CD45^-$ $CD31^+$, VEGFR-2^+, ~1.7-fold increase). Moreover, perfusion cultured constructs showed a uniform spatial distribution of endothelial structures with increased size over the time in culture, an improved cell proliferation, and a higher release of pro-angiogenic factors (e.g VEGF). Perfusion-based culture also accelerated the *in vivo* construct vascularization: the vessel length density was ~5.5- and ~1.4-fold higher than statically cultured constructs at 3 and 7 days, respectively. Moreover, after perfusion implanted human cells showed a superior survival and directly participated to the formation of functional blood vessels, as either endothelial cells or pericytes, after 28 days *in vivo*.

DISCUSSION & CONCLUSIONS: Our findings suggest a strong influence of the culture method over the *in vitro* prevascularization and *in vivo* angiogenic potential of the engineered tissue by shifting the initial SVF population toward a pericytic-like phenotype. Further investigations are needed to understand the *in vitro* mechanism by which perfusion enhances the pericytic component of the SVF and the specific role of the pericytes in the *in vivo* acceleration of angiogenesis.

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Guiding stem cell fate *in vitro* and implant osteointegration *in vivo* by tuning the geometrical disposition of integrin-binding ligands on Ti

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INTRODUCTION: Anchoring of receptor-binding peptidic ligands on implant surfaces is a versatile strategy to achieve better osteointegration and longer life span of orthopaedic devices by promoting bone regeneration directly on the surface of the device. In detail, customization is readily obtained by combining synergic or complementary peptidic sequences. The Arg-Gly-Asp (RGD) and Pro-His-Ser-Arg-Asn (PHSRN) sequences from the integrin-binding site of fibronectin (FN) are good candidates for this multiple functionalization, as they synergically bind integrin α5β1 [1], which has been demonstrated to be important in the osteogenic differentiation of stem cells [2]. Nonetheless, unless tight control of the presentation of the ligands is achieved, functionalization might result in poor improvement of the material performance. In the case of RGD/PHSRN, the distance between the motifs is crucial to preserve the affinity for α5β1. By using a custom synthesized double-branched molecule to coat Ti surface, we propose a straightforward solution to achieve chemical control of motifs presentation.

METHODS: A double-branched peptidic platform (PTF) to present RGD and PHSRN was synthesized [3] and covalently anchored to smooth titanium by silanization with APTES (Figure 1). Functionalization was characterized by contact angle and XPS. The response of human mesenchymal stem cells (hMSCs) was studied by immunofluorescence, proliferation assays, RT-PCR and calcium deposition (Alizarin Red S). To verify the ability of the PTF ligand to foster bone growth *in vivo*, PTF-coated implants were tested in a partial thickness calvarial model in rat.

RESULTS: Contact angle results and XPS analysis revealed that functionalization was successful and stable. hMSCs cultured on PTF-coated Ti have increased area and vinculin staining compared to the random equimolar mix of RGD and PHRSN (MIX) and to uncoated Ti (CTRL). Furthermore, cells cultured on the PTF deposited highly mineralized matrix and expressed more integrin α5β1 and osteogenic marker Runx2, compared to MIX and CTRL. *In vivo*, bone growth in the calvarial defect adjacent to the PTF-coated Ti implant was higher compared to CTRL.

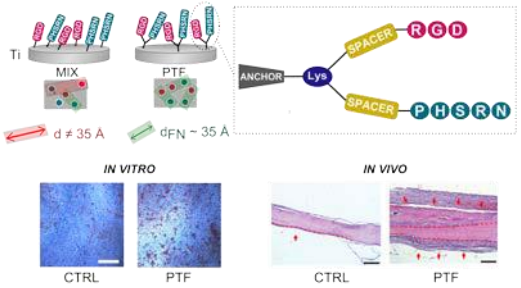

Fig. 1: Schematic representation of the synthetic platform (PTF) and part of the in vitro *(staining of calcium deposits, scale bar 500 μm) and* in vivo *results (H&E, scale bar 150 μm). d_{FN} refers to the distance between RGD and PHSRN in FN.*

DISCUSSION & CONCLUSIONS: The rationale design of the synthetic ligand to mimic the presentation of RGD/PHSRN encountered in FN proved successful: We verified that, only by controlling the disposition of motifs on the surface, MSC osteogenic differentiation is induced and, accordingly, bone growth fostered *in vivo*. This work demonstrates that fine chemical control of peptide presentation with such platform is a straightforward strategy to tailor biomaterials for the delivery of precise biological signals.

ACKNOWLEDGEMENTS: This study was funded through project MAT2012-30706 and a Marie Curie Career Integration Grant (FP7-PEOPLE-2012-CIG, No. 321985). RF thanks the Government of Catalonia for financial support through a pre-doctoral grant (FI-AGAUR).

Cell instructive hydrogel-systems for functional cartilage tissue engineering

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INTRODUCTION: Cell-based tissue engineering is a promising approach for treating cartilage lesions but the optimal cell-scaffold combination for hyaline cartilage regeneration has yet to be identified. Novel hydrogels allow including tailored tissue type specific modifications with physiologically relevant peptides, by this selectively influencing the cell response. Aim of this study was to modify a poly(ethylene glycol) (PEG)/heparin hydrogel by functionalization with cell instructive peptides introducing matrix-metalloprotease (MMP)-degradability, the cell adhesion motif RGD, or collagen binding motifs (CKLER, CWYRGRL) to improve cartilage matrix deposition in tissue engineering constructs.

METHODS: The hydrogels were formed by mixing thiol-endfunctionalized (MMP-insensitive) starPEG or starPEG-MMP-conjugates carrying MMP-sensitive peptides at every arm and maleimide-functionalized heparin [1] in the presence or absence of cell instructive peptides. Human mesenchymal stromal cells (MSC) or porcine chondrocytes were grown in the hydrogels for up to 4 weeks *in vitro* under chondrogenic conditions, and *in vivo* in subcutaneous pockets of immunodeficient mice.

RESULTS: MMP-sensitive and –insensitive starPEG/heparin hydrogels supported chondrogenic differentiation of MSC according to induction of COL2A1, BGN and ACAN mRNA expression. Enhanced MMP-sensitivity and therefore degradability increased cell viability and proliferation. Modification with RGD, CKLER or CWYRGRL strongly promoted cell spreading with intense cell network formation in MSC- and chondrocyte-seeded hydrogels. Peptide-modification resulted in less collagen type II deposition by MSC suggesting reduced chondrogenesis of spread cells. In contrast, matrix deposition in chondrocyte-containing peptide-functionalized hydrogels remained high. The instructive effect of the hydrogels on chondrocytes appeared stronger *in vivo* where the merely pericellular cartilaginous matrix deposition was overcome in RGD-functionalized starPEG/heparin hydrogels.

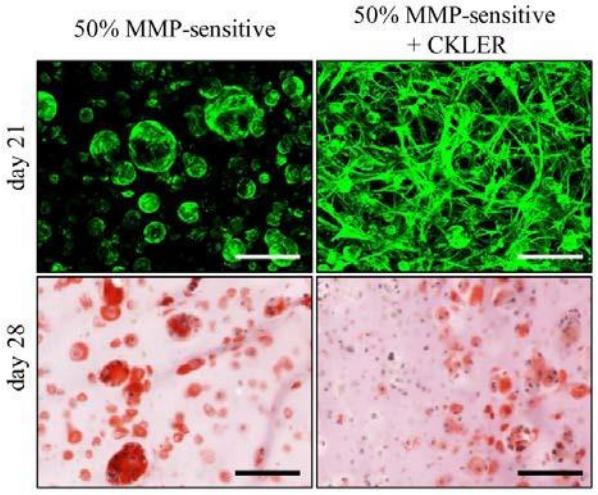

Fig. 1: Cell morphology (upper panel) and collagen type II staining (lower panel) of MSC in 50% MMP-sensitive starPEG/heparin hydrogels without and with CKLER. Scale bar = 100 µm.

DISCUSSION & CONCLUSIONS: Peptide-functionalized starPEG/heparin hydrogel altered cell morphology, proliferation and differentiation with MSC being more sensitive to cell-matrix interaction cues than articular chondrocytes. Thus, careful screening for the right pairing of cell type and modular hydrogel composition makes cell instruction valuable for functional cartilage tissue engineering.

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

Non-destructive imaging of cell viability and physical properties within 3D constructs with phase-resolved optical coherence tomography

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INTRODUCTION: State-of-the-art imaging technologies developed for 2D investigations often perform poorly in dense scattering tissues or within dense artificial substrates (i.e 3D polymeric substrate). *In vitro* or *in vivo* tissue imaging by confocal has been restricted by light scattering to superficial investigations and to small areas of interest. In vivo the potential of multiphoton microscopy for stem cell niche imaging is promising and has been exemplified during hair follicles regeneration, to depth of 100micrometer, illustrating the need to monitor the niche at cellular resolution in tissue context. Here, we explore an alternative label-free technology, phase-resolved optical coherence tomography, to address the unmet need to quantitatively monitor complex stem cell niche physical (biomechanics, fluid flow) and biological (viability) properties at the tissue level with a typical 1mm penetration depth. Optical coherence tomography (OCT) is an interferometry imaging modality that bridges the gap between microscopy and ultrasound achieving micrometer scale resolution at mm scale penetration depths in highly scattering tissues.

METHODS: We developed novel algorithms to process time-lapse quantitative phase information to derive parameters of interest for tissue engineering and regenerative medicine[1-2]. A bespoke phase-resolved optical coherence tomography microscope was developed in our lab. The superluminescent light source, centred at 930nm with a FWHM of 90nm was coupled to an inverted microscope through a custom scanning head in a common path configuration. The light backscattered from the sample was then analysed in a spectrometer (Callisto OCT engine (Thorlabs)). First, the spectra was linearly resampled in k-space, then a fast Fourier transform was performed to retrieve both the in-depth intensity and phase. A custom laser scanning protocol was implemented to increase phase stability and to allow for the recording of minute phase fluctuations.

RESULTS: We showed that cell viability can be retrieved in depth within tissue engineering constructs (scaffolds, gels, spheroids) from the analysis of phase fluctuations originating from intracellular cell activity[3]. In addition similar algorithms based on phase sensitive time-lapse acquisition allowed the measurement of cell physical properties such mechanical properties, fluid flow and shear stress.

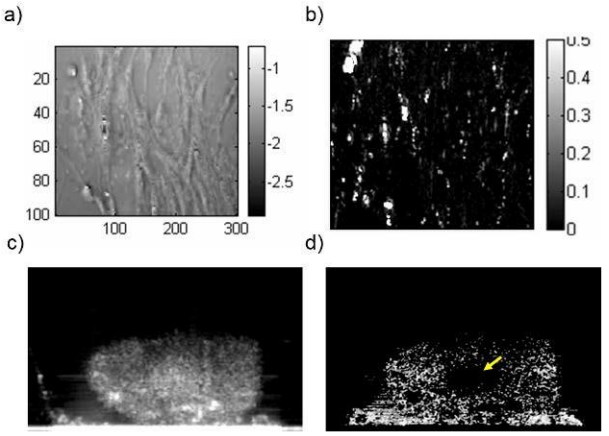

Fig. 1: 2D (a,b) and 3D (c,d) optical coherence phase microscopy of cell viability. a) X-Y OCPM optical intensity section of mesenchymal stem cells growing on a 2D tissue culture plastic substrate; b) X-Z in-depth intensity cross-section of cancer cell spheroid (MCF-7). b) and d) shows phase fluctuations imaging mode that correlated with cell viability. Note the delineation of the necrotic core within the spheroids (arrow).

DISCUSSION & CONCLUSIONS: Phase sensitive OCT has been demonstrated in our group to measure in depth within 3D engineered constructs 1) mechanical properties of artificial and biological tissues 2) microcirculation and shear stress in tissue engineering, 3) live cell motility

ACKNOWLEDGEMENTS: We would like to thank the UKRMP hub for engineering and exploiting the stem cell niche for funding.

Effect of direct current low voltage electrical stimulation on amputated rat limb regeneration

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INTRODUCTION: Limb loss is a disability with devastating consequences[l]. While current treatments such as, reattaching the amputated limb, reconstructing the defect with autologous tissues, prosthetics and limb allotransplantation, can successfully restore differing degrees of function, each is associated with drawbacks and complications. The ideal solution would be to augment the body's existing regenerative capabilities and regrow or rebuild new limbs using tissue engineering approaches. Multiple studies have shown that electrical stimulation (ES) is an effective tool for controlling cell behavior and has been used successfully to treat dermal wounds, nerve injuries, and large bone defects in the clinical setting for more than 30 years[2]. The goal of this research was to analyze the effect ES has on regeneration of limb tissues. We exposed amputated rat limb stumps to low voltage ES and assessed its effect on regrowth of different limb tissues.

METHODS: The right limbs of 58 Sprague-Dawley rats were amputated at the middle third of the humerus, and an bimetallic ES device was implanted. Animals were randomized into 3 groups: 1) active ES device (experimental group, n=20); 2) deactivated ES device (sham group, n=18); and 3) no device (control group, n=20). 8-10 rats in each group were sacrificed at 7 and 28 days post amputation and the stumps were fixed and stained for histological and immunohistochemical analysis.

RESULTS: Histomorphometric analysis demonstrated significant increases in cartilage and bone formation in electrically stimulated samples at day 28 (Figure 1). The presence of new vessel growth was signicantly higher in the ES treated stumps. No new nerve formation was detected in any of the three groups. However at 28 days in the control and sham treated animals we observed neuroma formation at the distal part of the stump. In contrast no such neuroma formation was detected in ES treated stumps.

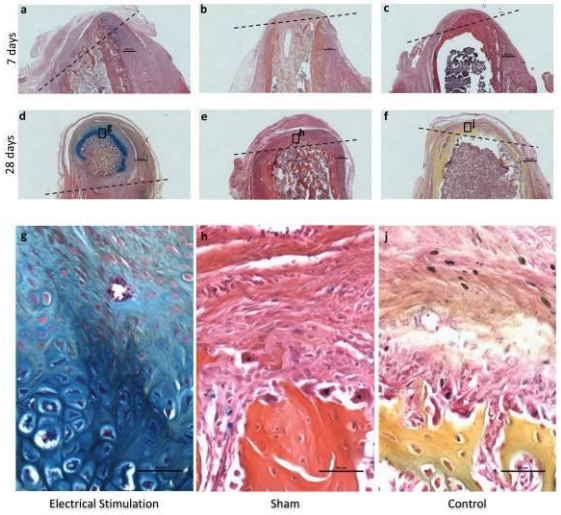

Fig. 1: Longitudinal histological sections of rat limb stumps at days 7 (first row) and 28 (second and third rows) post amputation (Alcian Blue Orange G staining). Scale bar - 50µM. a-d-g electrical stimulation; b-e-h sham; c-f-j control

DISCUSSION & CONCLUSIONS: This study demonstrates that low voltage DC electrical stimulation promotes growth of specific tissues in the stump of our rat limb amputation model. We hypothesize that: 1) low voltage DC electricity contributes to a shift in the balance towards tissue regeneration (seen in ES treated stumps) in select tissues, versus healing with scar formation (seen in controls), and 2) electrical stimulation causes the observed effect by stimulating bone marrow stem/progenitor cells to generate highly vascularized osseocartilaginous centers in the zone of injury. Future studies will allow us to better understand the role of ES in limb regeneration and better control it to improve and expand its use in the clinical setting.

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Self-assembled liver organoids recapitulate hepato-biliary organogenesis *in vitro*: a novel model to test drug teratogenesis?

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INTRODUCTION: Bioengineering of a fully functional tissue requires precise recapitulation of normal tissue development. Specifically for the liver, one may use bipotential human liver progenitor cells (hFLCs) capable of differentiation into hepatocytes and cholangiocytes. The goal of the current study was to develop a system that would efficiently recapitulate embryonic development of hepatic parenchymal tissue and bile ducts, using decellularized liver extracellular matrix (ECM) as scaffolds[1].

METHODS: hFLCs were seeded on decellularized liver ECM discs (300 µm thickness, 8 mm diameter) and were cultured for up to 3 weeks in presence of hepatic differentiation medium. Immunofluorescence microscopy was used to determine the extent of progenitor cell differentiation into hepatocytes and cholangiocytes. Urea, albumin and drug metabolism were quantified as paramaters of liver function. Furthermore, a γ-secretase inhibitor was added to the culture media and bile duct and hepatocyte development was monitored.

RESULTS: hFLCs seeded on acellular liver ECM discs differentiated into hepatocytes and cholangiocytes. The cells showed predominant albumin expression along with loss of α-feto protein (AFP) expression at 3 weeks (Fig. 1D,E). The cells also expressed other mature hepatocyte markers like HNF-4α, α-1-antitrypsin and cytochrome P450 1A2, 2A and 3A (Fig. 1B-E). The cells in the ductular structures expressed bile duct specific markers like CK19, SOX9, EpCAM, ASBT, β-catenin and the presence of apical primary cilia (stained with α-acetylated tubulin), thus demonstrating differentiation towards cholangiocyte lineage along with maintaining apico-basal polarity (Fig. 1B-E). Urea and albumin secretion was higher in the liver disc organoids compared to control hFLCs cultured in petri dishes. Several metabolites of the drugs diazepam and 7-ethoxycoumarin were also detected by LC-MS/MS, showing broad cytochrome P450 activity in these organoids. The addition of a γ-secretase inhibitor severely impacted the number of bile ducts formed and their maturation, mirroring a biliary atresia model.

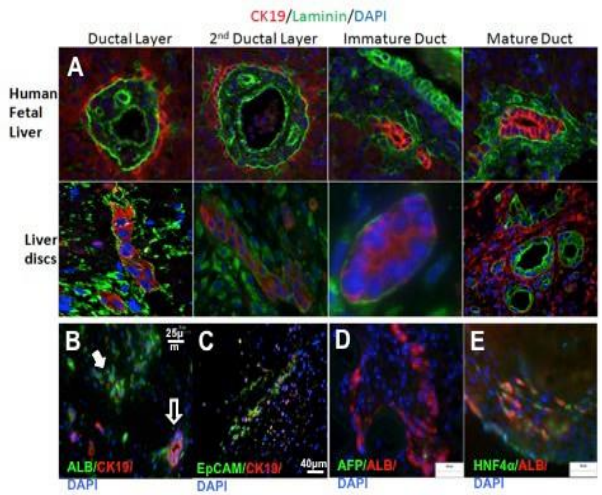

Fig. 1: Hepatoblast differentiation in decellularized liver matrix mimics hepatic development. (A) hFLC seeded on acellular disc mimic bile duct development process as seen during fetal development. Top panel shows various stages of bile duct development during fetal liver organogenesis. Bottom panel shows similar developmental stages in liver organoids. (B) Bipotential differentiation of hFLC into Alb+ hepatocytes and CK19+ bile ductular structures. (C) A bile duct spawning for 200µm in length on a whole-organ scaffold. (D) Mature hepatocytes showing Alb+ staining and absence of AFP. (E) Hepatocytes showing Alb+ and HNF4α+ staining. Scale bar is 20um unless specified otherwise.

DISCUSSION & CONCLUSIONS: Our results demonstrate the efficient generation of bioengineered human liver tissue with hFLC that recapitulates stepwise development of hepatocyte and bile duct formation (Fig. 1A). Altogether, this study demonstrates the potential of this technology to study and mimic human liver development. These models provide novel approaches for liver bioengineering, drug discovery and toxicology (including drug teratogenesis evaluation in vitro), and ultimately for the treatment of liver disease.

From fibres to bone: intelligent additive manufacturing of functionalised scaffolds for in situ tissue engineering

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INTRODUCTION: Constructs of cells or tissues that have been modified to repair, regenerate or replace human tissues are classified as advanced-therapy medicinal products (ATMP). Thus, they have to be evaluated for safety and efficacy like any other conventional drug, which means long timescale and high costs until experimental products might reach clinics. Therefore, this study targets a scaffold design suitable to enable cellular ingrowth, vascularisation and tissue reconstruction *in situ*. The requirements for a fibre based scaffold for bone regeneration covers 1) the use of a cytocompatible biomaterial suitable for fibre generation, 2) application of an additive manufacturing method, 3) implementation of an open interconnected porous 3D structure, 4) functionalisation with osteoconductive materials to enhance scaffold stiffness and accelerate osteogenic differentiation, and 5) presentation of human platelet derived growth factor (PDGF) to enhance invasion of mesenchymal stromal cells (MSC) and osteoprogenitor cells as well as acceleration of vascularisation.

METHODS: Transgenic silkworms were generated to functionalise silk proteins with hPDGF. Silk fibres were spun presenting hPDGF or osteoconductive ceramic materials (hydroxyapatite, HA and beta-tri-calcium-phosphate, ß-TCP). For scaffold design and realisation of a homogenous distribution of interconnected open pores the 3D structure was modelled using the software GeoDict. Applying short fibre based Net Shape Nonwoven (NSN) technology, interconnected porous 3D constructs were manufactured. Scaffolds were characterized in terms of porosity, compressive strength, and cyclic load. Cytocompatibility was evaluated by monitoring viability of MSCs and human umbilical vein endothelial cells (HUVEC). Osteogenic differentiation was determined by realtime PCR. Spatial distribution and vascularization were visualized by laser scanning microscopy.

RESULTS: The modelled open porous structure (Fig.1A) could be transferred into manufacturing of 3D scaffolds using the NSN technology (Fig.1B). Silk fibre based 3D scaffolds showed cytocompatibility and hMSC and Huvecs covered the scaffold fibres (Fig.1C). MSC cultured on HA- and ß-TCP/HA-functionalised silk scaffolds expressed higher levels of osteogenic marker genes Runx2 and ALP. Capillary-like structures could be observed in all scaffold types.

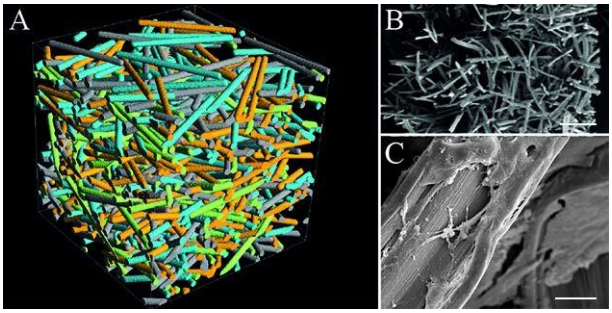

Fig. 1: A) Modelled scaffold structure; B) REM picture of interconnected pores of a NSN scaffold (bar = 1 mm); C) MSCs and HUVECs co-cultured on silk fibres of a NSN scaffold (bar = 20 µm).

DISCUSSION & CONCLUSIONS: The presented additive manufacturing technique in combination with modelling and prediction of a 3D scaffold structure is suitable to realise scaffolds for *in situ* tissue engineering. In combination with functionalisation of silk fibres with hPDGF and osteoconductive ceramic material the presented textile scaffolds were proved as promising biomaterials for bone regeneration *in situ*.

ACKNOWLEDGEMENTS: This research was funded by: Federal Ministry for Economic Affairs and Energy, following a decision of the German Bundestag (Project number: KF 2340102AJ3) and Deutsche Forschungsgemeinschaft (DFG-CH174/24-2)

A human 3D skin microtissue model for testing anti-skin aging therapies

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INTRODUCTION: The Mechanisms of skin aging including intrinsic aging and photoaging are complex reaction, so the anti-aging candidates have been developed to treat each step. Though development of anti-aging agent is increasing, limited in vitro models such as UVB-irradiated 2D-cultured cells and skin equivalent are currently used. However, skin equivalent is relatively complicated preparation process, which is impossible approach for high throughput screening and should be considered cost-effect. Although UVB-irradiation models are possible for HTS, but these models grown in monolayered culture are not able to express the complicated in vivo microenvironment. In this study, we demonstrated that a three-dimensional cell mass (3DCM)[1] formed with human fibroblast (FB) was dermis-like ECM composition and evaluated the potential of the 3DCM as a micro-tissue model for testing skin anti-aging therapies.

METHODS: The FB 3DCM was prepared by using bio-engineered artificial extracellular matrix MBP-FGF), which interacts with heparin sulfate proteoglycan, to regulate cell-matrix adhesion strength [1]. Briefly, FB was seeded to MBP-FGF fusion protein coated wellplates. After that, the FB 3DCM was formed spontaneously for 1 d incubation. The formed 3DCMs were collected at 1, 3 and 5 days. The 3DCM formation of adherent FBs was observed by phase contrast microscope. The viability of FB 3DCMs was evaluated by a live and dead assay. ECMs and MMPs expressions were evaluated by PCR, western blot and immunostaining. For drug testing study, retinoic acid, abietic acid, and TGF-β1 were used as model drugs. The drugs were added to culture media after or before FB 3DCM formation. FB 3DCMs and the culture media were harvested after 1, 3 and 5 days for MMP-1 analysis.

RESULTS: FB was aggregated to form 3DCM spontaneously within 1 day on MBP-FGF coated substrate. The average diameters of FB 3DCMs decreased as increasing the size of cultured well. In a live and dead assay, dead cells in FB 3DCM were not observed over 5 days. MMP-1, a skin-aging inducer, was expressed dramatically higher in FB 3DCM than in 2D-cultured FB. In immunofluorescence staining analysis, collagen type I positive area decreased with an increase of culture in FB 3DCM. From western blot analysis, we found that collagen type I was degraded continuously in FB 3DCMs up to 5 days during the culture period. In drugs testing, drugs efficacy was different depending on culture method (2D vs. 3D) and drug treatment time.

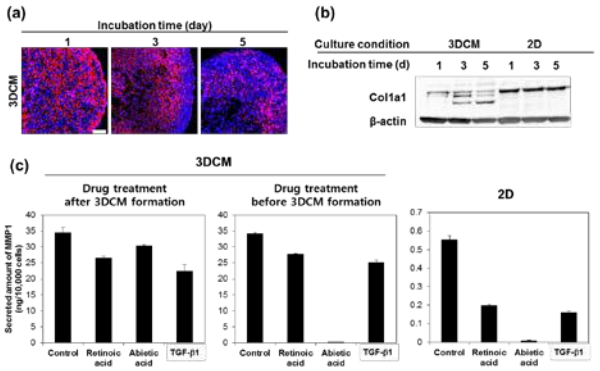

Fig. 1: (a) Immunofluorescence images of FB 3DCM stained with collagen type I, (b) western blot analysis of collagen type I, and (c) Drug screening study by using ELISA

DISCUSSION & CONCLUSIONS: Human FB was cultured as a 3D microtissue, which composed of ECMs similar to human dermis, on a self-organizing material. hFB 3D microtissue could be suggested as a skin-aging model because of the properties of MMP-1 synthesis and collagen degradation. Drugs efficacy was changed depending on different assay protocols due to their therapeutic mechanisms and/or physical properties, probably. Such differences must be taken into consideration when developing drug.

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Bone Tissue Engineering using mesenchymal stem cells and endothelial progenitor cells in the large animal model sheep

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INTRODUCTION: For decades remarkable improvements have been made in generating functional bone substitutes and optimizing current therapeutic options for bone defects. Nevertheless, the reconstruction of large and poorly vascularized bone defects remains a major challenge and there is still the urgent need for further optimization of generating vascularized bone tissue.

METHODS: Mesenchymal stem cells (MSC) and endothelial progenitor cells (EPC) were isolated from sheep bone marrow/blood. Characterization was performed using differentiation, protein/gene expression analyses, tube formation/sprouting and acLDL uptake. For combination with the arterio-venous (AV) loop model for vascularized bone TE [1], a sheep tibia defect model (defect size 3cm) was established. Defects were filled with the clinical gold standard autologous bone. Using 3D imaging (MRI/µCT), x-ray and IHC vascularization and bone formation was visualized over 12 weeks. In ongoing experiments MSC-EPC co-cultures are seeded on different bone substitutes (NanoBone® block, easy-graft® CRYSTAL) and incubated in a perfusion bioreactor. Different flow rates and the effect of rhBMP-2 and VEGFA will further be evaluated.

RESULTS: MSC showed typical stem cell properties, such as CD44+/CD29+/CD166+ and differentiation into osteogenic, chondrogenic and adipogenic lineages. EPC showed positive staining of endothelial markers CD34, lectin and uptake of acLDL (Fig. 1a), formed tubes and sprouted in a 3D matrix. The tibia defect model was successfully established and is optimally suited for further pre-clinical testing of tissue-engineered constructs. Analyses of tibia defects after 12 weeks filled with the clinical gold standard, revealed insufficient bone formation in the defect site confirming the urgent need for optimizing this therapeutic option. Recently we optimized the sheep AV loop model for generation of vascularized bone tissue [1] which should further be combined with the tibia defect model. Preliminary results of the bioreactor show that MSC-EPC co-cultures adhered and proliferated in different bone substitutes over time (Fig. 1b).

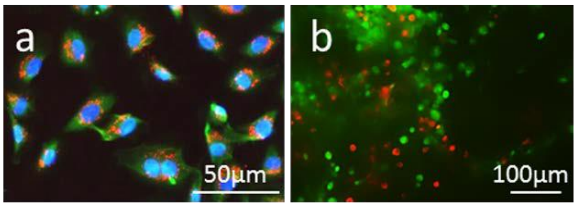

Fig. 1: (a) Lectin (green), acLDL (red), DAPI (blue) staining of EPC. (b) MSC (green)-EPC (red) co-culture on NanoBone® block.

DISCUSSION & CONCLUSIONS: In the present study we could demonstrate that MSC and EPC are suitable cell sources for bone tissue engineering purposes. We assume that MSC and EPC co-cultures with supplemental growth factors can lead to improved vascularization and bone formation in tissue engineered constructs. In future studies a bone substitute in combination with MSC, EPC and growth factors will be implanted in the AV loop model for further vascularized transplantation into the bone defect site to gain important insights for early clinical application.

ACKNOWLEDGEMENTS: This study was funded by Staedtler Foundation, Else-Kröner Fresenius Stiftung, Baxter Innovations GmbH, Xue Hong and Hans Georg Geis Foundation.

DISCLOSURES: T Gerber is Executive Director of Artoss GmbH, the distributor of NanoBone®. He was involved in the design of the study. All other authors confirm that there are no conflicts of interest.

Novel fibrinogen hydrogels for cell encapsulation and delivery

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INTRODUCTION: Delivery of cells to a site of injury may facilitate the regeneration of damaged tissue. Hydrogels are promising carriers for cell delivery as they may be injected into an injury site. We report here on a novel fibrinogen hydrogel produced using a proprietary fibrinogen polymerisation agent (FPA). FPA is composed of the fibrinogen-binding peptide (GPRP) bound to a soluble carrier. When added to fibrinogen, FPA causes fibrinogen to polymerise into a multi-branched fibrin-like gel. The structure and properties of the fibrinogen hydrogel can be modified by altering the concentration of FPA used for gelation. The aim of this research was to investigate the potential of these novel hydrogels for encapsulation and culture of chondrocytes and mesenchymal stem cells (MSCs).

METHODS: Fibrinogen hydrogels were formed by mixing solutions of 34 mg/ml fibrinogen with a range of concentrations of the FPA. Commercial fibrin gels were used as a comparator and formed according to the manufacturer's instructions. For cell encapsulation, either bovine chondrocytes or MSCs were resuspended in fibrinogen prior to gelation by FPA or thrombin for fibrin gels. The cell encapsulated hydrogels were incubated under chondrogenic conditions for up to twenty one days. Cell viability was determined using resazurin dye [1] and extracellular matrix (ECM) formation was assessed using 1,9-dimethylmethylene blue [1] to determine glycosaminoglycan (GAG) content. Statistical analysis was by one-way ANOVA with a Tukey post-test.

RESULTS: Chondrocytes and MSCs exhibited good survival and proliferation in all fibrinogen and fibrin hydrogels. After twenty one days of incubation, chondrocytes remained encapsulated and showed deposition of GAGs indicating ECM formation. In contrast, MSCs rapidly degraded and migrated out of all hydrogels after three days of encapsulation. Aprotinin inhibited hydrogel degradation and the resultant MSC migration under the culture conditions used. In the presence of aprotinin, deposition of GAGs in the hydrogels indicated ECM formation. The data indicated that 2.66 mg/ml FPA was optimal for chondrocyte encapsulation and 0.7 mg/ml FPA for MSC encapsulation. The fibrinogen hydrogels gave similar results to the commercial fibrin hydrogels.

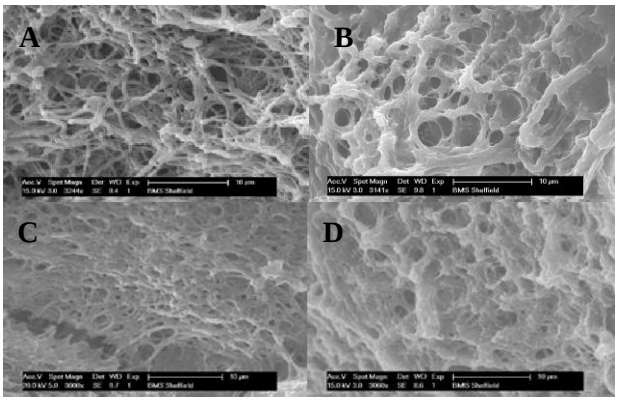

Figure 1: *Scanning electron microscopy images of cell-free hydrogels. A) Fibrin. B-D) FPA concentration: B 2.66 mg/ml C 1.33 mg/ml D 0.7 mg/ml*

DISCUSSION & CONCLUSIONS: The novel fibrinogen hydrogel system supported cell survival and ECM formation for chondrocytes and MSCs. The MSC-induced degradation and their migration from the hydrogels was most likely due to the activation or production of a serine proteinase. This property could allow MSCs to migrate from the hydrogel into the injured tissue. The addition of aprotinin provided greater control over the degradation by MSCs. The fibrinogen gels were comparable to commercial fibrin hydrogels but have the advantage of altering the concentration of FPAs present during gelation. Therefore, the cross-linking density of the structure can be readily modified enabling optimisation of the hydrogel.

In conclusion, this novel fibrinogen hydrogel has a strong potential for use in cell encapsulation and delivery for the regeneration of injured tissue.

ACKNOWLEDGEMENTS: We thank the MRC for funding and Haemostatix Ltd for kindly providing the fibrinogen polymerisation agent.

Multifactorial approaches for cell phenotype maintenance and function

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INTRODUCTION: Cell-based therapies require removal of cells from their optimal in vivo tissue context and propagation in vitro to attain suitable number. However, bereft of their optimal tissue niche, cells lose their phenotype and with it their function and therapeutic potential. Biophysical signals, such as surface topography and substrate stiffness, and biochemical signals, such as collagen I, have been shown to maintain permanently differentiated cell phenotype and to precisely regulate stem cell lineage commitment [1, 2]. Herein, we developed and characterised substrates of variable rigidity and constant nanotopographical features to offer control over cellular functions during ex vivo expansion.

METHODS: PDMS substrates with varying ratios of monomer to curing agent (0:1, 1:1, 5:1) were fabricated based on established protocols. Grooved substrates were created using a silinated wafer with groove dimensions of 2µm x 2µm x 2µm; planar control groups were created using flat glass. The aforementioned PDMS solutions were poured onto the wafer/glass, cured at 200 ºC and treated with oxygen plasma. Substrates were then investigated with/without collagen I coating. (0.1, 0.5, and 1 mg/ml). Atomic force microscopy (AFM) and optical profilometry were used to assess the topographical features of the substrates. Dynamic mechanical analysis (DMA) was used to determine the mechanical properties of the substrates. The simultaneous effect of surface topography / substrate rigidity on cell phenotype and function was assessed using human permanently differentiated cells (tenocytes) and stem cells (human bone marrow stem cells) and various morphometric and gene / protein assays.

RESULTS:

PDMS substrates of varying stiffness (1000 kPa, 130 kPa, 50 kPa) can be made by varying the Sylgard ratio, while maintaining topographical features. Human tenocytes attach, align and elongate on the grooved PDMS substrate surface of all 3 stiffnesses.

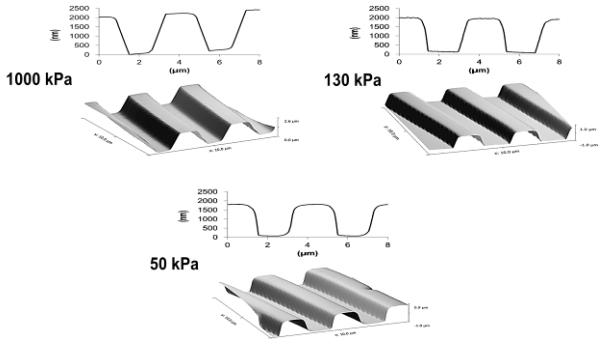

Fig. 1: AFM analysis revealed that topographical features were maintained across all 3 different stiffnesses.

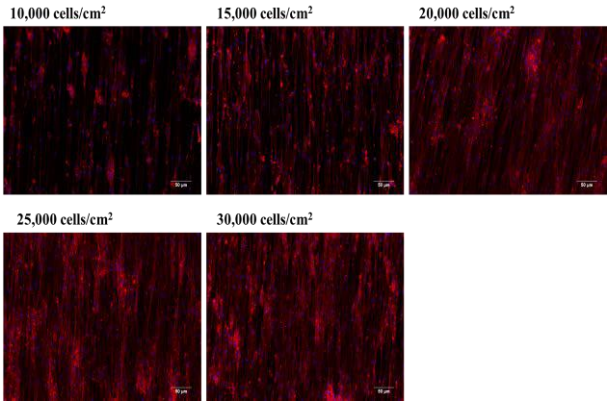

Fig. 2: Human tenocytes attach, align and elongate on grooved substrates at a range of cell seeding densities up to day 14.

DISCUSSION & CONCLUSIONS: Preliminary in vitro data indicate that surface topography and substrate stiffness play crucial role in maintaining cell phenotype and the prevention of phenotypic drift in vitro.

ACKNOWLEDGEMENTS: CURAM Science Foundation Ireland (SFI) Grant Number 13/RC/2073. Centre for Microscopy & Imaging funded by NUI Galway and PRTLI, Cycles 4 and 5, National Development Plan 2007-2013

A novel platform for screening hematopoietic stem cell niche factors in human bone marrow models *in vivo*

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INTRODUCTION: Bone marrow transplants are the only available therapy for many cancers involving blood cells, such as leukemia or lymphoma. A major limitation of this treatment is the availability of hematopoietic stem cells (HSCs). Their expansion *ex vivo* remains a major challenge in medicine today. HSCs grown *in vitro* rapidly lose their regenerative capacity likely due to the lack of niche-derived signals comprising molecular and cellular components [1]. Identification of critical hematopoietic niche components necessitates the generation of more tractable *in vivo* models. An attractive solution is to combine a blank biomaterial with different growth factors and cell types known to be present in the niche *in vivo*. To date, novel approaches for heightened throughput of screening such systems *in vivo* are lacking. We present a novel device, which allows the screening of multiple unique conditions in a single mouse, and have applied it for use in discovery of critical HSC niche factors.

METHODS: Human mesenchymal stem cells (hMSCs) were encapsulated in functionalized biomimetic polyethylene glycol (PEG) hydrogels [2] with or without bone morphogenetic protein-2 (BMP-2). These PEG gels were polymerized directly in eight individual wells of a novel multiplexing polydimethylsiloxane (PDMS) device. Then, the screening devices containing PEG hydrogels were subcutaneously implanted in immunocompromised mice (4x per animal), resulting in 32 unique conditions per mouse (Fig 1). After 8 weeks, the devices were harvested and analyzed for bone and bone marrow formation by microCT, histology, and FACS.

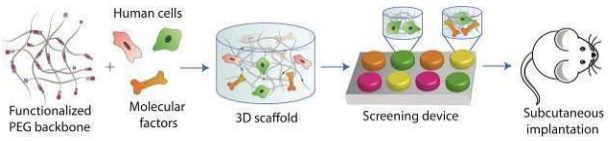

Fig.1: In vivo niche screening device. Modular PEG hydrogels, growth factors and cells are employed to create microenvironments of variable composition in the 4x 8-well screening devices that are subcutaneously implanted per mouse.

RESULTS: Bone formation as assessed by microCT revealed the presence of mineralization in all wells containing gels with hMSCs and BMP-2 (Fig 2A), but not in those lacking the growth factor (green circle). Histological analysis corroborated these findings. Hydrogels containing hMSCs and BMP-2 developed into a bone-marrow like structure enclosed by a bone shell filled with marrow containing trabecular bone structures (Fig 2B). FACS analysis on the murine hematopoietic population of the cell-laden and BMP-2 constructs showed enrichment of long term HSCs compared to the constructs without BMP-2 or cells (Fig 2C).

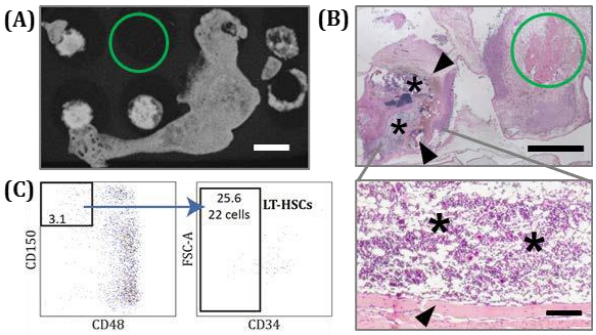

Fig. 2: Evaluation of HSC niche. (A) MicroCT and (B) H&E staining show ectopic ossicle formation (arrow) and bone marrow-like cavities (*) in cell-laden gels loaded with BMP-2, or fibrous tissue formation in absence of BMP-2 (circles). (C) FACS analysis of ossicles shows presence of long term HSCs. Scale bars: top 2mm, bottom 100µm.

DISCUSSION & CONCLUSIONS: Hydrogel conditions, cell type, and soluble factors were screened to optimize conditions for bone marrow niche formation *in vivo*. Results indicated a functional ectopic niche in BMP-2 + hMSCs gels. This device represents a powerful new tool for heightened *in vivo* screening of tissue engineering constructs with a broad range of applications.

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Crosstalk between BMP signaling and mechanotransduction: mechanical parameters determine crosstalk sensitivity

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INTRODUCTION: In musculoskeletal tissues like bone, cells are exposed to mechanical forces resulting from load bearing and muscle activity. During tissue regeneration cellular functions like progenitor cell differentiation depend on coordinated signals arising from both mechanical forces as well as growth factors signaling. During bone formation and healing Bone Morphogenetic Protein 2 (BMP2) is an indispensable growth factor which is applied clinically. Interestingly, we found recently that mechanical signals feed positively into the BMP signaling pathway by enhancing signaling events [1]. In this study we aimed at a deeper understanding what mechanical signals are most effectively supporting the BMP signaling pathway. We further examined whether the cytoskeleton is involved in mediating the crosstalk between mechanotransduction and BMP signaling.

METHODS: Human fetal osteoblasts (hFOBs) were seeded in 3D macroporous collagen scaffolds which provide optimal oxygen and nutrient supply. Cell seeded constructs were transferred into a custom made bioreactor system [2] where they were stimulated by mechanical loading (cyclic uniaxial compression), BMP2 (5nM) or a combination of both. Phosphorylation of Smad1/5/8, an early signaling event in the BMP pathway, was investigated after mechanical loading of different frequencies (f= 0.03Hz, 1Hz, 10Hz) using western blot.

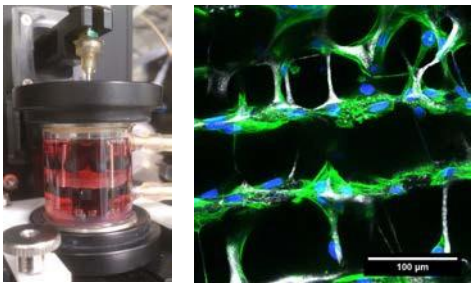

Fig. 1: Collagen scaffold cultured in the bioreactor chamber (left). Confocal image showing a scaffold seeded with hFOBs. Actin (green), nuclei (blue), collagen scaffold (white).

Mechanistic studies were performed using an actin filament stabilizing drug during the loading experiment to reduce cytoskeletal remodelling.

RESULTS: Smad phosphorylation level and duration under concurrent BMP2 stimulation were found to be strongly affected by the loading frequency. Stimulation with f= 1Hz and 10Hz enhanced the Smad1/5/8 phosphorylation already after 30 min, reaching significant differences after 90 min and remained at the same level up to 120 min. In contrast, f= 0.03Hz led to a reduced synergistic effect at 30 min that quickly vanished over the time course of 90 min. After treatment with a cytoskeletal inhibitor that stabilizes the actin filaments, the synergistic effect of mechanical loading (f=1Hz) and BMP signaling vanished, suggesting that cytoskeletal adaptation to loading and related processes are crucial for the crosstalk.

DISCUSSION & CONCLUSIONS: The results suggest a quick cellular adaptation to low frequency loading after the initial mechanical trigger, while higher frequencies require a continuous cell adaptation leading to a sustained crosstalk. Mechanical forces were found to be a relevant regulator influencing BMP signaling in a frequency-dependent manner. In a clinical setting mechanical signals might be utilized to further increase the effectivity of BMPs at the site of injury. This would allow a reduction of the necessary dose and lower treatment costs as well as the risk of side effects.

ACKNOWLEDGEMENTS: This work was funded by the German Research Foundation (DFG), research group FOR 2165 (# PE 1802/1-1).

A full-layer bladder wall patch by mincing both porcine bladder mucosa and detrusor in a natural-synthetic scaffold

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INTRODUCTION: Full layer bladder regeneration has remained a challenge in bladder tissue engineering due to lack of or incomplete regeneration of smooth muscle cells, especially in large area defects [1, 2]. To create a suitable tissue transplant for bladder augmentation, we developed a tissue-engineered construct that includes 1) autologous urothelial cells for good barrier function, 2) autologous smooth muscle cells to regenerate a muscular layer, and 3) a biodegradable support that allows take of the transplant, tissue ingrowth and meets the need of low-pressure high-compliance storage function.

METHODS: An electrospun PLGA sheet with optimized porosity [3] was placed on a semi-gel collagen inside a mold and covered by a second collagen hydrogel followed by distribution of minced detrusor from porcine bladder on top. Thereafter, third collagen hydrogel layer was added and minced mucosa of porcine bladder was seeded on top. The whole construct underwent plastic compression (PC), and was incubated for 1, 2, and 4 weeks *in vitro* for electron microscopy, histology and immunoassay studies.

RESULTS: SEM imaging was performed on both surfaces seeded by minced tissue. Imaging the top collagen surface demonstrated a complete coverage with proliferated urothelial cells after two (confluent) and four (multi- layered) weeks. SEM imaging on the middle-seeded surface also showed that, spindle-shaped smooth muscle cells completely covered the surface after two weeks.

Histology and immunoassays demonstrated that successful proliferation of both urothelial and smooth muscle cells was achieved. It was seen that cells migrated from the minced tissue specimens (either on top or inside the construct), proliferated, reorganized and formed a multilayer epithelium (4 weeks) as well as muscle cells. Furthermore, immunostaining for MNF116 and SMA indicated epithelial cell origin and smooth muscle cells respectively.

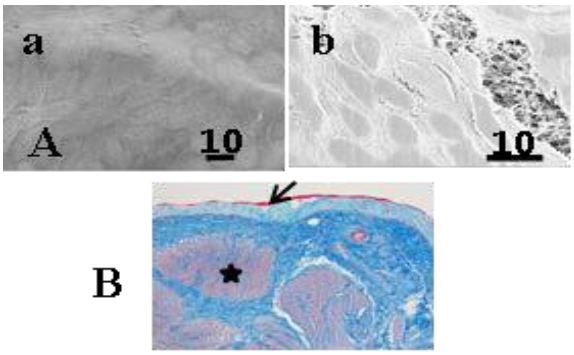

Fig. 1: A) SEM images (a) confluent urothelium, (b) smooth muscle cells and B) Cross-section of PLGA-PC collagen construct seeded with minced mucosa and detrusor after 2 weeks (Trichrome x10 magnification, (arrow) shows urothelium, (*) denotes muscle cells.

DISCUSSION & CONCLUSIONS: With our previous achievements, based on mincing bladder mucosa to regenerate urothelium [3], we continued to develop the technique to regenerate bladder muscle, a tissue that is considered more challenging to expand. We successfully regenerated both urothelium and muscle cells in a hybrid nano-micro, natural-synthetic scaffold of compressed collagen-electrospun PLGA. In a clinical setting the construct is easy to make and all steps: harvesting, mincing and constructing the hybrid scaffold could be performed just before transplantation in a one-stage procedure.

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In toto cultivation of repopulated whole hearts under 3D biomechanical stimulation of the left ventricle

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INTRODUCTION: Herein we demonstrate the impact of three-dimensional (3D) left-ventricular stretching of tissue engineered whole heart constructs.

METHODS: Decellularized rat hearts were repopulated (repop) with neonatal cardiomyocytes, and subjected to coronary perfusion with or without biomechanical stimulation of the left ventricle for 6 days. 3D repopulation, viability and cellular alignment were studied by confocal microscopy, complemented by a modified WST-1 Assay. Quantitative RT-PCR (qRT-PCR) was used to analyze markers of cardiomyocyte maturation. In addition, SDS PAGE gave a simple and non-specific outline of the overall protein apparatus of constructs complemented by Western blotting.

RESULTS: We observed vital cell population throughout the 3D vicinity of the left ventricle of recellularized whole hearts with an increase in viability after six days of cultivation (Fig. 1). In selected regional analysis we confirmed increased cellular alignment in stretched constructs similar to previously shown data for murine myoblasts[1]. qRT-PCR revealed a significant 3-fold increase of Cx43 expression after stimulation versus controls, reaching 60±19 % of the expression level observed in native neonatal rat hearts (vs.9.8±5 % in controls) (Fig. 2). The SDS PAGE band profile demonstrated a remarkable similarity to that of native control, with one distinctive peak at 55 kDa, most likely representing desmin.

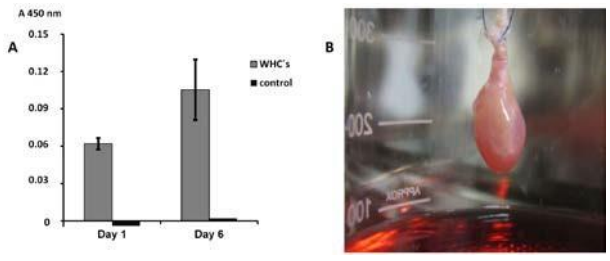

*Fig. 1: **A:** Cellular viability as measured by WST-1 cell viability assay and adapted to whole heart constructs. **B:** Macroscopic view of a repop. heart after six days of cultivation. The repopulated area exhibits a clear increase of optical density.*

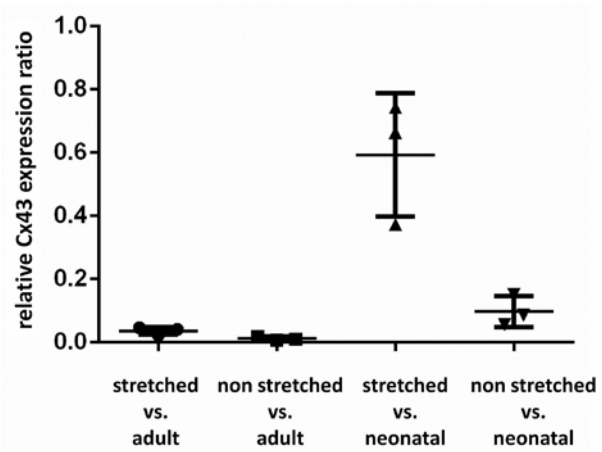

Fig. 2: Cx43 expression of repop. whole hearts with or without 3D stimulation was compared to native adult or neonatal myocardium.

DISCUSSION & CONCLUSIONS: The impact of whole left ventricle stretching of repopulated whole hearts has a significant impact on cardiac gene expression and protein constitution. Here, 3D stimulation promoted cellular alignment and 3D organization. On RNA and protein level an adaption to the absorption and transmission of mechanical force was demonstrated. Thereby this study proves the crucial role of sophisticated bioreactor systems as tools for improved stimulation and cultivation of tissue engineered tissues and organs.

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Osteoinductive properties of nanostructured hydroxyapatite-functionalized gelatins

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INTRODUCTION: Autologous bone graft represents the gold standard for the treatment of bone defects, but its application is strongly limited by poor bone supply and donor site morbidity. Biomimetic gelatin nanocrystalline hydroxyapatite porous scaffolds with tailored properties were previously developed [1] and demonstrated suitable structural and biological properties. The aim of this study was therefore to study the osteoinductive ability of the scaffold: *in vitro* to assess the osteogenic differentiation of human bone marrow mesenchymal stromal cells (hMSC) or *in vivo* in an ectopic site to determine new bone formation.

METHODS: hMSC were seeded on gelatin (GEL) or gelatin-hydroxyapatite 10% (GEL-HA10) scaffolds. Osteogenic differentiation was assessed *in vitro* by the analysis of alkaline phosphatase activity and gene expression. *In vivo* experimentation was conducted under the observance of the Italian Law on animal experimentation (Law by Decree 26/2014), transposition of the EU directive 2010/63/UE. The experimental protocol was approved by the Ethical Committee of Rizzoli Orthopaedic Institute and by the Italian Ministry of Health. Osteoinductivity was assessed in nude mice. Briefly, GEL or GEL-HA10 scaffolds, with or without hMSC, were implanted in dorsal subcutaneous pockets. After 8 weeks, implants were retrieved for microtomographical (microCT), histological and gene expression analyses.

RESULTS: The scaffolds sustained the proliferation and osteoblastic differentiation of hMSC, as demonstrated by the assessment of ALP activity and gene expression analysis. In particular, *SP7* (Osterix) and *BGLAP* (osteocalcin) were significantly upregulated in GEL and even more in GEL-HA10 samples. *VEGFA* expression increased at 3 weeks in GEL-HA10 samples only. *In vivo*, microCT showed the osteoinductive potential of both GEL and GEL-HA10 scaffolds. The materials seeded with hMSC showed osteoinductive characteristics only with the presence of HA. Histological analysis of explants revealed a good integration of the scaffold and no connective tissue capsule around the implants. In MSC-seeded scaffolds cellular infiltration and production of extracellular matrix were uniform in every implant, otherwise cells were only in the superficial layers. Finally, the analysis of gene expression in retrieved implants showed that the levels of murine *Col1a1* or *Bglap* did not change among different groups. The expression of *Runx2* was significantly lower in GEL-HA10+hMSC than in GEL-HA10, while the expression of *Alpl* was higher in GEL+hMSC than in GEL. The effect of HA was more pronounced for human cells. Indeed, except for *COL1A1*, the expression of osteoblastic genes markedly increased in GEL-HA implants compared to GEL, with a mean fold increase of 271.36 for *RUNX2*, 9.41 for *ALPL* and 95.82 for *BGLAP*.

DISCUSSION & CONCLUSIONS: The results demonstrated an osteoinductive potential for both GEL and GEL-HA10. Moreover, gene expression analysis revealed that human cells were more responsive to the presence of HA than murine cells. Further studies should be performed to evaluate the ability of the scaffolds to repair critical size defects.

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DISCLOSURES: The authors declare no conflict of interest.

Fluid flow regulation of revascularization and cellular organization in a bioengineered liver platform

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INTRODUCTION: Modeling of human liver organogenesis, especially cellular distribution and the mechanisms underlying it, is fundamental for studying liver development and disease, yet there are no reliable models that fully mimic these processes *ex vivo*. Using an organ engineering approach[1], we designed a perfusion system that delivers discrete mechanical forces inside an acellular liver extracellular matrix scaffold in order to study hepatic tissue organization, including the effects of mechanical stimulation.

METHODS: Scaffolds were seeded with 30 million liver cells (HepG2) and endothelial cells (MS1) via the portal venous system and then maintained in the bioreactor for up to 7 days. Experiments were performed at five different flow rates, ranging from 3-40 ml/min, to determine the effect of flow rate on cell delivery and tissue formation. The seeded bioscaffold was characterized after 1 and 7 days by analyzing cell penetration into the scaffold, percentage of the scaffold seeded with cells, cellular proliferation, cellular stress, vascular structures' re-vascularization, and cellular organization through histochemistry and immunofluorescence, with subsequent image analysis. Furthermore, selective inhibition of nitric oxide synthase (NOS) was performed. The same analytical parameters indicated above were evaluated in recellularized liver scaffolds after 1 and 7 days.

RESULTS: We observed a fluid flow rate dependent response in cell distribution within the liver scaffold, with vascular pressures slightly above physiological portal levels showing superior cell penetration and distribution, revascularization and tissue formation. Since nitric oxide is a major mediator of fluid flow effects on endothelial cells, we hypothesized that it played a vital role in the observed cellular processes. Hence, by selective inhibition of NOS, we observed a significant impairment of both neovascularization and liver tissue organization (Fig. 1B-D). Similar results were observed in bioengineered livers grown under static conditions, possibly to the lack of mechanical stimulation. Finally, mechanical stimulation was vital to significantly increase the lining of vascular structures with EC (Fig. 1C).

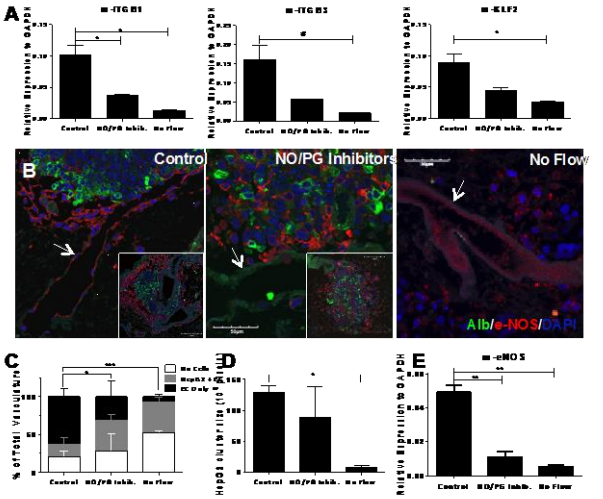

*Fig. 1: NO inhibition reduces vascular and hepatocytic organization. (A) Gene expression of seeded scaffolds using standard 9 ml/min perfusion conditions, showed lowered expression of integrin β1 (m-ITGB1), integrin β3 (m-ITGB3) and KLF2 (m-KLF2) with addition of NO and PG synthesis inhibitors (NO/PG inhibitors), and no flow conditions. (B) Furthermore, a significantly higher number of vascular structures were lined by EC in control conditions (white arrows point to vascular structures covered with EC). (C) Analysis of cell types covering the vascular structures at 7 days post cell seeding. (D) HepG2 cluster size at 7 days post cell seeding (E) Expression of m-eNOS. n=3. # p<0.1, * p<0.05, ** p<0.01, *** p<0.001.*

DISCUSSION & CONCLUSIONS: Overall, we were able to unveil the potential central role of discrete mechanical stimulation through the nitric oxide pathway in the revascularization and cellular organization of a bioengineered liver. Lastly, we believe that the created whole organ bioengineering platform can contribute significantly to the identification of physiological mechanisms of liver organogenesis and regeneration and improve our ability to bioengineer livers for transplantation.

Macroporous alginate scaffold as an in vitro research model for the investigation of ovarian carcinoma progression mechanisms

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INTRODUCTION: Ovarian Carcinoma **(**OC), the leading cause of death from gynecological cancers in western countries, is characterized by primary solid tumors, solid metastases, and effusions to the peritoneal and pleural cavities as the tumor progresses. Exosomes, 30-100 nm vesicles, contain various lipids, proteins, and nucleic acids, exhibit paracrine bioactivities as well as distant transfer of regulatory messages to other cells. The presence of OC derived exosomes accelerated cancerous pathologies in mice, therefore a worthy target for OC therapy. The precise mechanism of the biogenesis and release of exosomes has yet to be elucidated; however, several studies have revealed the involvement of NSMASE2, TSAP6, and RAB27A/B genes in these processes [1]. In order to study the regulatory mechanisms of exosome secretion, an in vitro model must be established that mimics the disease various stages and forms. The aim of the present study was to establish and characterize a three dimensional (3-D) ovarian carcinoma cell culture based on artificial alginate scaffolds and to further utilize this culture as a model for elucidating the secretory mechanism of exosome release in OC.

METHODS: Macro-porous alginate scaffolds were fabricated by a freeze-dry technique. OC ES2 cells were seeded into porous scaffolds and cultured for two weeks. OC cells were either seeded into these scaffolds or cultured as spheroids. After two weeks of cultivation, cell constructs were analyzed for mRNA expression levels of TSAP6, and RAB27A/B genes. The profile was compared to that of OC samples, derived from primary, metastatic lesions and from effusion derived OC cells.

RESULTS: Our results indicate that ES2 cells grown in alginate scaffold form organoids that resemble solid tumors in their gross morphology. Moreover, mRNA expression levels of TSAP6 and RAB27A in ES2 cell constructs grown within alginate scaffold are significantly lower compared to those in ES2 spheroid cultures, emulating the gene profile in solid tumors vs. effusions in OC (Fig 1, a-b, T-test, $p<0.05$). In OC samples, TSAP6 and RAB27A mRNA expression levels are significantly higher in effusions vs. solid tumors (Fig.1 c,e Kruskall-Wallis, $p<0.05$); while the protein expression levels of these genes are significantly lower in effusions vs. the solid samples in these very same samples (Fig.1 d,f Kruskall-Wallis, $p<0.05$). This is explained by the presence of these proteins in effusion fluid-derived exosomes.

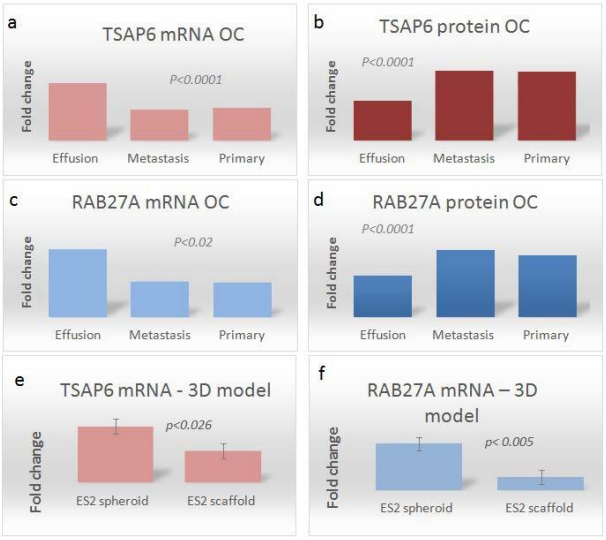

Fig. 1: q-PCR and Western Blot analyses for gene and protein expression levels of TSAP6, RAB27A genes, involved in exosome biogenesis and release; in vitro model (a,b), OC samples (c-f).

DISCUSSION & CONCLUSIONS: Our 3D *in vitro* model for various forms of OC will enable studying exosome secretion mechanism in OC, and the development of potential novel therapies.

A potential solution to the world-wide chronic organ shortage: warm perfusion

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INTRODUCTION: Transplantation is the therapy of choice for end-stage organ failure. In the case of kidneys, transplantation is the only therapy that allows a patient whose kidneys have failed to regain the ability to lead a normal life. Although transplantation is the therapy of choice, relatively few transplants occur each year. The reason so few patients are transplanted is due the demand for organs being significantly larger than the available supply. This large unmet medical need is due to today's narrowly defined kidney donor criteria. Of the traumatic injury death population each year, less than 4% meet the current donor criteria: brain death on life support; the salient factor being minimal ischemic damage. Complicating the situation is that renal allograft preservation times are limited clinically to just 24 hours. A limitation that must be overcome if we are to be successful in transplanting ischemically damaged kidneys from the 96% of patients dying each year from a traumatic injury. The only near term solution to the kidney shortage is to use tissue engineering to repair ischemically damaged kidneys.

METHODS: A historical review of the field of kidney transplantation will focus on the role of kidney preservation technology and the impact of ischemic injury; the two factors limiting criteria for organ donation. In 1980 with the goal of achieving long-term kidney preservation, technology was developed in animal models to prolong renal allograft preservation times. A period of hypothermic machine perfusion in combination with a short period of intermittent warm perfusion was used. Later the goal of developing optimized ex vivo preservation times involved using a heart-lung machine to conduct the warm perfusion. Simultaneously, a program was instituted at the University of Maastricht to revisit the potential of transplanting kidneys with a limited ischemic injury. This work was first conducted using a canine kidney transplant model and was later transitioned to the clinic.

RESULTS: Over six days the cold preservation period was successfully interrupted with four hours of warm perfusion. These four hours of warm perfusion resulted in life sustaining function in a canine transplantation model. This work was the first to demonstrate the beneficial effect of

warm perfusion and the ability to achieve longer renal allograft preservation. Studies using the heart-lung machine were also successful. However, the complexity of its use and the associated costs made routine clinical use of the technology prohibitive. A longstanding clinical program has been developed at Maastricht that has successfully transplanted kidneys procured from patients deceased by cardiac criteria (DCD) rather than brain death to address the organ shortage. Critical to the success of any attempt to utilize ischemically damaged renal allografts is the development of an effective viability test that can distinguish damage that is reversible from irreversible damage that will result in primary non-function. However, such efforts to develop reliable prognostic testing were initially disappointing. A major result of our efforts to develop a DCD kidney transplantation program has been the development of the Maastricht classification that categorizes ischemic damage following cardiac arrest that is still in use today [1].

Exsanguinous Metabolic Support (EMS) technology was also developed in Maastricht and has demonstrated the potential to accomplish long term ex vivo near-normothermic perfusion. Continued metabolism during EMS of sufficient magnitude to support synthetic functions holds the potential perform effective viability testing.

DISCUSSION & CONCLUSIONS: The first kidney transplant was performed 62 years ago between identical twins. The field has grown beyond original expectations. However, the future of the field will be limited unless more organs become available to meet the huge demand. Regenerative medicine may hold the key to the future of transplantation.

DISCLOSURE: The author has nothing to disclose.

Delivery of miRNA Therapeutics and Anabolic Genes for Bone and Cartilage Engineering

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INTRODUCTION: Bone is a dynamic tissue that requires coordinated homeostasis of bone-forming osteoblasts and bone-resorbing osteoclasts to maintain constant bone mass and volume. Osteoporosis arises from the dysregulation of bone turnover such that bone resorption exceeds bone formation, leading to reduction of bone mass and micro-architectural deterioration. MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression. MiRNAs modulate diverse biological processes, including osteoclastogenesis, osteogenesis, bone formation and can alter the bone phenotype. However, little is known about the roles of miRNA in osteoporosis. Baculovirus (BV) is a non-pathogenic insect virus and can transduce BMSCs and adipose-derived stem cells (ASCs) at efficiencies exceeding 90%. We hypothesized that miRNA expression in osteoporotic BMSCs is dysregulated, hence impairing the ability of BMSCs to differentiate into osteoblasts for bone repair.

METHODS: We created osteoporotic rat models by ovariectomy (OVX) and analyzed the miRNA expression profile in the OVX-BMSCs. We uncovered that miR-140* and miR-214 were overexpressed in OVX-BMSCs, and thus constructed Cre/loxP-based BV harboring miRNA sponges, in order to persistently knock down miR-140* or miR-214. Whether suppressing miR-140* or miR-214 was able to restore the osteogenesis of OVX-BMSCs and inhibit the osteoclast maturation was evaluated in vitro. We further assessed whether the BV-engineered, miRNA sponge-expressing OVX-BMSCs, with or without BMP2 co-expression, were able to heal critical-size bone defects in osteoporotic rats.

RESULTS: Engineering OVX-BMSCs with the hybrid vectors persistently attenuated the cellular miR-140*/miR-214 levels, which promoted the OVX-BMSCs osteogenesis and augmented the ability of OVX-BMSCs to repress osteoclast maturation in vitro. Notably, suppressing miR-214 exerted more potent osteoinductive effects. In the osteoporotic rat models with a critical-size bone defect at the femoral metaphysis, implanting the OVX-BMSCs ectopically expressing BMP2 failed to heal the defect, which underscored the difficulty to heal osteoporotic bone defects. Nonetheless, allotransplantation of the miR-214 sponges-expressing OVX-BMSCs healed the defect and ameliorated the bone quality (density, trabecular number, trabecular thickness and trabecular space) at 4 weeks post-implantation. Co-expressing BMP2 and miR-214 sponges in OVX-BMSCs further synergistically substantiated the healing.

DISCUSSION & CONCLUSIONS: To date, the majority of drugs for osteoporosis management either promote bone formation or inhibit bone resorption. The OVX-BMSCs-based cell therapy in conjunction with miR-214 sponge expression, with or without BMP2 expression, not only stimulates bone formation, but also mitigates excessive bone resorption, thus paving a new avenue to the treatment of osteoporotic bone defects.

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imaging skeletal muscle regeneration after stem cell application using diffusion tensor imaging (DTI) and magnetisation transfer (MT) measurements

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INTRODUCTION: Urinary incontinence compromises a patient's quality of life and inflicts tremendous health care costs[1]. Recent advances using cell therapy such as satellite stem cells (SSCs) show promising results towards correcting the underlying etiology[2] but evaluating the success of such treatments *in vivo* is difficult. In this study, we show that Magnetic Resonance Imaging (MRI) properties enable a monitoring of adult stem cell myogenic differentiation in a mouse model.

METHODS: We have isolated, characterized and expanded human SSCs followed by injection into a tibialis anterior muscle crush mouse model. We followed up the *in situ* differentiation via MRI (4.7 T scanner) for 21 days focusing on Magnetization Transfer Ratio (MTR) and Diffusion-Tensor Imaging (DTI) properties of the *de novo* tissue and confirmed the results by histology, immunohistochemistry, western blot and real time PCR.

RESULTS: MT measurements showed an initial MTR decrease before increasing steadily and approximating the MTR values of reference skeletal muscle tissue. DTI revealed that *de novo* generated muscle fibers are orientated in the same direction as the surrounding fibers which were not affected by the initial muscle crush injury. Cell differentiation and myofibers formation could be confirmed by increased muscle specific markers.

DISCUSSION & CONCLUSIONS: Human SSCs form muscle tissue *in situ* and MT-MRI allows to directly assess muscle fiber formation as a measure of myogenic differentiation. DTI there while highlights the direction of the newly formed fibers. These results will be transferable to the clinical setting as a non-invasive biomarker for the assessment of muscle tissue regeneration in patients.

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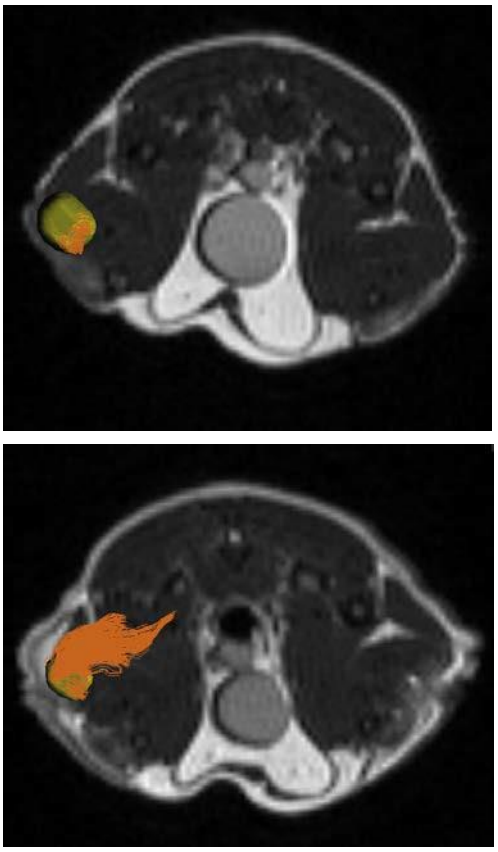
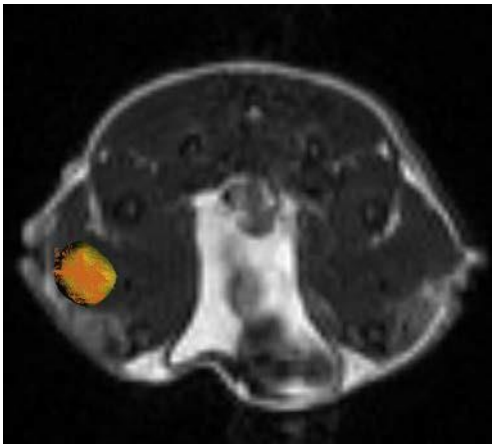


Fig. 1: Top: Healthy skeletal muscle Middle: Decrease in fiber number within the injury site 1 day after surgery. Bottom: Fibre distribution after 21 days



bioartificial liver in vitro: an innovative method of hepatogenic differentiation

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INTRODUCTION: End-stage liver disease (ESLD) is a global health problem. Current therapy is far from being satisfactory and mortality remains high. *In vitro* testing for novel therapies is difficult to establish because mature hepatocytes in culture lose their specific functions. Recently, pluripotent stem cells have been promoted as an alternative source for differentiated cells. We investigated the differentiation potential of human adipose-derived stem cells (ASC) toward hepatocyte phenotype on a commercially available 3D collagen scaffold in order to model the *in vivo* microenvironment, where cells may better maintain their functional and differentiation properties.

METHODS: ASCs were isolated from adipose tissue obtained by liposuction or fat excision and were expanded on cell culture plastic. After expansion, cells were seeded and differentiated in 3D collagen scaffolds. Differentiation was induced by specified growth factors[1] and a 2D cell culture served as control. The differentiation potential of ASCs, as mesenchymal stem cells (MSC), was verified by the presence of mesenchymal markers by flow cytometry (FACS), by their ability to turn into adipogenic and osteogenic phenotypes and to form clones in 2D culture. Adipogenic and osteogenic differentiation was evaluated by OilRedO staining and by alkaline phosphatase staining, respectively. Hepatogenic differentiaton was assessed by glycogen storage and by immunohistochemistry (IHC) of albumin, α-fetoprotein, Cytokeratin 18 and CYP450 isoform 3A4 and 1A2. Expression at mRNA level of hepatogenic markers was evaluated at different time points by qPCR during culture. Urea and albumin in the medium were analyzed by colorimetric assays.

RESULTS: ASCs confirmed their mesenchymal phenotype by differentiation into adipocytes and osteocytes, by forming clones and by retaining mesenchymal cell markers on their surface. PAS staining after 3 weeks of 3D culture in hepatogenic differentiation media showed high glycogen accumulation, which did not change during two months of static culture. In contrast, in the 2D control, for hepatogenic differentiation[1], weak glycogen positivity was found from 3 weeks on. Cells displayed high metabolic activity throughout the scaffold during each time point of culture. IHC of 3D versus 2D culture showed the presence of intracellular albumin, cytoplasmatic cytokeratin 18 and CYP450, isoforms 3A4 and 1A2, in the cells on the scaffold. These markers were absent in 2D cultures for up to 3 weeks, although cells on plastic showed a morphology similar to hepatocytes. FACS data indicated ASCs differentiation since markers typical for MSC got lost during culture. Compared with 2D cultures, albumin and urea concentrations were higher in 3D, indicating an improved functionality of the cells. qPCR of 3D scaffolds indicated expression of mRNAs for the the hepatogenic markers such as HNF4 α, albumin, CYP450 isoform 3A4 and 1A2, OATP1 and OATP2.

DISCUSSION & CONCLUSIONS: Results obtained revealed an improved differentiation of ASCs into hepatocytes in a 3D collagen structure compared to the 2D plastic. Accordingly, 3D cultures on collagen provided an optimal environment for differentiation of the cells. This method represents a novel tool for culture of hepatocytes *in vitro*. In future, it may be used for *in vitro* drug testing and to investigate liver regeneration in animal models of ESLD.

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ECHO: computer model explains cartilage development and homeostasis

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INTRODUCTION: An intricate network of regulatory processes determines the chondrocyte cell fate both during development and maintains tissue homeostasis. The exact regulation of these cellular processes by this network during development are yet unknown, hampering cartilage and bone tissue engineering initiatives. To generate stable articular cartilage we need to mimic the regulatory processes in their original state. However, because of the inherent complexity of regulatory networks, they cannot be efficiently analysed and understood without computational assistance. To obtain insight into the function of such complex networks we developed a dynamic computational model of chondrocytes, the **Executable CHOndrocyte** or **ECHO,** figure 1. In ECHO cell fates corresponding to hypertrophy or transient cartilage as well as permanent articular chondrocytes can be investigated. We used ECHO to mimic all steps of the development of stable articular cartilage.

METHODS: ECHO is a computational model of the articular chondrocyte, built in ANIMO[1,2]. In it we summarize current experimental knowledge on articular chondrocyte signal transduction and gene expression. ECHO consists of 7 signal transduction pathways important for chondrocyte development and cartilage maintenance: IGF, PTHrP, BMP, FGF, TGFβ, WNT, IHH. The model can autonomously evolve to a hypertrophic state or to an articular chondrocyte state. qPCR experiments on mesenchymal stem cells and articular chondrocytes were used to validate the model.

RESULTS: Using ECHO we analysed the most influential pathways, and performed *in silico* experiments to obtain insight into the molecular mechanisms of cartilage development. For example, we investigated the cell's response to addition of single growth factors or cytokines or combinations of growth factors and cytokines. The model showed a dose-dependent response to (combinations of) external stimuli. qPCR validated this dose-dependency in MSC and chondrocytes. In addition, we performed *in silico* double knockout and overexpression experiments to predict which signals could cause an hypertrophic chondrocyte to switch back to an articular state. These experiments provide potential therapeutic clues for better cartilage tissue engineering. Laboratory experiments are currently being carried out to validate these findings.

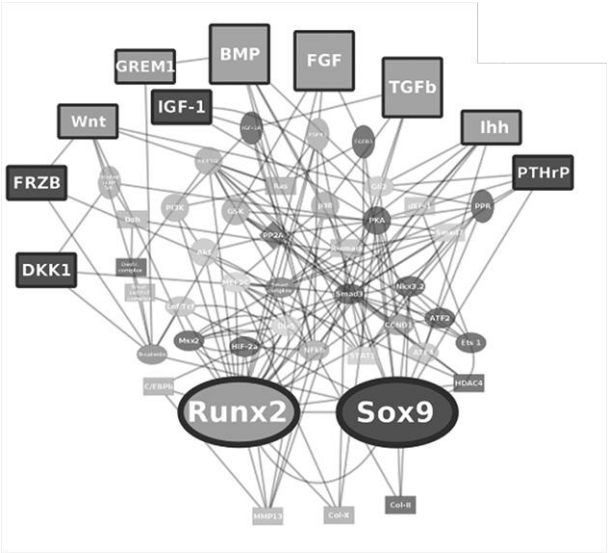

Fig. 1. ECHO is a network of 7 signal transduction pathways with 92 nodes and 123 interactions.

DISCUSSION & CONCLUSIONS: We show how ECHO was used to mimic biological scenarios during chondrocyte development. We obtained insight into healthy chondrocyte development and chondrocyte hypertrophy, and identified factors that are crucial to the decision of cell fate.

Mechanical modulation of stem cell chondrogenesis

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INTRODUCTION: Unlocking the potential of mesenchymal stem cells (MSCs) for musculoskeletal tissue engineering requires understanding how environmental factors regulate their fate. Cartilage tissues engineered using MSCs will be subjected to a complex mechanical environment once implanted into damaged synovial joints. This talk will summarise our work using bioreactor systems to investigate how various mechanical stimuli regulate chondrogenesis, hypertrophy and endochondral ossification of MSCs.

METHODS: We have developed 3 distinct bioreactor systems to subjected cell laden hydrogels to either cyclic hydrostatic pressure (HP), dynamic compression (DC) or dynamic tension (DT). We have applied HP (10MPa; 1Hz) to MSC laden hydrogels for up to 42 days in the presence of transforming growth factor (TGF)-β3. We have also applied DC and DT (5-10% strain; 0.5/1Hz) to MSC laden hydrogels for similar periods of time in the presence or absence of TGF-β3. Following the application of loading, we have examined changes to the cytoskeleton and markers of chondrogenesis, hypertrophy and endochondral ossification.

RESULTS: Tensile loading, in the absence of soluble differentiation factors, was found to enhance osteogenesis and supress markers of adipogenesis in MSCs. When applied in the presence of TGF-β3, tensile loading was found to enhance markers of endochondral ossification. In contrast, the application of cyclic hydrostatic pressure was found to enhance chondrogenesis[1-3] and supress markers of endochondral ossification[4].

The response of MSCs to dynamic compression (DC) is more complex. The early application of DC was found to supress chondrogenesis of MSCs; however the late application of this mechanical stimulus can enhance chondrogenesis of MSCs if applied 3 weeks after TGF-β3 mediated differentiation[5]. Interestingly, the long-term application of drive chondrogenesis of MSCs within hydrogels that would normally support myogenesis[6].

The application of appropriate levels of mechanical loading can also be used to engineer more functional articular cartilage-like grafts. By modulating both the mechanical environment and oxygen levels through the depth of developing cartilage constructs, it is possible to suppress MSC endochondral progression and to engineer tissues with zonal gradients mimicking certain aspects of articular cartilage[7]

DISCUSSION & CONCLUSIONS: Once implanted into the musculoskeletal system, MSCs will be subjected to a complex mechanical environment. These mechanical cues will play a key role in determining the ultimate phenotype of implanted MSCs. By understanding the role of these environmental cues in regulating MSC fate, we can better design scaffolds and engineered tissues to fulfil their desired function.

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Age matters - the role of the matricellular protein Sparc in aging tendon

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INTRODUCTION: The ability of tendons to withstand stress generally decreases with age, often resulting in increased tissue degeneration [1,2]. However, our understanding of the complex mechanisms underlying tendon ageing is far from complete. In this work, we sought to identify the molecular determinants of age-associated changes in healthy aged mouse Achilles tendon tissue. Our findings reveal profound changes in the expression of ECM-related proteins and a previously unknown role of Secreted protein acidic and rich in cysteine (Sparc; also known as BM-40 or osteonectin) in tendons.

METHODS: A suppression-subtractive-hybridization (SSH) screen comparing cDNA libraries generated from Achilles tendons of mature-adult (3 months) and old (18 months) female C57BL/6 mice was conducted. Subsequently, the differential expression of the identified genes was validated by RT-qPCR and selected genes were then further analysed by immunohistochemistry and Western blot. To investigate age-related structural alterations in the collagenous extracellular matrix we applied SHG-microscopy and TEM. Further, we characterized young and old tendon derived stem/progenitor cells (TSPCs) by 2D and 3D cell culture assays.

RESULTS: Approx. 170 cDNA clones were isolated and based on sequencing data functionally annotated using DAVID. Several of the identified gene ontology (GO) terms related to genes encoding proteins important for processes such as cell adhesion, cytoskeletal re-arrangements, cell migration, and extracellular matrix (ECM) deposition. Generally, the expression of ECM and ECM-remodeling genes was significantly reduced in old tendon tissue and tendon stem/progenitor cells (TSPCs) isolated from Achilles tendon. Further, we observed a significantly reduced expression of the collagen-binding matricellular protein Sparc in healthy aged tendons.

Tendons of Sparc-/- mice were generally thinner and TEM revealed thinner collagen fibrils and a larger interfibrillar area with a concomitant increase in fibril number. SHG-microscopy revealed minor changes in collagen fibril alignment for Sparc-/- animals. Further, TSPCs of old and Sparc-/- tendons formed thinner in vitro tendon constructs and display altered cell-ECM adhesion and cell migration properties when compared to young wildtype cells. Biomechanical analysis revealed a reduced load to failure and changes in elastic stiffness for Sparc-/- tendons. Further, we provide evidence suggesting that Sparc is important for age-related changes of tendon-resident stem/progenitor cells.

DISCUSSION & CONCLUSIONS: The decreased expression of ECM proteins and modulators thereof in old tendons in combination with structural changes is most likely associated with an increased risk of tendon injury in the elderly, since structure and composition of the tendon are directly related to its function. Further, Sparc seems to be an important molecular player in tendon cell and tissue senescence, potentially leading to tendon degeneration.

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Gene activated scaffolds for orthopaedic tissue engineering

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INTRODUCTION: Over 2.2 million bone graft surgeries are performed each year with autologous bone harvesting representing the current gold standard despite recognised limitations (1). Within our group, a variety of collagen-based scaffolds have been developed as viable bone graft substitutes (2). This study aims to develop a next generation gene activated scaffold via the incorporation of star-shaped poly-(L-lysine) (star-PLL) polypeptides loaded with pro-osteogenic genes e.g. Bone Morphogenic Protein-2 (BMP-2) and pro-angiogenic genes e.g. Vascular Endothelial Growth Factor (VEGF) into these scaffolds. Star-PLLs are attractive bio-inspired non-viral vectors with an inherent ability to complex and condense pDNA into nano-sized cationic polyplexes (3).

METHODS: A series of collagen scaffolds comprising either collagen alone, collagen-glycosaminoglycan (chondroitin sulphate) (GAG), hyaluronic acid (HYA), nanohydroxyapatite (nHA) or hydroxyapatite (HA) were formed via an established freeze-drying technique (4). Star-PLL-pDNA polyplexes were characterised using nanoparticle tracking analysis, zeta potential measurement and MTT cell viability assays. Star-PLLs complexed with the reporter plasmids luciferase (pGLuc) and green fluorescent protein (pGFP) as well as the therapeutic plasmids BMP-2 and VEGF were used to establish transfection efficacy of the star-PLL system in mesenchymal stem cell (MSC) monolayer. Lead star-PLL-pDNA polyplexes were loaded onto collagen scaffolds and utilised to transfect MSCs in 3D thereby forming a functional gene activated scaffold.

RESULTS: Three star-PLLs (01,02 & 03) were utilized to successfully prepare star-PLL-pGLuc polyplexes of varying N/P ratios. Cell viability assays determined a substantial decrease in cell viability above N/P10. MSC transfection identified star-PLL (03) at N/P5 with a 2ug pDNA cargo as a lead non-viral system (size 190±42.4nm, charge +30mV, transfection efficiency 23%). This lead formulation was capable of producing nanogram quantities of BMP-2 (85.07±6.78ng) and VEGF (2.22±0.09ng) in 2D at day 7. 3D pGLuc transfection studies demonstrated a 21 day transgene expression profile which was statistically higher in a collagen-(HYA) scaffold compared to a collagen only, collagen-GAG, collagen-nHA and collagen-HA scaffold at days 7 & 14 (P<0.001).

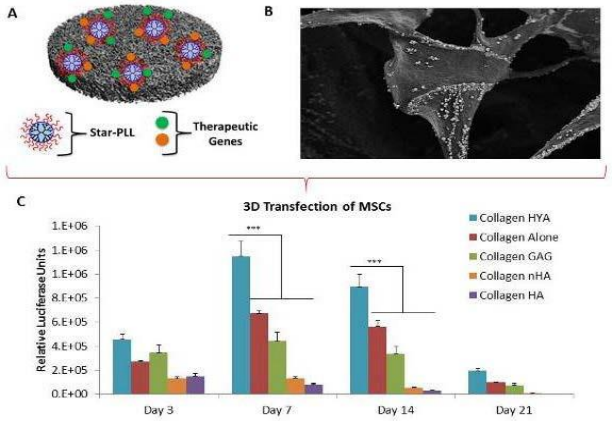

Fig. 1: (A) Schematic of a star-PLL gene activated scaffold; (B) Scanning electron micrograph of star-PLL-pDNA polyplexes incorporated into a collagen-GAG scaffold; (C) Transgene expression profile of star-PLL-pGLuc on a collagen alone, collagen-HYA, collagen-GAG, collagen-nHA & collagen-HA scaffold.

DISCUSSION & CONCLUSIONS: The N/P ratio of the formulation determined the polyplex size, charge, cell viability and transfection efficiency in both 2D and 3D environments. Star-PLL 03 N/P5 2ug pDNA was identified as a lead system from 96 potential combinations. This formulation was capable of efficiently delivering both reporter (pGLuc and pGFP) as well as therapeutic (BMP-2 & VEGF) plasmids. Star-PLLs demonstrated transfection capability in 3D with sustained transgene expression up to 21 days in all scaffolds tested, with markedly higher expression in a collagen-HYA scaffold. These novel, gene activated scaffolds therefore possesses immense potential for orthopaedic tissue regeneration.

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Stem cells: players in tendon physiology, disease and repair

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INTRODUCTION: In contrast to the other musculoskeletal tissues, tendons are poorly investigated, which correlates with the lack of efficient tendon-specific therapeutics [l]. To counter this lag in the tendon field, we have established over the years valuable in vitro and in vivo models that enable deciphering the roles of stem cells in tendon tissue physiology, disease and repair. The following examples will be provided:(i) on molecular factors that we have identified to regulate the tendon stem cell niche [2-3]; (ii) on the alteration of stem cell behaviour upon tendon aging and degeneration [4]; and (iii) on stem cell-based approach to repair tendon injury using a clinically relevant animal model. In this approach we evaluated if human mesenchymal stem cells (hMSC), which were forced into tendon progenitors via direct programming with Scx, can be used for repair of Achilles tendon rupture in a rat experimental model.

METHODS: After generating a 3 mm tendon defect in rat Achilles tendon, we implanted a scaffold-free cell pellet (1 million cells). We analysed the tendon repair after 16 weeks, a time point corresponding to the remodelling phase of the healing process. We compared the regenerative potential of uncommitted hMSC from bone marrow (Mock group) to hMSC expressing Scx cDNA (Scx group). An additional study group consisted of defects, which did not receive any cells (Empty Defect group, ED). Each of the three groups contained 10 animals. Our study comprised of various histological and immunohistological methods, X-ray analyses, precise cell morphometry and in vivo tracking.

RESULTS: The measurement of tendon length and width demonstrated that the ED group had the largest regenerates, while the Scx group exhibited the closest size to the native tendon. The in-tendinous calcification, assessed by X-ray and osteopontin staining, showed large ossified areas in the ED group which were almost reabsorbed in the other groups. Next, PAS staining revealed that the mucopolysaccharide content in the ED and Mock groups was higher than Scx group. The safranin O results showed remnant cartelageous areas only in the ED group, which was additionally confirmed by alcian blue staining. The regenerated tendon-like tissues in the Mock and Scx groups showed higher birefringence of aligned collagen fibres under polarized light microscopy. Cell morphometry clearly demonstrated in Scx group an enrichment of cells exhibiting cell shape factor and alignment characteristics similar to that of non-injured tendon. Finally, two different methods for tracking of the implanted cells showed their long-term survival and integration in vivo.

DISCUSSION & CONCLUSIONS: Altogether, the above results suggested an advanced stage of the tendon repair in the Scx group, hence proposing our approach as of scaffold-free delivery of tendon progenitors as promising way to threat tendon injuries. Moreover, we believe that (i) understanding the exact functions of stem cells in tendon tissues can be very helpful in addressing different basic scientific and clinical questions; and (ii) that research efforts in this field can tremendously enrich our knowledge and can result in major breakthroughs in the management of tendon diseases.

ACKNOWLEDGEMENTS: I acknowledge the Germany Research Foundation (DFG 1414/1-1, 1414/3-1, 1718/1-1), the AO Research Foundation (S-10-74D, S-07-18D) and the Bavarian Research Foundation (FORZEBRA TP1WP2, DOK-100-08) for their support to tendon research over the years.

Mesenchymal stem cell secretome; immunomodulation and oxygen dependency

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INTRODUCTION: Mesenchymal stem cells are in widespread clinical trials for multiple pathologies. In spite of their broad application an enigma remains surrounding their precise mechanism of action; functional incorporation or paracrine signaling? A universal mechanism of action is unlikely where functional incorporation may be better suited to the musculoskeletal milieu but where intravenous delivery and application for immunological indications likely rely on secretome signaling. The precise nature of the secretome factors and their modulation by physiological conditions remains to be determined.

METHODS: Human mesenchymal stem cells (hMSC) were isolated according to a previously published methodology[1]. Multipotent differentiation capacity and ISCT-compliant immunophenotype were established for all hMSC where secretome was evaluated. Total protein concentration was established using the Bradford Assay and secretome cytokine composition with Human Cytokine ELISA Plate Array (Signosis).

Immunological cell line activation was achieved with the addition of PMA/PHA and quantified via IL2 secretion (Jurkat, T-cell), Benzidine incorporation (K562, erythrocyte), CD10 expression (K562, megakaryocyte), and TNFα (THP, M1 macrophage) or IL10 (THP, M2 macrophage) expression and secretion.

RESULTS: We explored the effect of physiological normoxia (2% O2; pathological (PN) vs. chronic (CN)) vs. hyperoxia (21% O2; (HYP)) on global secretome concentration. PN was achieved via multi-user tri-gas incubator, CN via a hermetic workstation. hMSC were cultured for 24 hrs in serum-free media (SFM) to condition media (CM). CM was then collected and total protein concentration determined. PN SFM (350 ng/ml) had significantly higher protein levels than CN (250 ng/ml) and HYP (110 ng/ml) and CN than HYP (Figure 1). Cytokine arrays revealed characteristics broadly consistent with total concentration except anti-inflammatory cytokines in HYP CM and pro-inflammatory cytokines in CN.

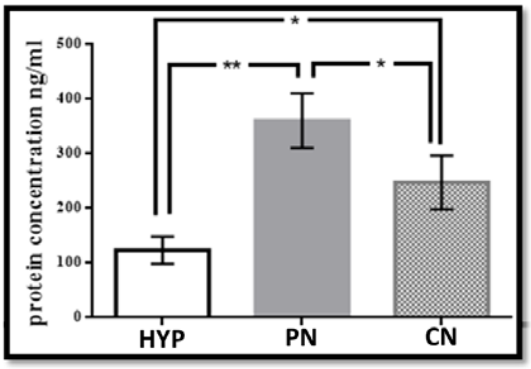

*Fig. 1: Protein concentration in hMSC conditioned media. See main text body for legend. Error bars = +/- 1SD. * indicates p<0.05, ** indicates p<0.001.*

We next sought to evaluate the role of SF-CM from HYP and PN on a range of haematological originating cell lines; Jurkat (T cell), THP-1 (monocyte), and K562 (bipotent myeloid progenitor) and their activation into T cells, macrophages, and megakaryocyte/erythrocytes, respectively. SF-CM from PN and HYP blocked Jurkat activation. Similarly THP-1 macrophagic activation was strongly promoted by CM from both. PN-CM and HYP-CM abrogated all erythrocytic differentiation. Finally, K562 megakaryocytic differentiation was blocked in both PN and HYP vs. control activation conditions.

DISCUSSION & CONCLUSIONS: Taken together we have demonstrated that oxygen-signaling plays a role in modifying hMSC secretome components but that key immunomodulatory components may be an intrinsic feature of hMSC biology.

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New developments in tissue engineered wound healing and scar models

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INTRODUCTION: Since most cutaneous wounds heal with scar formation, it is a major challenge for scientists and clinicians to develop methods to prevent adverse scar formation after severe injury (e.g. burns) and to understand why some individuals form adverse scars e.g. keloids after relatively minor injury. Furthermore it is necessary to understand why one wound will readily heal whereas another will remain resistant to current therapies (ulcer). Since animals do not form scars similar to humans, due to differences in skin physiology and immune system, it is necessary to develop human physiologically relevant in vitro models to investigate wound healing and scar formation. These models can then be used to identify novel drug targets and to test new therapeutics**.**

METHODS: Many different models exist to study scar formation ranging from simple 2D monocultures, to co-cultures to 3D organotypic tissue engineered models [1-3]. In addition to the choice of cell types to include in the model, the wound healing parameter to be assessed needs to be considered. This may be e.g. cell proliferation, in vitro wound closure, stimulation of blood vessel sprouting, initiation or inhibition of an inflammatory response, deposition of granulation tissue etc. It is most important to introduce the level of complexity required to meet the research question being asked and determine whether a low, medium or high throughput model is required.

RESULTS: A battery of in vitro wound healing assays have been developed for addressing i) chronic wound healing where the aim is to identify actives which will revitalize the chronic wound bed and ii) adverse scar formation (hypertrophic and keloid) where the aim is to understand the disease pathology in order to identify new drug targets for reducing fibrosis.

DISCUSSION & CONCLUSIONS: New developments in tissue engineered wound healing and scar models are enabling novel drug targets to be identified and tested. However, important questions are still difficult to address. For example: why does one wound heal whereas another does not, why does a hypertrophic scar form after severe trauma instead of a normal scar and what causes a keloid scar to form rather than a normal scar or hypertrophic scar in some individuals. Also, how is the genetic pre-disposition of the individual and the immune system involved? Understanding the differences in disease pathology is essential if we are to identify new drug targets and develop optimal strategies in the future to prevent adverse scar formation. Recent developments within the multi-disciplinary field of ‘’organ on a chip’’ and in particular’’ skin on a chip’’ may possibly move the field forward into the much needed immune competent disease models.

ACKNOWLEDGEMENTS: The Dermatology Laboratory (VUMC) and Oral Cell Biology (ACTA) research teams.

Human platelet lysate-based hydrogels provides a novel 3D-matrix for the optimization of large-scale expansion of mesenchymal stem cells

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INTRODUCTION: Stem cell expansion in standardized and animal-free conditions represents a major challenge for the production of cell therapy products. To date, many cell culture laboratories still use Fetal Bovine Serum (FBS), as a cell culture supplement. Replacing bovine serum with human Platelet Lysate (hPL) allows for expansion of stem cells without the risk of xenogeneic immune reactions or transmission of bovine pathogens[1]. Biomaterials have impact on proliferation and differentiation of cell preparations. Stem cells are routinely cultured on conventional tissue culture plastic (TCP). In these dishes, plastic adherent cell growth is restricted to two dimensions and it occurs preferentially at the rim of colonies due to contact inhibition. Furthermore, adherent cells need to be loosened from the substrate for passaging – usually by enzymatic treatment with peptidases such as trypsin.

METHODS: We evaluated the use of hPL-hydrogels as a 3D-matrix for large-scale expansion of Mesenchymal stromal cells (MSCs). AlamarBlue cell viability reagent was used to quantify MSC proliferation on hPL-gel over a period of 15 days. Differentiation of MSCs into adipogenic and osteogenic lineage on hPL-gel was analyzed by staining with BODIPY and Alizarin Red S, respectively.

RESULTS: We demonstrated that cells cultured on hPL-hydrogels reveal much higher proliferation rates in comparison to MSCs cultured on tissue culture plastic (TCP). Further, the frequency of initial fibroblastoid colony forming units (CFU-f) increased on hPL-gel. MSCs grew in several layers at the interface between hPL-gel and hPL-medium without contact with any artificial biomaterials. Moreover the viscous consistency of hPL-gel enabled passaging with a convenient harvesting and reseeding procedure by pipetting cells together with their hPL-matrix – this method does not require washing steps and can easily be automated. The immunophenotype and in vitro differentiation potential toward adipogenic, osteogenic, and chondrogenic lineage were not affected by culture-isolation on hPL-gel.

DISCUSSION & CONCLUSIONS: Taken together, hPL-gel has many advantages over conventional plastic surfaces: it facilitates enhanced CFU-f outgrowth, increased proliferation rates, higher cell densities, and non-enzymatic passaging procedures for culture expansion of MSCs for cell therapy purposes.

DISCLOSURES: RWTH Aachen University Medical School has applied for a patent for hPL-gel. PL BioScience GmbH has acquired an exclusive license for the hPL-gel products. For further information, please contact info@pl-bioscience.com.

A textile platform using mechanically reinforced hydrogel fibres towards engineering tendon niche

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INTRODUCTION: Tendon injuries can result from tendon overuse or trauma, resulting in substantial pain and disability. Given that natural or surgical repair of tendons lead to a poor outcome in terms of mechanical properties and functionality, there is a great need for tissue engineering strategies. Textile platforms enable the generation of biomimetic constructs [1]. Therefore, the main goal of this study is the development of cell-laden hybrid hydrogel fibers reinforced with a mechanically robust core fiber and their assembly into braided constructs towards replicating tendon mechanical properties and architecture.

METHODS: To fabricate mechanically reinforced hydrogel fibres, a commercially available suture was coated using a cell-hydrogel mixture of methacryloyl gelatine (GelMA) and alginate. Composite fibres (CFs) were obtained by ionic crosslinking of alginate followed by photo-crosslinking of GelMA. CFs were assembled using braiding technique and the mechanical properties of single fibres and braided constructs were evaluated. Different cells were encapsulated in the hydrogel layer, including MC-3T3, mesenchymal stem cells (MSCs) and human tendon-derived cells (TDCs). Cell viability and metabolic activity were evaluated by LIVE/DEAD staining and presto blue assay of metabolic activity. The expression of tendon-related markers and matrix deposition were also investigated.

RESULTS: CFs were fabricated with a GelMA:alginate hydrogel layer and using multifilament twisted cotton or biodegradable suturing threads. The biocompatibility of this system was evaluated on encapsulated cells (Fig.1a). Cells (MC-3T3, MSCs and TDCs) were homogeneously distributed along the hydrogel layer, being viable up to 14 days in culture. In addition, TDCs were spreading inside the hydrogel after less than 48 h. Moreover, to further improve the mechanical properties of CFs, braided constructs were generated (Fig. 1b). Braiding CFs together enhanced their tensile strength and the process did not affect the viability of encapsulated cells.

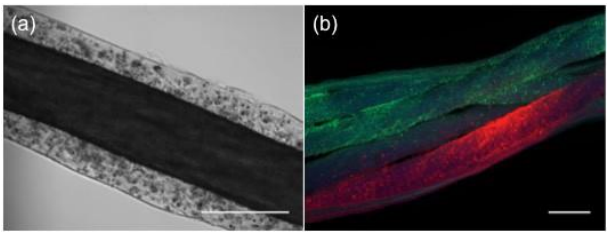

Fig. 1: Composite fibres. (a) Optical microscope images of CFs with MSCs encapsulated after 14 days. (b) Braided CFs with fluorescent beads.

DISCUSSION & CONCLUSIONS:

CFs were generated with a load bearing core and a hydrogel layer towards mimicking both mechanical properties and the matrix-rich microenvironment of tendon tissue. Accordingly, cell behaviour can be further modulated by modifying the hydrogel composition or, ultimately, through the addition of bioactive cues. Finally, braiding CFs together allows tuning the mechanical properties of developed constructs to match those of native tendon tissues.

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Scaffold-free Bio-3D Printing for Solid organ fabrication

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Fabrication of transplantable 3D tissue or organ in vitro is one of the major goals in regenerative medicine. Several scaffold-free systems have been developed to avoid potential side effects caused by scaffold mainly used to build three-dimensional tissue construct. They seemed to be still unable to produce fine structures without contamination from exogenous biochemical materials.

Inspired from bone fracture treatments in orthopedic surgery, we established a simple method to fabricate 3D scaffold-free cell construct. This method use spheroids and temporal fixator which enable placement of various types of three-dimensional cells into desired xyz positions without need of hydrogels or biochemical reactive materials. We also developed a robotic system for scaffold-free cell construction.

By using this “Bio 3D printer”, we successfully fabricated cartilage, blood vessels, liver, and so on. Also some of pile-lines are already start IN VIVO study.

Near future, we may be able to build living organs for autologous transplantation by using this scaffold free Biofabrication system. And this multi-cell construct may be useful research tools for drug development.

Biomaterial-based therapy for ischemic diseases

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INTRODUCTION:

Biomaterials are no longer considered innate structures and using functionalisation strategies to modulate a desired response whether it is a host or implant is currently an important focus in current research paradigms. Fundamentally, a thorough understanding of the host response will enable us to design proper functionalisation strategies. The input from the host response needs to be weighed in depending on the host disease condition. In addition biomaterials themselves provide immense therapeutic benefits, which needs to be accounted for when using functionalisaton strategies. Using biochemical functionalisation strategies such as enzymatic linking systems, we have been able to link biomolecules to different structural moieties. The programmed assembly of biomolecules into higher-order self-organised systems is central to innumerable biological processes and development of the next generation of functionalised scaffolds. Recent design efforts have utilised a developmental biology approach toward both understanding and engineering supramolecular protein assemblies. Structural moieties have taken a variety of different forms such as nanofibers and nanoparticulate. This approach has resulted in functionalisation of micro and nanoparticles with biomolecules that include designed peptide motifs, growth factors and a multitude of gene vector systems. In addition, nature itself has abundant structural complexity that can be harnessed for targeted clinical applications.

This talk will elucidate some of these ongoing strategies used in the EU funded AngioMatTrain project to promote angiogenesis. Therapeutic angiogenesis, which aims at inducing the formation of new blood vessels, involves a complex series of molecular and cellular interactions. The AngioMatTrain project uses a multidisciplinary approach to achieve the design of sophisticated recombinant biomaterial carriers as therapeutic delivery systems to facilitate the controlled release of optimum doses of angiogenic factors within the microenvironment. In order to achieve this objective and fully exploit the potency of angiogenic factors for therapeutic purposes while avoiding its deleterious effects, AngioMatTrain incorporates these fundamental principles in the design of novel biology-oriented therapeutic strategies.

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Biomimetic spherical polymeric devices to support tissue regeneration

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The encapsulation or attachment of cells in spherical matrices has been widely explored in cell and tissue engineering. Distinct strategies have been employed in our research group to prepare hybrid soft systems containing cells, using using natural-based polymers.

In the first strategy, biomimetic superhydrophobic surfaces have been used as a platform to fabricate spherical hydrogel beads in mild conditions. This technology permits to encapsulate living cells and other biological cargo with high efficiency. Examples are shown involving the production of polysaccharide-based beads with different sizes and with hierarchical organization [1]. Such biomaterials encapsulating mesenchymal stem cells may be used as implantable devices to regenerate bone [2]. Alternatively, in order to avoid diffusion limitation of nutrients to the cells location sites, hydrogel particles may be use to support cellular organization over their surface, acting as cells supports for injectable scaffolds. By decorating the surface with antibodies these particles are able to recruit specific cell populations, enhancing the therapeutic potential of such system [3].

In another strategy, capsules are fabricated using the so-called layer-by-layer technology, where the consecutive layers are well stabilized by electrostatic interactions or other weak forces. 3D devices may be produced that could be useful in biomedicine, including in tissue engineering and regenerative medicine. By using spherical templates containing cells it is possible to produce liquefied capsules that may entrap viable cells. The presence of solid microparticles inside such capsules offers adequate surface area for adherent cell attachment (with controlled mechanical properties) increasing the biological performance of these hierarchical systems, while maintain both permeability and injectability. The liquid environment allows for a free-organization in the space of the cells towards the formation of new microtissues. The compartmentalization of distinct cell types (including mesenchymal stem cells and endothelial cells) may enhance the osteogenic capability of this system, that could be useful in bone tissue engineering applications[4].

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Development and clinical outcomes of an autologous canine engineered skin

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INTRODUCTION: A canine engineered skin model was developed by Serra et al. in 2007 [1,2]. However, its grafting capability and usefulness in clinical practice has not been demonstrated yet. The aim of this study was to develop an autologous canine engineered skin, useful for the treatment of large, deep or delayed wounds in dogs.

METHODS:

Dogs and cell cultures: Two dogs suffering of full-thickness skin wounds were included in the present study. Canine fibroblasts and keratinocytes cultures were established after a double enzymatic digestion from a small biopsy of each dog, as previously described [2]. After culture expansion of cutaneous cells, a large surface of canine engineered skin was developed by seeding keratinocytes onto the surface of a dermis scaffold based on canine clotted plasma with embedded fibroblasts.

Graft: Dogs were anaesthetised and canine autologous skin constructs were fixed over the wound and covered with clotted canine plasma. A Robert Jones-like bandage was performed in both cases in order to fix the skin construct to the wound bed. After wound closure, biopsies from centre and edge of the wound were obtained from both dogs for histological evaluation. Biopsies were fixed in 10 % buffered formalin and embedded in paraffin wax. Sections (4 μm) were cut and stained by routine methods with haematoxylin and eosin (H&E).

RESULTS: The engineered skin successfully grafted in both patients and epithelisation was permanent in both cases (*Figure 1* shows case 1). Epithelial loss or blistering injuries were not observed. The complete healing of both cases was achieved after 25 and 40 days, respectively. Aesthetic and functional results were acceptable. Histology from the centre and the edge of the wounds did not reveal differences. A mature dermis and completely stratified epithelium were always present (*Figure 2*).

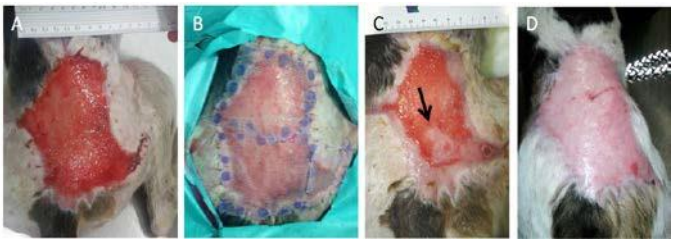

Fig 1: Skin wound before transplantation (A) and after 0 (B) and 10 days (C) of grafting. Complete healing was achieved at day 25 (D).

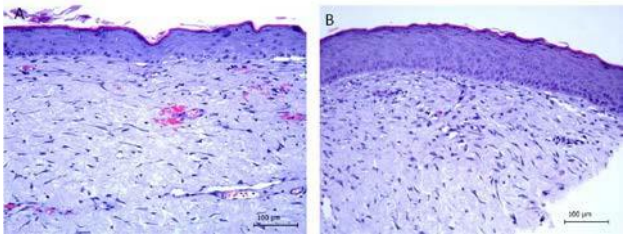

Fig 2: Histological study of biopsies obtained from the center (A) and the edge (B) of the wound after total closure (day 25).

DISCUSSION & CONCLUSION: An artificial complete skin (dermis and epidermis) has been successfully developed and used for treatment of large skin defects in dog. One of the limitations in the treatment of large skin injuries is the insufficient extent of autologous skin donor sites to cover the wound. This problem could be solved by tissue engineering. In the present study a large surface of autologous artificial skin was developed from a small skin biopsy.

Designing 3D cell niches exploiting self-assembling peptides

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INTRODUCTION: Over the past two decades, there has been significant effort to develop soft materials exploiting the self-assembly of short peptides for biomedical applications.[1] β-sheet forming peptides have been shown to allow the design of highly stable hydrogels with potential application is a range of fields.[2-5] In particular, they offer a flexible platform for the design of 3D cell niches.

METHODS: We have developed a library of understanding to design of hydrogels with tailored properties and functionalities exploiting the self-assembly of short (4-10 amino acids) β-sheet forming peptides. The design of these peptides is based on the alternation of hydrophilic and hydrophobic residues. These materials have been characterized using a variety of techniques including small angle scattering and rheology.

RESULTS: The self-assembly process of these peptides was investigated and is represented schematically in Figure 1. We are able to control the properties of these scaffolds (e.g.: modulus and functionality). by altering the primary structure of the peptide, its formulation and processing conditions. Moreover we were able to design injectable, as well as sprayable, hydrogels that can be used for 3D cell culture as well as *in-vivo* cell delivery.

DISCUSSION & CONCLUSIONS: We have developed a platform for the design of 3D scaffolds whose properties can be tailored to accommodate different cells' needs. We have used the library of novel materials we have prepared for the culture of a variety of cells including chondrocytes[5], osteoblasts, fibroblasts as well as embryonic stem cells. For the latter cell type we showed that their pluripotency is retained when embedded within the gels in 3D. We have gone on to demonstrate how to control and direct the differentiation of these cells into a number of different lineages. Our results clearly demonstrate that our peptides offer great promise for the design of specific cell niches due to their low immunogenicity and the ability we have to control and tailor their properties.

ACKNOWLEDGEMENTS: The authors acknowledge EPSRC and EU-FP7 (BIOSCENT) for funding.

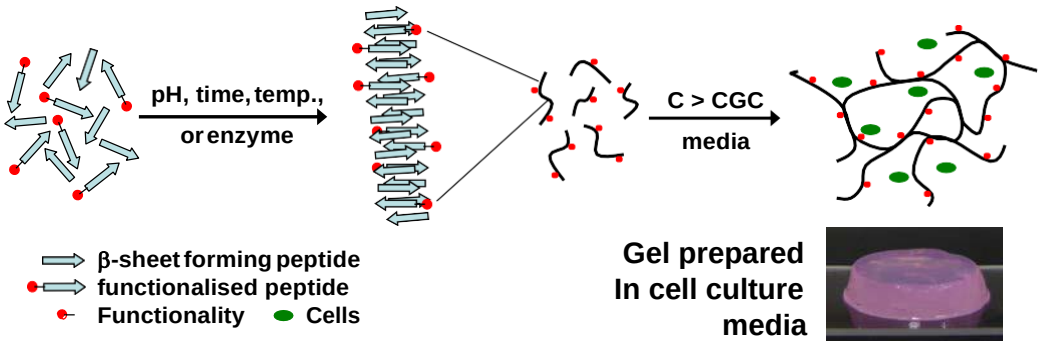

Figure 1 *Schematic representation of the self-assembly process of our peptides.*

A tissue engineered pulmonary heart valve using natural materials

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INTRODUCTION: Tissue engineering (TE) offers the potential to develop replacement heart valves (HV) that can grow and remodel with the patient; however many TE approaches have failed due to cell-mediated contraction, which prevents valve leaflets maintaining a tight seal over time. The aim of this study was to create a functional TE pulmonary HV through dynamic conditioning of a natural composite biomaterial scaffold of fibrin, collagen and glycosaminoglycan (FCG). Specifically, we aimed to (1) reinforce a fibrin gel with a porous CG matrix to create a scaffold capable of resisting cell-mediated-contraction, (2) design and validate a manufacturing method to create FCG in an anatomically relevant HV shape and (3) examine the effect of dynamic conditioning on TE of a functional pulmonary valve.

METHODS: We initially reinforced a fibrin gel with a porous CG matrix to create a FCG sheet scaffold capable of resisting this cell-mediated-contraction [1]. Creating a repeatable manufacturing process for HV shaped scaffolds was a significant challenge. Subsequently, we designed and validated a novel manufacturing process to upscale this material in HV shape. Following crosslinking to stabilise the material's mechanical properties, smooth muscle/fibroblast cells extracted from the vein of human umbilical cords were encapsulated within the FCG construct [2]. The constructs were statically cultured for 7 days, followed by 14 days of dynamic conditioning in a custom-made perfusion bioreactor which mimicked the ventricular pumping action of the heart [2]. Valve functionality was evaluated using high speed video and ultrasound; histological staining was used to assess extra cellular matrix deposition by cells. Burst strength of the construct was recorded using custom built equipment and valve performance was measured in a custom built flow loop according to ISO 5840 (Cardiovascular implants- cardiac valve prostheses).

RESULTS: The FCG-based TEHV demonstrated excellent coaptation of valve leaflets following dynamic conditioning (*Fig.1 A*). Alignment of α-smooth muscle actin and deposited collagen III confirmed appropriate remodelling of the scaffold (*Fig.1 B, C*), with staining of fibronectin showing remodelling in process throughout the construct. Analysis of burst strength showed a significant two-fold increase in both HV leaflet and HV wall tissue. This valve passed the acceptance criteria for regurgitation fraction, mean pressure difference and effective orifice area for pulmonary valves according to ISO 5840.

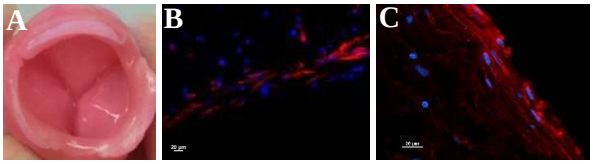

Fig.1 Heart valves (HV) following conditioning (A) showing excellent coaptation of leaflets; (B) Aligned α-smooth muscle actin; (C) Alignment of deposited collagen III within HV leaflets.

DISCUSSION & CONCLUSIONS: This study is the first demonstration that a functional pulmonary HV that achieves ISO requirements can be engineered through dynamic conditioning of a natural biomaterial scaffold of fibrin, collagen and glycosaminoglycan. As well as therapeutic applications in HV repair, this TEHV can also be used as an *in vitro* platform for drug screening, device testing or analysis of vascular cell interactions and disease states – opening up a myriad of applications.

ACKNOWLEDGEMENTS: Shane Mulderrig, Gerard Cooney, Nian Shen, the Irish Heart Foundation & the European Research Council (FP7/2007-2013).

Therapeutic potential of stem cells in spinal cord injury and ALS

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INTRODUCTION: Stem cells were investigated for their therapeutic potential in spinal cord injury (SCI), and animal models of amyotrophic lateral sclerosis (ALS). We compared human mesenchymal stem cells (MSCs) from bone marrow, a conditionally immortalized human stem cell line derived from fetal spinal cord (SPC-01) [1] and human induced pluripotent stem cell-derived neural precursors (iPS-NPs) [2] for their capacity to migrate towards lesion sites, differentiate and induce better regeneration.

METHODS: SCI was induced using a balloon-induced compression lesion in rats; ALS was studied in transgenic rats [3]. Transplanted MSCs, SPC-01 or iPS-NPs were labeled in culture with iron-oxide nanoparticles for MRI tracking. Animals were tested using motor and sensory tests. Moreover, various biocompatible synthetic or natural hydrogels have been developed for bridging tissue defects and for their use as stem cell carriers.

RESULTS: In SCI, the best functional improvement was found after iPS-NP transplantation, followed with MSCs and SPC-01. *In vivo* MRI proved that SPC-01 or iPS-NPs migrated into the lesion, where they survived for several months and differentiated into motoneurons and glia. However, MSCs rarely differentiated into neurons, but reduced the expression of inflammatory cytokines and modulate formation of glial scar.

Improved motor and sensory scores in chronic SCI were found after the implantation of biomaterials seeded with MSC or SPC-01. Recently, we also used extracellular matrix hydrogels, which facilitated neural tissue regeneration.

Transplantation of human MSCs led to improved function as well as prolongation of lifespan and decreased motoneuronal loss. Quantitative analyses of cervical and lumbar levels of the spinal cord revealed significantly greater numbers of perineuronal nets in the MSC-treated animals.

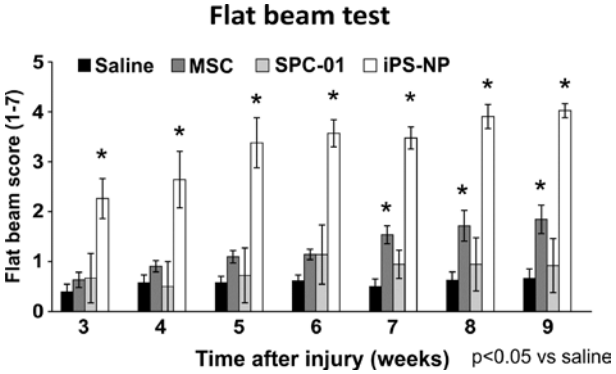

Fig. 1: In tests requiring more advanced locomotor skills, such as the Flat beam test the iPS-NP and MSC treated animals demonstrated significantly higher performances compared to the SPC-01 and saline injected rats.

DISCUSSION & CONCLUSIONS: The iPS-NP treatment of SCI led to the greatest recovery of locomotor function due to the robust graft survival, tissue sparing, reduction of glial scarring and increased axonal sprouting. On the other hand, MSCs attenuate immune reaction in both, SCI and ALS animals, which is in agreement with their supposed anti-inflammatory properties. Combination of stem cells with hydrogel scaffolds is promising strategy for bridging the lesion as well as for successful cell survival after transplantation. Cell therapy may be a new possibility to cure ALS by providing neurotrophic support to host motoneurons and/or replace dysfunctional glial cells.

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A bilayered scaffold derived from growth plate and articular cartilage extracellular matrix for osteochondral tissue regeneration in a caprine model

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INTRODUCTION: Articular cartilage (AC) is a soft tissue lining ends of bones in synovial joints. Even minor lesions affecting AC and underlying bone show poor long term endogenous regeneration capacity and can cause pain, impaired mobility and progress to osteoarthritis, a disease affecting millions of adults worldwide [1]. Despite previous attempts to repair osteochondral defects, its regeneration still remains a significant clinical challenge [2], motivating the development of new tissue engineering strategies for repair of this complex interface. In recent years there has been increased interest in the use of decellularized extracellular matrix (ECM) [3] as it may contain the structural and functional molecules needed to facilitate AC and bone repair [4]. In our lab, a bilayered decellularized ECM scaffold has been developed, which supports osteochondral-like tissue deposition *in vitro* [5]. The objective of this study was to evaluate this scaffold in a goat model for osteochondral defect repair.

METHODS: Two different ECM, AC and growth plate (GP), were harvested from porcine joints, decellularized, homogenized and freeze-dried to produce a bilayered scaffold (Fig. 1A). The scaffold was then press fit cell-free into a defect (6 Φ x 6 mm deep) within two different load bearing sites (condyle and trochlear ridge) of a goat knee for 6 or 12 months (n=8 per group). Mechanical testing, microCT and histological analysis was used to assess the repaired tissues. An empty defect and a commercial scaffold (MaioRegen™) [6] were used as controls.

RESULTS: Macroscopically, cartilage surface of ECM scaffold treated groups appeared smooth and cartilage-like after 6 months. MicroCT analysis demonstrated abundant bone deposition in osseous phase (Fig 1B). AC ECM maintained cartilage thickness while GP ECM appeared to enhance endochondral bone remodelling (Fig 1C).

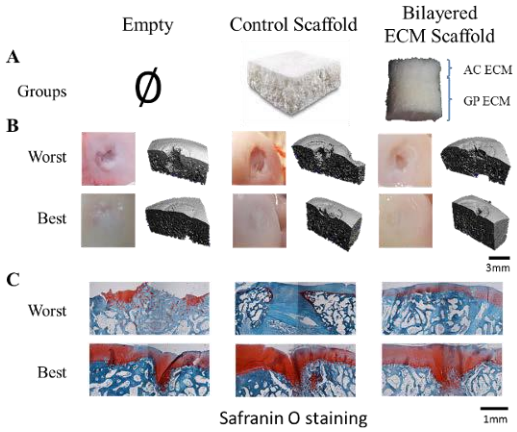

Fig. 1: (A) Scaffold macroscopic appearance, day 0. (B) Macroscopic image from the top (left) and its microCT reconstruction (right). (C) Safranin O staining: AC in red and bone in blue. Worst/best samples for empty, control and Bilayer ECM Scaffold at 6 months in the condyle defect site.

DISCUSSION & CONCLUSIONS: There was evidence of significant improved repair within ECM scaffold treated sites over controls at 6 months. Further histological analysis is currently being used to assess the quality of the regenerated tissue at 12 months. This bilayered ECM derived scaffold demonstrates potential as an effective material for osteochondral defect repair.

ACKNOWLEDGEMENTS: Surgical team in University College Dublin. Enterprise Ireland (EI), European Research Council (ERC), Irish Research Council (IRC) & Science Foundation Ireland (SFI) funding.

MSC fate following non-viral transfection with therapeutic genes strongly depends on the choice of gene delivery vector

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INTRODUCTION: Controlling the phenotype of MSCs is a central challenge in tissue engineering (TE). This could potentially be achieved *in vivo* through the delivery of regulatory genes. Essential to effective gene delivery is the choice of gene carrier. Non-viral delivery vectors have been extensively used in TE but their intrinsic effects on MSC differentiation remain relatively unexplored. Therefore the objective of this study was to investigate the influence of three different non-viral gene delivery vectors (polyethylenimine [1] (PEI), nanohydroxyapatite [2] (nHA) and RALA peptide [3]) on modulating stem cell fate after the delivery of genes encoding for chondrogenic and osteogenic growth factors.

METHODS: Porcine MSCs were transfected using PEI, nHA or RALA particles complexed to 2µg of pDNA encoding for reporter (pGFP) and therapeutic genes (pTGF-β3 and pBMP2). After assessing gene transfection and therapeutic protein production, differentiation of MSCs in 2D and in pellet culture was evaluated. Alizarin red and alcian blue stainings were performed to assess calcification and sGAG production. Immunohistochemistry was used to determine the presence of collagen type II and X.

RESULTS: All three gene carriers showed similar MSC transfection efficiencies of ~20% at day 3. Gene delivery of therapeutic factors (TGF-β3 and BMP2) resulted in effective protein production (Fig.1.A and B), with higher levels of protein produced using the RALA and PEI vectors. In spite of this, nHA-mediated TGF-β3 or BMP2 transfection promoted greater mineralization after 14 days of culture (Fig.1.C). This osteoinductive effect of the nHA nanoparticles was also evident when attempting to promote chondrogenic differentiation of MSCs, where it lead to the development of a hypertrophic phenotype as evident by collagen type X deposition (Fig.1.D). On the contrary, RALA-mediated gene delivery resulted in the promotion of a more stable hyaline cartilage-like phenotype, with strong staining for GAG and collagen type II deposition (Fig.1.D).

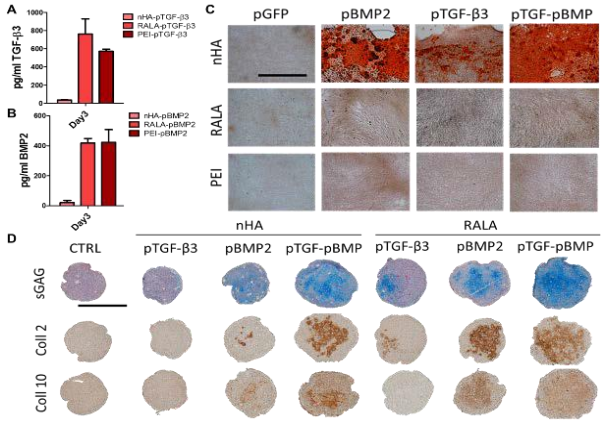

Fig. 1: MSC-mediated expression of (A) TGF-β3 and (B) BMP2 at day 3. (C) Alizarin red staining of MSCs transfected with pTGF-β3 and/or pBMP2 using nHA, PEI and RALA after 14 days of 2D culture. (D) Alcian blue (sGAG) staining and collagen type II and X immunostaining of transfected MSCs with pTGF-β3 and/or pBMP2 using nHA or RALA, after 21 days of pellet culture. Scale bars = 0.5 mm.

DISCUSSION & CONCLUSIONS: Delivery of either TGF-β3 and/or BMP-2 genes using nHA promoted robust osteogenesis and hypertrophy of MSCs. Even though transfection with both the RALA and PEI resulted in higher production of these growth factors, they failed to drive comparable levels of osteogenesis. Co-delivery of TGF-β3 and BMP-2 using the RALA peptide did however result in enhanced chondrogenesis of MSCs. This study highlights the importance of the choice of gene carrier in modulating MSC fate and demonstrates its importance for cartilage and bone tissue engineering.

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Regenerative therapies for lameness in horses

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AUTOLOGOUS CONDITIONED SERUM: Autologous conditioned serum (ACS) is an example of an available treatment that may become more targeted in the future when clearer indications are developed. It uses the idea of specific inhibition of deleterious cytokines and mediators using anti-inflammatories inflammatory substances produced by the animal's own blood cells. Controlled data on the efficacy of ACS in clinical cases are currently lacking in the horse, although in the CSU OA model, treated horses showed a significant improvement in lameness and improvement in some parameters of articular morphology[1].

The original indication for the use of ACS was degenerative joint disease that failed to respond to intra-articular corticosteroids. However, clinical impressions in horses with chronic lameness have been disappointing. Therefore ACS is used predominantly in cases of mild synovitis and early OA, particularly in patients where corticosteroids may be contraindicated. IRAP/ACS has also been suggested as a potentially effective biological treatment for tendon and ligament injury with possible anabolic effects mediated by growth factors. However, the clinical data for use of IRAP in tendon injury are lacking, and the experimental data fail to show any beneficial effect.

PLATELET RICH PLASMA: Recently, platelet-rich plasma/concentrate has gained popularity in the treatment of tendon, ligament and joint injuries. PRP contains high levels of growth factors sequestered in platelets, most notably platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-beta1). PRP can be prepared for clinical use by either centrifugation or filtration. Centrifugation systems tend to concentrate platelets less efficiently compared to filtration but are more efficient at eliminating white blood cells. Evidence of efficacy in comparison to other treatments is still lacking although some experimental work in equine tendons has shown significant improvements in tissue organisation and mechanical characteristics[2]. Evidence for the efficacy of PRP for the treatment of naturally developing performance-limiting diseases in horses is typically anecdotal or determined on the basis of results of uncontrolled clinical case series. A recent report suggests that the use of PRP in equine joints is safe although activation with bovine thrombin was considered undesirable because of a sustained increase in synovial leukcoyte concentrations.

MESENCHYMAL STEM CELLS: There has been even more interest in the potential therapeutic benefits of mesenchymal stem cells (MSCs) in regeneration of functional tendon and ligament tissue. The clinical efficacy of MSCs was recently reported in an adequately-powered large study of SDFT injuries in Thoroughbred racehorses treated with autologous MSCs in bone marrow supernatant[3]. The authors demonstrated significantly reduced re-injury rates compared to two published case series of horses with SDFT injuries, which had undergone a variety of other treatments. However, there was no contemporaneous control population and no indication of a mechanism for the action of implanted MSCs on the healing of the damaged tendon matrix. Mesenchymal stem cells have also have been used to treat joint lesions via intra-articular administration. Treatment of soft tissue injuries in the femorotibial joint with arthroscopy and concurrent intra-articular injections of MSCs has produced unexpectedly favourable results (Ferris et al. 2009)

3D printing of a calcium phosphate cement under mild conditions – strategies for growth factor loading during scaffold fabrication

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INTRODUCTION: Additive manufacturing enables the fabrication of scaffolds with defined architecture. Versatile printing technologies such as extrusion-based 3D plotting allow in addition the incorporation of sensitive biological components. We have recently presented a method to fabricate calcium phosphate cement (CPC) scaffolds by 3D plotting of a storable CPC paste based on a water-immiscible carrier liquid [1]. Scaffold plotting and hardening is carried out under mild conditions allowing the combined processing with hydrogels [2] and the precise and local integration of growth factors (GF). For GF loading, we have developed different strategies: (i) an encapsulation approach based on microparticles which can, in freeze-dried state, be mixed into the CPC paste and (ii) the combined plotting of the CPC paste with GF-laden hydrogels – either by using a biphasic or a core/shell strand scaffold design (Fig. 1).

METHODS: 3D plotting was performed using the multichannel BioScaffolder 2.1 from GeSiM (Grosserkmannsdorf, Germany). The CPC paste was provided by InnoTERE (Radebeul, Germany), alginate, gellan gum and chitosan in different concentrations were applied as hydrogels for plotting. To generate core/shell strands, self-made coaxial needles were used. Bovine serum albumin (BSA), lysozyme and vascular endothelial growth factor (VEGF) were used as model proteins, quantification of released proteins was done by Bradford assay or ELISA. Microparticle encapsulation was carried out by complex formation of the protein with dextran sulphate and coacervation of chitosan [3]. Setting of the plotted CPC scaffolds was conducted for three days in water-saturated atmosphere to prevent early loss of GF by leaching [3]. Scaffold characterization was carried out by light and electron scanning microscopy, µ-CT, helium-pycnometry and mechanical testing. For biological characterization, direct and indirect cell culture experiments with mesenchymal stromal cells (MSC) as well as human dermal microvascular endothelial cells (HDMEC) were carried out.

RESULTS: Mixing of GF into the CPC paste was possible in form of GF loaded chitosan-based microparticles in freeze-dried state – in this way immediate start of cement setting, which would compromise printing, was avoided. In case of core/shell plotting, the core material was a low concentrated hydrogel which came in contact with the CPC paste only during extrusion allowing the deposition of CPC strands containing a GF-loaded hydrogel core without adverse effects. Two-channel plotting was used to combine the CPC paste and high concentrated hydrogel pastes, loaded with GF, within one scaffold according to a biphasic design (Fig. 1A). The release profile was depending on the loading strategy: whereas small quantities were released from the microparticle-loaded and the core/shell CPC scaffolds, higher amounts especially in the initial phase where released from the biphasic scaffolds. For all three approaches, maintenance of the biological activity was proven for VEGF.

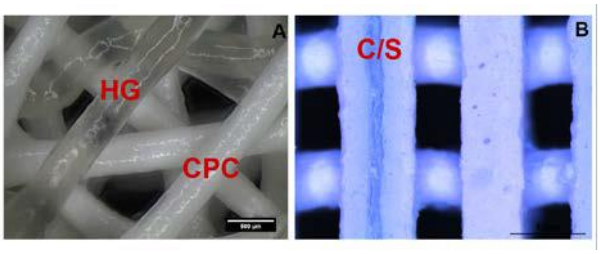

Fig. 1: ***A.*** *Biphasic scaffold consisting of CPC and hydrogel (HG) strands;* ***B****. CPC scaffold with core/ shell (C/S) strand (section through topmost layer)*

DISCUSSION & CONCLUSIONS: 3D plotting of CPC scaffolds enables not only the realization of specific geometries but also the integration of GF. We have developed different loading strategies and demonstrated their suitability.

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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 tissue engineering.

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.

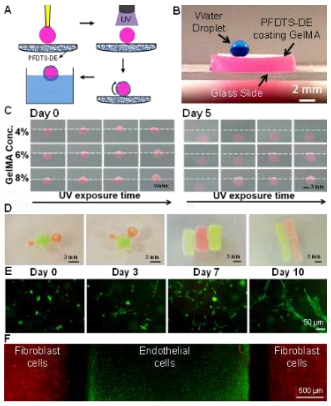

Fig. 1: (A) Schematic illustration of the method to produce the Hhydro. (B) A water droplet on top of Hhydro. (C) Floating ability of Hhydro prepared under different conditions at time 0 and 5 days. (D) Floating hydrogels self-assembling. (E) Live/dead of encapsulated cells at days 0, 3, 7, and 10. (F) 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.

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Bottom-up cell µ-aggregate assemblies for efficient endochondral bone tissue engineering

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INTRODUCTION: Current bone tissue engineering (TE) strategies are increasingly exploring endochondral bone formation via generation of a cartilage intermediate in vitro that is able to form bone in vivo [1]. However, by culturing large 3D constructs in vitro, phenotypic inhomogeneity and cell death in the constructs is observed, limiting their potential. We hypothesize that improved differentiation efficiency and bone formation in vivo may be achieved by adopting a bottom-up approach [2], where small hypertrophic modules are fused to gradually form larger tissue constructs.

METHODS: 3%w/v agarose (Invitrogen) micro-wells with a diameter of 200µm were formed using PDMS moulds. $2.5*10^5$cells/ml human periosteum derived cells were seeded in each well resulting in ~250cells/aggregate (Fig 1A). These µ-aggregates were cultured in chondrogenic medium over 4 weeks. Size, DNA, RT-qPCR and histology was analysed for every week. DAPI-Phalloidin staining was imaged with confocal microscope (Fig. 1B). ~$3*10^3$ mature µ-aggregates ($7.5*10^6$ seeded cells) were fused in a PDMS mould for 24 hours and implanted ectopically in nude mice for 4 weeks. Pellets ($7.5*10^6$ self-aggregated cells) cultured in chondrogenic medium during equal time were implanted as control. Micro-CT was used to analyse mineralized tissue.

RESULTS: Early chondrogenic gene markers Sox9, ACAN were upregulated 3 and 10-fold until week 2, after which they were downregulated 2 and 10-fold, respectively. Col II and the hypertrophic gene marker Col X were upregulated 10^4 and 10^3-fold respectively until week 3 while no significant change was seen week 4. ALP was upregulated 20-fold at 2, 3 weeks but a 10-fold downregulation was detected at week 4. Ihh was upregulated 30-fold at week 3. Alcian Blue (AB) and Safranin O (Saf.O) staining demonstrated presence and gradual increase of cartilaginous matrix over time and throughout the fused constructs (Fig. 1C). The control pellet had positive AB and Saf.O staining only at its periphery. After 4 weeks implantation, a shell-like structure of mineralized tissue was detected for the pellets while mineralized tissue was present throughout the µ-aggregate constructs (Fig. 1D).

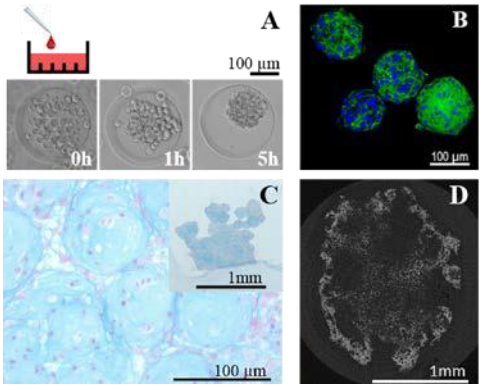

Fig. 1: Self-aggregation of single cells into µ-aggregates (A) DAPI (blue) and Phalloidin (green) stain day 14 (B). Alcian blue stain (day21) (C), and in vivo mineralization (4 weeks) (D) of the fused hypertrophic µ-aggregate construct.

DISCUSSION & CONCLUSIONS: We observed the onset of hypertrophy at week 3 based on the gene markers Col X, Ihh and ALP as well as morphologic observations of hypertrophic cells in histologic sections. At this time-point, a homogenous cartilaginous matrix was observed throughout the µ-aggregates leading to a homogenous macro-assembly. However, for the control pellet, AB and Saf.O positive matrix was limited to its periphery, a phenomenon attributed to diffusion limitations. This resulted in differences regarding to quantity and distribution of the observed implant mineralization *in vivo*. This strategy demonstrated a clear improvement in the efficiency of *in vitro* cartilaginous matrix obtained (per cell), as well as the extent of *in vivo* implant mineralization.

ACKNOWLEDGEMENTS: ERC Grant Agreements n.279100 and 249191, Research Foundation Flanders (FWO).

A 3D bioprinted bone marrow niche to study bone-residing malignancies

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INTRODUCTION: The bone tissue is intricately connected to the bone marrow, constituting a niche needed for haematopoiesis. This niche supports and regulates the haematopoietic stem cell, and is also the preferred location of several malignancies, among which multiple myeloma (MM) [1, 2].
Incoming MM cells depend on the bone marrow niche to self-renew and massively proliferate [2]. At present, these primary cells do not survive for a prolonged period of time in vitro. In order to gain better insight in the cellular and molecular interactions taking place in the bone marrow niche, there is a need for a reliable culture model. In this project we developed a reconstituted bone marrow microenvironment that can be used as a culture model, using three-dimensional (3D) bioprinting technology. The optimal composition of this bone marrow microenvironment was analysed for MM cells.

METHODS: Pasty calcium phosphate ceramic (p-CPC), Matrigel and multiple cell types were combined at varying ratios using 3D bioprinting. MSCs were used undifferentiated, adipogenically differentiated for 21 days (A-MSC) or osteogenically differentiated for 21 days (O-MSC). MM cells were traced within the culture using DiI, DiO or DiD. Cell viability was assessed at different time points. MM cells were further assessed for their expansion and migration towards specific sub niches.

RESULTS: The effect of MSCs (either differentiated or non-differentiated) on the proliferation speed of different MM cell lines was only significant when co-cultured with EPCs. For all cell lines tested, either MSCs and EPCs or O-MSCs and EPCs tested at different ratio's showed a significant expansion of MM cells (Fig.1). Interestingly, the combination of both MSCs, O-MSCs and EPCs diminished this effect. Because of this, a scaffold was designed with a separate bone department (O-MSCs on top of p-CPC) and a vascular BM hydrogel culture (undifferentiated MSCs with EPCs) (Fig.2).

DISCUSSION & CONCLUSIONS: A 3D bioprinted bone marrow niche was reconstituted, providing a tool to propagate MM cell lines as well as primary multiple myeloma cells. Such an animal-free model is valuable for the study of cell-cell interactions and signals that promote tumor growth. Further validation to show that this model is representative of the patient situation is needed, before investigating MM cell induced phenotypic changes of the bone marrow.

ACKNOWLEDGEMENTS: This study was funded by the UU Life Sciences Seed Grant.

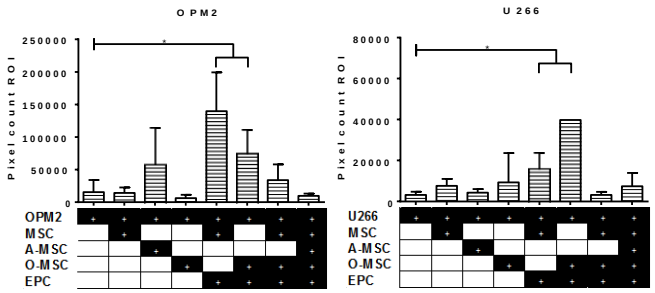

Fig. 1: *Expansion of OPM2 and U266 cells after 10 days, co-cultured in 3D with different types of supporting cells. MSC = mesenchymal stromal cell, A-MSC = adipogenic differentiated MSC, O-MSC= osteogenic differentiated MSC, EPC = endothelial progenitor cell.*

Fig. 2: *3D printed ceramic scaffolds (A), which can be functionalized with osteogenic differentiated MSCs, acting as a bone-like compartment to the soft bone marrow culture (in pink, B, C.)*

Bone regeneration of rabbit femur through the implant of a hydrogel based on novel bioactive elastin-like recombinamers (ELRs)

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INTRODUCTION: Due to the enormous number of bone injuries through the global population, there is a great interest in developing new therapies to achieve an optimal bone tissue repair. Within them, we can find biomaterial-based regenerative therapies, which include elastin-like recombinamers (ELRs), trying to improve regeneration of newly formed bone and to decrease healing time. Because of their recombinant nature, bioactive ELRs can be easily achieved [1]. In this case, an injectable hydrogel-forming ELR previously described [2] was modified to include RGD cell-adhesion sequences in one case, and the BMP-2 osteogenic protein [3] in other case, both of them including an elastase cleavage sequence for biodegradation. This mixture has been tested in a model bone defect in rabbits, showing good regenerative properties after 3 months.

METHODS: Recombinant DNA techniques were used for the synthesis of ELR-RGD and ELR-BMP-2, both of them elastase sensitive, which were expressed and bioproduced in *E. coli*. Physicochemical characterization of the material included SDS-PAGE, MALDI-TOF and differential scanning calorimetry (DSC) to determine gelation temperature (Tt). *In vitro* cell

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Nichoid substrates fabricated by two-photon laser polymerization promote maintenance of function during expansion of adult and embryonic stem cells

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INTRODUCTION: Expansion of pluripotent stem cells in defined media devoid of animal-derived feeder cells and exogenous conditioning factors, such as the leukocyte inhibitory factor (LIF), would be advantageous for many regenerative, biological or disease-modelling studies. Here we tested the possibility to use a substrate mimicking some geometric constraints of the three-dimensional (3D) native niche to maintain stem cell differentiation ability during expansion.

METHODS: The "nichoid" substrate was fabricated by two-photon laser polymerization using a zirconium/silicon hybrid sol–gel [1]. We expanded human bone marrow-derived mesenchymal stromal cells (hMSC) and R1 mouse embryonic stem (mES) cells for two weeks on the nichoid substrates, without using soluble conditioning factors. In hMSC, we evaluated multipotency by expression of CD146 and of osteogenic/adipogenic lineage commitment by Alizarin Red/Oil red O and by expression of RUNX2 and Bone Sialoprotein (BSP). In mES, we evaluated pluripotency by expression of OCT4 and lineage commitment by expression of the endodermal marker GATA-4 and mesodermal marker alpha-SMA. We used one-way ANOVA to assess statistical significance between groups.

RESULTS: In hMSC expanded in the nichoids, cell density and colony diameter were greater by 36% and 15%, respectively, than in flat-cultured controls. The expression of RUNX2 and BSP after osteogenic conditioning was greater than in flat-cultured controls. The number of adipocytes and their lipid content obtained after adipogenic conditioning were comparable to non-expanded cells. In mES cells expanded in the nichoids, the expression of OCT4 was significantly greater than in both flat-cultured controls and cells cultured on extracellular matrix obtained from de-cellularized renal tissue (Fig. 1). The expression of all the lineage commitment markers tested was significantly lower than in flat-cultured controls.

DISCUSSION & CONCLUSIONS: These results demonstrate the ability of the nichoid substrate to promote maintenance of pluri/multi potency of stem cells in expansion culture. Further studies are necessary to understand which features of the physical microenvironment of the artificial niche microstructures are critical to function. The nichoid substrate may help elucidate mechanisms of pluripotency maintenance while potentially cutting the costs and risks of xeno-conditioning for industrial-scale expansion of stem cells.

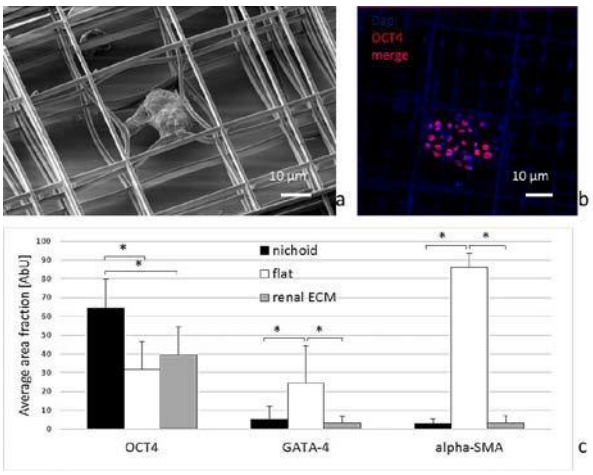

*Fig. 1: Mouse embryonic stem cells expanded in the nichoid substrate in the absence of a feeder-layer and of LIF, at 7 culture days. (a) SEM image of a cell aggregate adhering to the substrate, (b) immunofluorescence showing expression of the OCT4 pluripotency marker, (c) quantification of marker expression on immunofluorescence images; n=15, *p<0.01.*

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Cell-matrix interactions in hyaluronan-tyramine hydrogels matrix alter stem cell behaviour in 3D

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INTRODUCTION: Tyramine modified hyaluronic acid (HA-Tyr) conjugates have been introduced as scaffold materials for tissue regeneration [1,2]. HA-Tyr hydrogels are formed using horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) that generate covalent bridge formation between two tyramine (Tyr) radicals. Previous strategies of HA-Tyr synthesis achieved less than 8% Tyr substitution on HA, which limited final mechanical properties of HA-Tyr hydrogels [1,2]. The aim of this study was to increase the degree of substituted Tyr and investigate the influence of different Tyr substitutions on stem cell behavior in a 3D matrix.

METHODS: HA-Tyr was prepared by a recently described protocol using sequential DMTMM and Tyr addition [2]. The Young`s modulus of crosslinked HA-Tyr hydrogels was measured using unconfined compression testing [3]. Bone marrow derived human mesenchymal stem cells (hMSCs) (Freiburg, EK-326/08) were encapsulated in HA-Tyr (2.5wt%, $5x10^6$cells/ml) and cultured in growth medium. For CD44 blocking studies, hMSCs were treated with anti-CD44 monoclonal antibody before encapsulation. Calcein and Ethidium homodimer staining were visualized to quantify viability and aspect ratio`s of encapsulated hMSCs.

RESULTS: The Young`s modulus of crosslinked HA-Tyr hydrogels is increased with higher degree of Tyr substitution and H_2O_2 concentration (Fig.1). HMSCs in 9 kPa HA-Tyr gels with a wide range of substituted Tyr showed 87-98% viability over 4 days (Fig.2A). Interestingly, hMSC showed enhanced spreading in gels with higher degree of Tyr substitution (Fig.2B). CD44 blocking did not influence cell viability and early spreading.

DISCUSSION & CONCLUSIONS: We improved the mechanical features of HA-Tyr gels, which can still be controlled by the degree of Tyr substitution (Fig.1). Behavior of encapsulated hMSCs was altered by the degree of Tyr substitution, yet independent of CD44 mediated binding to HA at a given stiffness and H_2O_2 concentration (Fig. 2B). These findings highlight the potential of HA-Tyr to regulate stem cell fate in cell-binding peptide free HA constructs. Ongoing work is focused on identifying the mechanistic basis that may regulate MSC behavior and differentiation in HA-Tyr gels.

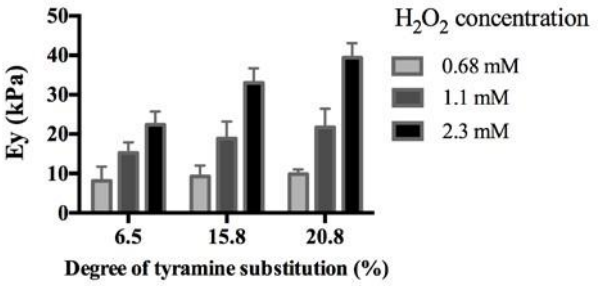

Fig.1: Young`s modulus (Ey) of HA-Tyr hydrogels as a function of tyramine substitution and H_2O_2 concentration (2.5wt%, 1 Unit/ml HRP, n=4).

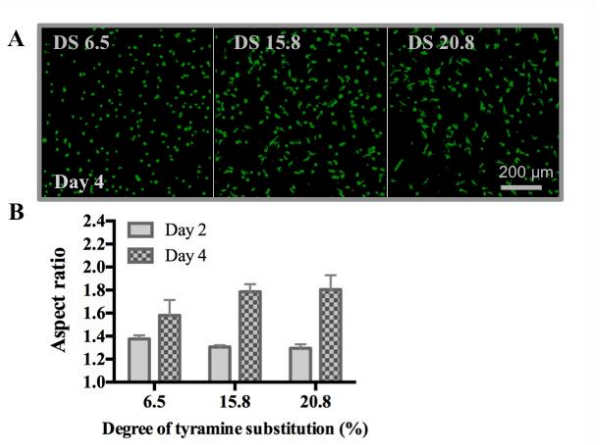

*Fig.2: **A** Live-dead images (day 4) and **B** aspect ratios of hMSCs (day 2 and 4) cultured in 9 kPa HA-Tyr hydrogels with various degree of Tyr substitution (0.68 mM H_2O_2, 2 Units/ml HRP 2.5wt%, n=3 loci, mean +/- SD).*

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A hyaluronic acid-based nerve guidance conduit for peripheral nerve repair

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INTRODUCTION: Tissue engineering has the potential to meet the demand of large gap peripheral nerve repair (PNR) and overcome the limitations of current treatments through the development of advanced nerve guidance conduits (NGC) as autograft substitutes [1]. Hyaluronic acid (HA) is a key component of the extracellular matrix of neuronal cells and laminin is found in the basal lamina of Schwann cells [2]. The overall objective of this study was to develop a NGC optimized for PNR with a luminal filler made up of HA and laminin. The specific aims of this study were to modify a HA-based biomaterial with laminin and create an NGC from it by integrating it into a collagen-based tubular scaffold as a luminal filler, to assess the biological response to the NGC both *in vitro* and *in vivo*, and to enhance its therapeutic capacity by the incorporation of nerve growth factor (NGF) and glial derived neurotrophic factor (GDNF), two neurotrophic factors which have been demonstrated to enhance PNR by synergistically enhancing the repair of both neurons and Schwann cells [3].

METHODS: X-ray photoelectron spectroscopy was used to verify the modification of the HA-based biomaterial. The NGC was fabricated by freeze drying of an outer collagen-based tubular scaffold with the HA-based biomaterial as an internal luminal filler. The biological response to the NGC was assessed *in vitro* using a Schwann cell line (S42) to assess its capacity to support attachment, a neural stem cell line (NE-4C) to assess whether it supports neurogenesis and gliogenesis, and dorsal root ganglia to assess if it supports the outgrowth of axons and Schwann cells. The performance of the NGC was then evaluated *in vivo* by assessing its capacity to repair a 10 mm sciatic nerve defect in rat. To enhance its therapeutic capacity for the repair of larger gaps, poly (lactic-co-glycolic acid) (PLGA) microparticles loaded with NGF and GDNF were incorporated into the NGC, and their release kinetics and bioactivity were determined.

RESULTS: A HA-based biomaterial was successfully modified with laminin. This modified HA-based biomaterial was seamlessly integrated into a cylindrical collagen-based tubular scaffold (Fig. 1a-c) as a luminal filler and freeze dried in order to create a NGC (Fig. 1b-d). The biological response to the HA-based biomaterial was excellent *in vitro* due to the modification with laminin: it supported enhanced S42 cell attachment, neurogenesis and gliogenesis of NE-4C cells, and the outgrowth of both Schwann cells and sensory axons from dorsal root ganglia. After 8 weeks *in vivo*, the NGC was capable of supporting the entry of Schwann cells and outgrowth of axons across the site of injury, in addition to returning functionality to the injured nerve. The bioactive release of NGF and GDNF was successfully controlled over 28 days and enhanced the outgrowth of Schwann cells and axons from dorsal root ganglia *in vitro*.

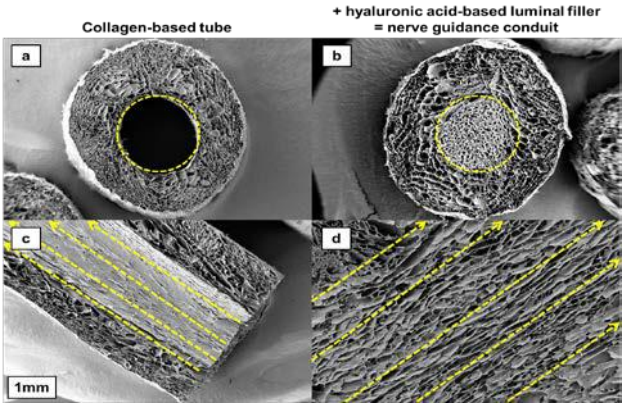

Fig. 1: Scanning electron micrographs of NGC

DISCUSSION & CONCLUSIONS: This study has led to the development of a NGC for PNR, with excellent performance both *in vitro* and *in vivo*, that potentially meets the demand of large gap repair through the tandem delivery of NGF and GDNF. This NGC marks a novel development in terms of its design, and its HA-based biomaterial represents a platform technology that may also have application in spinal cord injury.

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Engineered human-derived Kunitz-type protease inhibitor extends longevity of fibrin biomaterials

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INTRODUCTION: Fibrin is widely used in the clinic as a surgical sealant to assist hemostasis as well as tissue closure and healing. Although serving as a provisional material, a premature degradation of fibrin decreases treatment efficacy and can lead to recurrent bleeding and wound dehiscence. To prevent these complications, fibrinolysis inhibitors are often added to fibrin sealants, such as aprotinin. Aprotinin is a small Kunitz-type serine protease inhibitor derived from bovine source. In spite of its good efficacy, the xenogeneic origin of aprotinin has raised safety concerns [1]. In this study, we engineered a homologous human-derived Kunitz-type protease inhibitor domain (hKPI) from amyloid beta A4 protein precursor to prolong longevity of fibrin biomaterials and provide a competitive alternative to the use of aprotinin.

METHODS: hKPI sequences were inserted in pXLG. A cleavable his-tag was added at the N-terminus of the proteins sequences and the α_2PI_{1-8} or the $PlGF\text{-}2_{123\text{-}144A}$ domains were fused at the C-terminus. KPI variants were expressed in HEK293 cells and purified by his-tag affinity chromatography. AlexaFluor680-fluorescent fibrin gels were made as described elsewhere [2]; gels contained 10 mg/mL fibrin, 2 U/mL thrombin, 4 U/mL factor XIIIa, 5 mM $CaCl_2$, and 15 µM of inhibitors. After polymerization, gels were incubated in Tris-Buffered Saline pH 7.4, in presence of 2.5 nM plasmin. The buffer was daily replaced. Gel degradation was assessed by measurement of fluorescence using IVIS Spectrum Imaging System (PerkinElmer, Waltham U.S.). Commercial aprotinin (Roche, Basel, CH) was used as a control.

RESULTS: We assessed the ability of our engineered hKPI variants to inhibit plasmin-mediated fibrinolysis. We saw that complete degradation of hKPI-containing fibrin gels occurred after 5 days, similarly to fibrin without inhibitor. In contrast, gels supplemented with $hKPI\text{-}PlGF\text{-}2_{123\text{-}144A}$ degraded over 10 days, and those containing $hKPI\text{-}\alpha_2PI_{1-8}$ over 14 days, both overperforming aprotinin inhibitor.

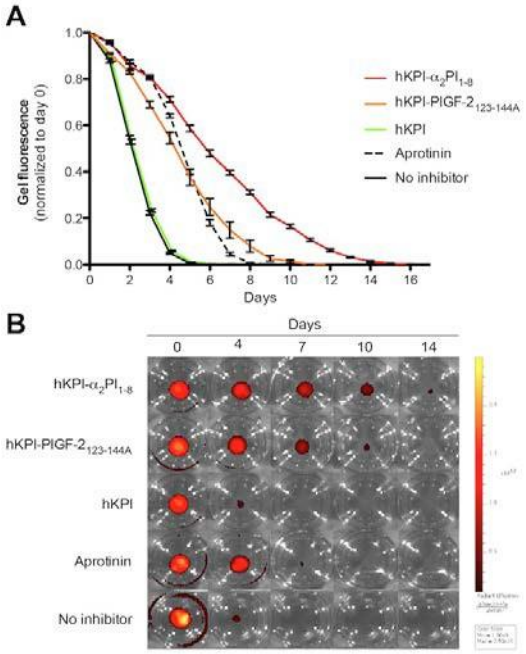

Fig. 1: Engineered hKPI variants prolong fibrin gels duration in vitro. (A) Quantification of fibrin degradation in function of inhibitors (n=4, mean ±SEM). (B) Images of fibrin gel degradation.

DISCUSSION & CONCLUSIONS: We hypothesized that hKPI engineered for increased retention into fibrin gels would have prolonged inhibitory effects on fibrinolysis. The $\alpha_2\text{-}PI_{1-8}$ transglutaminase substrate domain permits the enzymatic crosslinking of $hKPI\text{-}\alpha_2PI_{1-8}$ to fibrin [2], and so its covalently sequestration into gels. The $PlGF\text{-}2_{123\text{-}144A}$ domain displays a very high but non-covalent affinity to fibrin [3]. It also allows inhibitor retention into gels, but to a lower extent than $hKPI\text{-}\alpha_2PI_{1-8}$. Finally, the non-engineered hKPI diffuses out of the gels too quickly to offer any protection against fibrinolysis.

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For bladder tissue engineering a cell-produced extracellular matrix is more important than cells seeded within a scaffold

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INTRODUCTION: In the management of congenital and acquired bladder pathologies, needing reconstruction, bladder augmentation is commonly performed using vascularized digestive tract patches. This can result in severe complications, such as metabolic disturbances and even cancer [1]. Therefore, tissue engineering strategies using collagen-based grafts are a promising alternative. In this study, we designed experiments to address the question, if human *ex vivo* cultured cells or the human extracellular matrix (ECM) are more important for successful bladder regeneration. Since autologous cell therapies are associated with high costs, regulatory issues and added patient discomfort, an off-the shelf solution showing the same or even better tissue regeneration capacity would be a very attractive alternative for clinical use.

METHODS: We compared the bladder tissue regeneration *in vivo* of three different collagen-based scaffolds in 4 nude rats per scaffold type, with a specific focus on the cellular behavior. The three different scaffolds were: Acellular collagen-based scaffolds, collagen scaffolds with embedded human smooth muscle cells (hSMC), and "humanized" collagen matrices. The hSMC were labeled with lentiviral transduced with mCherry to being able to track the cells *in vivo* (Fig 1). The humanized collagen matrix was obtained by culturing hSMC within collagen scaffolds for 4 weeks, followed by decellularization and DNA removal. After a transverse hemi-cystectomy, the bladder was augmented with the respective collagen-based patches and the tissue regeneration capacity was analyzed four weeks post-surgery by macroscopic observations, histology and immunohistochemistry.

RESULTS: Four weeks post-surgery, all animals showed the same and normal bladder volumes as before surgery, and host cells had populated all the grafts. The cellular scaffolds and the humanized matrices were comparable, but showed significantly faster regeneration than the acellular scaffolds. Almost all human mCherry-transduced cells seeded within the scaffolds had disappeared in the scaffold and were replaced by host nude rat cells after 4 weeks.

DISCUSSION & CONCLUSIONS: Results obtained in this rat study, tell us that ex vivo cultured cells are not the most important fact from a regenerative perspective. We speculate that the scaffold's ECM is predominantly responsible, since we observed similar regenerative response in the transplanted rats if cells are present or not.

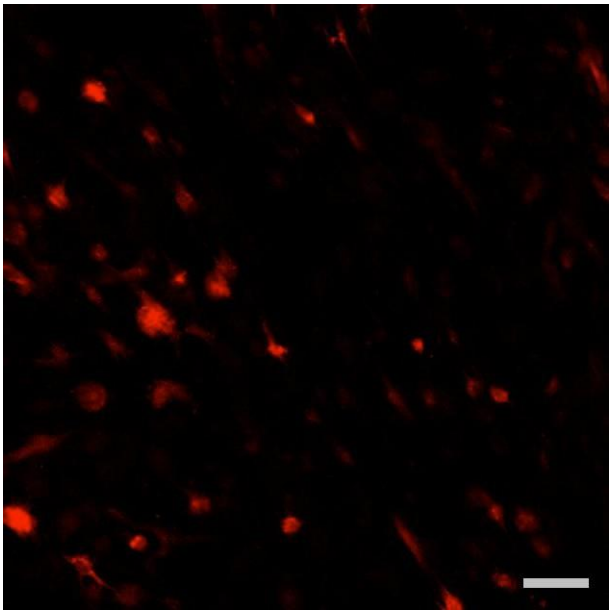

Fig. 1: Image of lenti-viral transduced mcherry-hSMC used in the study (Scale bar 50µm).

ACKNOWLEDGEMENTS: This research was supported by two CTI projects (project-14577.1PFLS-LS, 16627.1PFLS-LS)

A self-setting and injectable hydrogel for cell therapeutic treatment improves colonic radiation-induced damage

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INTRODUCTION: Healthy tissues surrounding abdomino-pelvic tumours may be impaired by radiotherapy and lead to chronic gastrointestinal complications with substantial mortality. Injection of Adipose-derived Mesenchymal Stromal Cells (Ad-MSC) represents a promising strategy for the treatment of radiation-induced intestinal lesions. However, systemic administration of Ad-MSC requires high number of cells and multiple injections due to low cell engraftment and cell death. Biomaterials that encapsulate Ad-MSC could be used to overcome these limitations. Our aim was to develop a cell-biomaterial strategy to improve the effects of Ad-MSC treatment on radiation-induced lesions.

METHODS: A silanized hydroxypropyl methylcellulose (Si-HPMC) hydrogel was synthesized and rheological measurements (viscosity, gel-point, elastic modulus (G')) were performed at a range of Si-HPMC concentrations (0.6 – 2%). SD rats were subjected to 29Gy colorectal irradiation. Three weeks after irradiation, Ad-MSC from GFP-SD rats were administered locally (1M+/-hydrogel) by colonoscopy. Seven days after injection, microscopic damage (MD), measurement of colonic paracellular/transcellular permeability (CPP/CTP), engraftment of cells, macrophages, apoptosis and proliferation assays were performed. Moreover, Ad-MSC viability was assessed on days 1, 7 and 14 *in vitro* and were analysed for secretomic molecules by ELISA assays. Statistical significance was assessed by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.

RESULTS: Rheological measurements determined that Si-HPMC at 1 and 1.5% could be injected through the specific catheter of the colonoscope. *In vitro* analyses determined that Ad-MSC were 70-80% viable within the hydrogel at 14 days and had the ability to secrete angiogenic and pro-regenerative molecules. Four weeks after colorectal irradiation of SD rats, CPP and CTP were significantly increased compared to controls (**$p < 0.01$). Treatment with local injection of Ad-MSC embedded in Si-HPMC significantly reduced colonic hyper-permeability and histological damages compared to cells injected alone (seven days after injection). Moreover, we observed, a large number of cells within the sub-mucosa following local injection with greater engraftment in Ad-MSC + Si-HPMC treated rats (*Fig. 1*). Proliferation/apoptosis assay demonstrated that cells were not proliferating but not apoptotic.

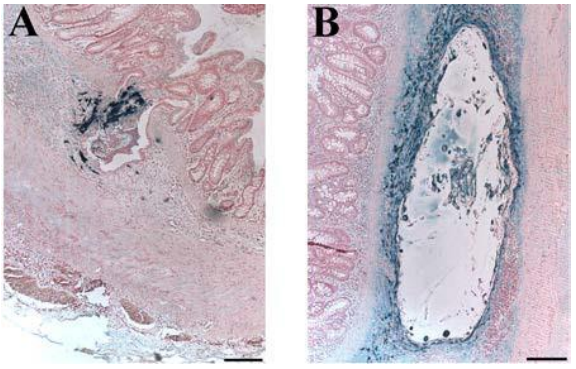

Fig. 1: Potentiation of Ad-MSC engraftment in colonic mucosa by Si-HPMC. (A) Ad-MSC engraftment in the sub-mucosa 7 days after injection and (B) Ad-MSC engraftment when embedded in Si-HPMC.

DISCUSSION & CONCLUSIONS: The combination of an Ad-MSC/hydrogel injectable treatment was able to reduce the severity of damage induced by colorectal irradiation. This approach improves the viability of Ad-MSC and allows their engraftment to the site of the lesion leading to better therapeutic benefit.

DISCLOSURES

Authors have no conflict of interest to disclose.

Engineering stem and progenitor cell microenvironments using modular designed synthetic hydrogels

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INTRODUCTION: The behavior of progenitor / stem cells is controlled by their natural microenvironment, consisting of neighboring cells, extra-cellular matrix components as well as soluble and matrix associated biological cues. It is hypothesized that the partial reconstruction of such microenvironments would be sufficient to elucidate and study stem cell functions as well as to form tissue mimetics. Synthetic materials, which are devoid of biological functions, provide an optimal basis for the rational design of microenvironments. We have earlier described the establishment and optimization of modular designed PEG-based materials [2]. Now we establish compatible growth factor immobilization and manufacturing procedures to engineer stem cell microenvironments and to create 3D tissue models.

METHODS: Building blocks (streptavidin and protein A), which are incorporated into forming PEG hydrogels and allow the creation of stable biological cues, were recombinantly expressed and purified [1-2]. Interleukine-4 (IL-4), platelet derived growth factor (PDGF), and bone morphogenetic protein (BMP) were tagged by recombinant expression or chemical modification, respectively [1-3]. Tissue progenitor and stem cells were 3D encapsulated and cultured in PEG hydrogels of different stiffness and proteolytic degradability. PEG hydrogels containing matrix-immobilized growth factors and cells were patterned using different techniques such as printing, layer-by-layer assembly or electro-chemistry. Capturing of growth factors / cytokines was determined by ELISA and western blotting. Cellular functions were determined by time-lapse microscopy, biochemical evaluations, and qPCR.

RESULTS: Hydrogel co-encapsulated MSCs and endothelial cells formed vascularized tissues which could be osteogenic differentiated, adipogenic differentiated, or kept undifferentiated [4]. Hydrogels containing affinity sites by efficiently capturing biotin or Fc-tagged growth factors could also be employed to form 3D-patterned cell-instructive matrices. Such patterned hydrogels were shown to specifically and locally stimulate cell signaling, spreading, proliferation, migration or differentiation [1-3].

DISCUSSION & CONCLUSIONS: We have employed PEG hydrogels as basis to generate various cell specific microenvironments, which control the local behaviour of cells. Such approaches have been used to study isolated tissue functions in vitro. However, the stable micro-patterning of different growth factors in specific locations holds great promise for the generation of 3D tissue models which allow to study more complex physiological and pathological tissue function in vitro.

REFERENCES: [1] M. Ehrbar et al. (2007)

ACKNOWLEDGEMENTS: Funding for this research was provided by the Peoples Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7 and the SNSF (Nos. 31003A_141051, CR2313_143766, CR3213_125426).

Modulation of growth factor signalling pathways for stable cartilage formation by adult mesenchymal stem cells

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INTRODUCTION: Mesenchymal Stem Cells (MSCs)-based tissue engineering is a promising strategy for cartilage repair. However, the propensity of the generated cartilage to undergo hypertrophic maturation when differentiation is induced by the classical TGFβ-based protocol, strongly limits their therapeutic application. During development, WNT and TGFβ signaling pathways regulate chondrogenic differentiation and hypertrophic maturation of the cells. Therefore, by using different interfering strategies, we investigate the role of WNT and the TGFβ pathways during chondrogenic differentiation of adult MSCs, with the aim to obtain a stable cartilage phenotype.

METHODS: Adult MSC were collected from bone marrow and expanded 2 to 4 passages. Silencing of the micro RNA miR-221 was performed via a siRNA-based approach prior chondrogenic induction. The WNT inhibitor IWP2 and the phospho-SMAD1/5/8 inhibitor dorsomorphin were used to modulate WNT or TGFβ pathway during chondrogenic differentiation. Chondrogenic capacity was evaluated *in vitro* (pellet culture) and *in vivo* (subcutaneous implantation of pellets [1] or osteochondral biopsies [2] in immunodeficient mice for 8-12 weeks) and monitored by RT-PCR, immunohistochemistry and micro-CT to detect undesired calcified extracellular matrix.

RESULTS: TGFβ is generally used to induce chondrogenic differentiation of MSCs [1,3], leading to hypertrophic cartilage formation. Here, we demonstrated that endogenous WNT signals and the SMAD1/5/8 intracellular TGFβ pathway are the main drivers of the hypertrophic maturation of MSC [1,3]. Although interesting this approach results laborious in a view of a possible clinical application. Then, we moved towards the investigation of a growth factor-free model to induce chondrogenesis (in absence of TGFβ). Knowing the role of miR-221 in blocking the expression of the chondrogenic transcription factor SOX9 in monolayer cultures [4], we evaluated the effect of a miR-221 silencing strategy on the chondrogenic capacity of MSCs. Interestingly, we observed that, both *in vitro* and *in vivo*, that the miR-221 silencing approach does not only induce chondrogenic differentiation of MSCs in absence of exogenous TGFβ, but also maintained very low the expression of hypertrophic markers [5].

DISCUSSION & CONCLUSIONS: Thanks to the role of miR-221 in the direct or indirect modulation of the WNT and the TGFβ pathway (Fig. 1), a silencing approach has the potential to be an innovative tool for the generation of stable cartilage, but also for targeting cells directly *in vivo*.

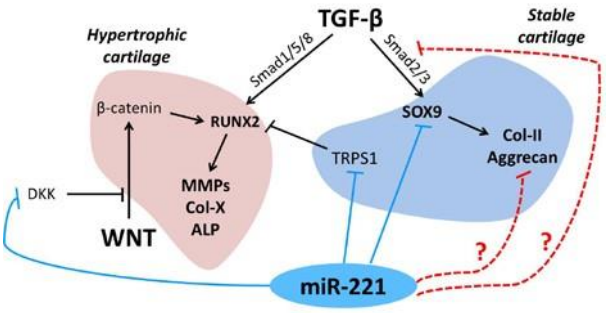

Fig. 2: Modulation of chondrogenic differentiation by TGFβ, WNT and miR-221. Black=known WNT and TGFβ interactions; Blue= known miR-221 interactions; Red=possible (unknown) interactions.

ACKNOWLEDGEMENTS: Funded by NIRM (0908), University of Ferrara ("fondo 5x1000 anno 2011") and VENI (13659).

Promoted two-photon induced cleavage of *o*-nitrobenzyl linkages in a hyaluronic acid based hydrogel by a two-photon initiator

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INTRODUCTION: The integration of **s**ingle and multiphoton cleavable linkages into hydrogel systems for 3D cell encapsulation allows for spatially defined real time manipulation of such matrices. Since intense focused fs laser pulses can provoke damage to cellular structures, two-photon (2P) induced processes in the presence of living cells need to be highly efficient to keep the impact of focused laser irradiation low. Our contribution investigates the role of the 2P photoinitiator **P2CK** [1] (*Fig. 1*) as photosensitizer in the 2P-patterning of a biocompatible hyaluronic acid based hydrogel system incorporating photo-labile *o*-nitrobenzyl (*o*NB) groups.

METHODS: Thiol functionalized hyaluronic acid (HA-SH) with a degree of substitution being 13% per repetition unit was prepared as described previously [2]. A photodegradable poly(ethylene glycol) (PEG) cross-linker with acrylate terminals was synthesized according to an established protocol [3]. HA-SH was dissolved in serum free medium and neutralized with 0.1 M NaOH to form an 18 mg/mL stock solution. The PEG-linker was dissolved in PBS giving a stock solution of 150 mg/mL. The solutions were combined in a stoichiometric ratio of functional groups and 40 µL droplets were formed.

RESULTS: A photo-degradable hydrogel system based on two orthogonal and biocompatible reactions, namely Michael addition of thiols to acrylates and radical-mediated cleavage of oNB units, was designed. Hyaluronic acid (HA) a prominent component of extracellular matrix (ECM) was chosen as backbone and functionalized with thiol groups to provide the possibility of 3D in situ cell encapsulation. To form the hydrogel a symmetrical PEG cross-linker containing photo-labile *o*NB moieties between the PEG chain and the acrylate terminals was added. After gelation (1.5 h) hydrogel droplets were swollen in serum free medium, PBS or, solutions of **P2CK** at room temperature overnight. Micro-channels were photodegraded into the hydrogels using an 800 nm fs pulsed laser beam with constant writing speed of 200 mm/s. The laser intensity was varied from 5–100 mW. The experiment demonstrates a significant improvement of 2P photodegradation at low laser intensities in the presence of **P2CK**.

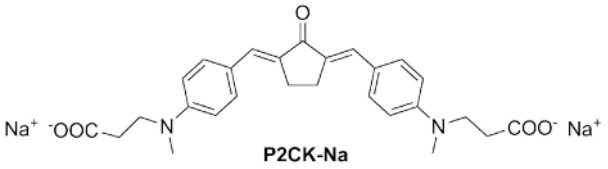

*Fig. 1: Molecular structure of the sodium salt of 2P photoinitiator **P2CK**.*

DISCUSSION & CONCLUSIONS: A novel photocleavable hydrogel system was formulated and photo-patterned. Significant decrease of the laser threshold intensity for 2P-degradation by the use of **P2CK** as photosensitizer could be demonstrated. These preliminary investigations illustrate the potential to apply this methodology in the presence of living cells.

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Vascular biology and translational medicine: from basic science to clinical applications

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INTRODUCTION: Therapeutic angiogenesis is a potentially useful strategy for the treatment of ischemic heart disease and peripheral arterial occlusive disease. It involves generation of new capillaries, collateral vessels or both in ischemic tissues using either recombinant growth factors or their genes. Regenerative processes, such as activation and stimulation of various types of progenitor cells can also be involved in the process. Mechanistically, both angiogenesis and arteriogenesis are involved, the latter of which is a process caused by increased shear stress at the arterial level resulting in the formation of large conduit vessels from pre-existing small vessels.

Most commonly used growth factors for therapeutic angiogenesis are members of the vascular endothelial growth factor (VEGF) family, fibroblast growth factors (FGFs), hepatocyte growth factor (HGF) and angiopoietins. Some other cytokines and growth factors can also have angiogenic effects.

Improved perfusion and functional parameters can be achieved by therapeutic angiogenesis in large animal chronic ischemia models and in man. Safety of the therapeutic angiogenesis in cardiovascular diseases has been excellent in human patients with long-term follow-up of up to ten years after the therapy. Most promising results have so far been obtained with direct catheter-based intramyocardial injections of VEGF-D genes with adenovirus and AAV vectors. Results from a recent phase IIa clinical trial with adenovirus-mediated intramyocardial catheter-mediated VEGF-D gene therapy in refractory angina patients will be discussed.

3D bioprinting of self-assembling hydrogels

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INTRODUCTION: Bioprinting, as a possible strategy of biofabrication, can be defined as application additive manufacturing processes for patterning and assembling living and non-living materials with a prescribed 2D or 3D organization in order to produce bioengineered structures serving in tissue engineering, regenerative medicine, pharmacokinetic and basic cell biology studies [1]. Among various techniques, robotic dispensing processes produce the most versatile fabricated hydrogel-based constructs with clinically relevant sizes, with customized external shapes and reproducible internal microstructures with engineered porosity and interconnectivity [2]. Ionic complementary peptide hydrogels represent an interesting building material as they combine biocompatibility with molecular self-assembly properties, and are tunable by varying external parameters such as pH [3].

Here we present the manufacturing process for fabrication of well-defined 3D construct using a phenylalanine-based nonapeptide, including its rheological characterization, and the FE model of the extrusion process.

METHODS: The shear rheology of FEFKFEFKK nonapeptide gel, at different concentrations and pH, was characterised using the Bohlin C-CVO rheometer. The FE model describes the fluid dynamic in the extrusion needle (both conical and cylindrical geometry) as a function of inner diameters, flow rate, pressure, concentration and pH. Simulations were performed with Comsol Multiphysics ® (COMSOL, Inc.). The PAM^2 system (Fig. 1A) was used to microfabricate complex structures [4], using a PVP/PVA hydrogel as support material. Both pressure- and piston-driven extruders were tested.

RESULTS: The gels are shear thinning, with a viscosity from kPa·s to mPa·s across a shear rate range from 10^{-2} up to 10^{3} s^{-1}. As the pH increases (from 3.7 up to slightly alkaline), the viscosity and stiffness of the gels increase: this behaviour allows the shape-retention during the printing procedure.

Extrusion experiments for printer calibration were carried out using several needles from 0.34 mm up to 0.8mm: the pseudoplastic behaviour determined a minimum extrusion flow rate (e.g. 0.4µl/s for 0.34 mm diameter) due to the high pressure drop (> 10^{6} Pa), as confirmed by FE model. Hydrogel strands extruded from smaller needles tend to swell up to a ratio 3:1 in respect to needle diameter. 3D scaffolds were fabricated by printing hydrogels directly into the supporting material, and adjusting the pH values to values higher than 6.5 (Fig. 1B). Finally, simulations indicate a piston profile of the hydrogel flow inside the needle for more than 70% of the section, thus decreasing the "shear-zone" that deeply affects vitality in cell-laden hydrogel printing.

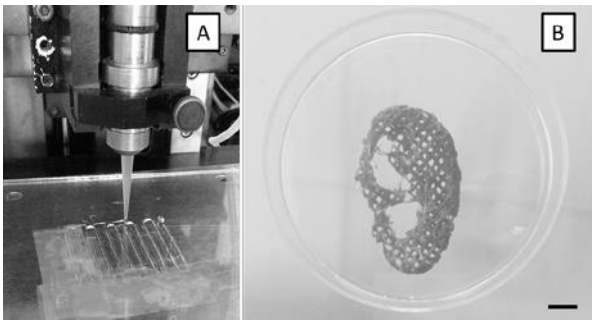

Fig. 1:fabrication snapshot using pressure-driven extruder (A); auricle-shaped scaffold, as example of clinically relevant size construct (bar 1cm) (B).

DISCUSSION & CONCLUSIONS: Bioprinting of 3D complex structures requires the accurate control of the flow rate, speed of the dispensing head, together with the appropriate choice of a suitable support material. The bioprinting of smart hydrogel with tunable properties is still an incompletely explored field of research, which can offer higher versatility for TERM applications.

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Intervertebral disc degeneration: role of migrating mesenchymal stem/stromal cells (MSCs) in extracellular matrix remodeling

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INTRODUCTION: Intervertebral disc (IVD) degeneration is characterized by loss of water content and loss of native matrix components. Simulated-degenerating IVDs were shown to release chemoattractors that can recruit human mesenchymal stem/stromal cells (hMSCs) [1]. In addition, hMSCs recruitment towards IVD can be potentiated by the injection of a stromal cell derived factor-1 (SDF-1)-delivery system in an ex vivo model of degenerating IVD [2]. Here we evaluate the potential of migrating hMSCs to repair/regenerate degenerated IVD [3].

METHODS: An IVD model of nucleotomized bovine discs was used [2]. Bone marrow-derived hMSCs were seeded on top of the cartilaginous endplate (CEP) ($1x10^6$ cells/disc). IVDs were maintained in culture during 21 days. Metabolic activity, cell viability and proliferation were assessed at day 21, as well as the presence of hMSCs in the IVD tissue. The contribution of the migrated hMSCs to IVD regeneration was assessed by the analysis of ECM main components such as aggrecan (Agg) and collagen type II (Col II) by immunohistochemistry and western blot. Collagens fibers density and organization was further analysed by transmission electron microscopy (TEM). The role of hMSCs in ECM synthesis was further complemented by the analysis of the growth factors produced during the culture using a growth factor array.

RESULTS: hMSCs seeded on the top of the CEP of nucleotomized IVDs migrated both to nucleus pulposus (NP) and annulus fibrosus (AF), and their presence and viability was confirmed after 21 days in culture, as well as the viability of IVD and hMSCs cells in organ culture. hMSCs were able to significantly increase Col II and Agg deposition particularly in NP and not so in AF of nucleotomized IVDs. Migrating hMSCs stimulated the production of growth factors namely fibroblast growth factor 6 (FGF-6) and 7 (FGF-7), platelet-derived growth factor receptor (PDGF-R), granulocyte-macrophage colony-stimulating factor (GM-CSF) and insulin-like growth factor 1 receptor (IGF-1sR).

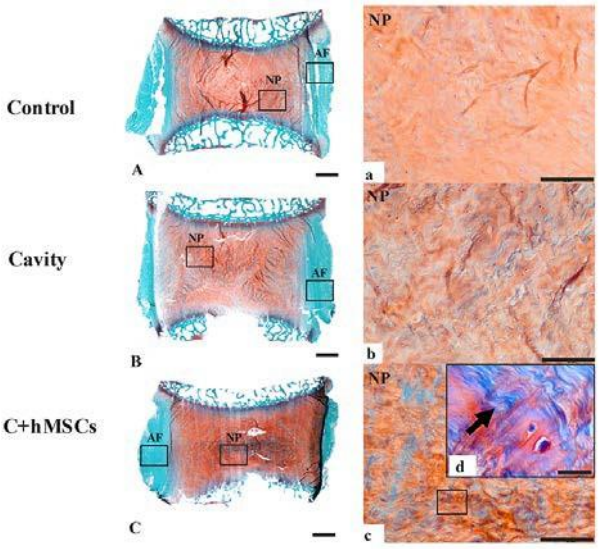

Fig. 1: Glycosaminoglycans (GAGs) deposition of IVD organ cultures with hMSCs seeded on the CEP. Safranin-O/Fast-green staining. In the first column (A-C), scale bar: 2000 μm, CEP, NP and AF regions of IVD can be distinguished. Magnifications of NP are presented (scale bar: 20 μm) in images from a-c. Collagens are visualized by green/blue (arrow) (d) color while GAGs are observed by orange color.

DISCUSSION & CONCLUSIONS: This work demonstrate that CEP can be an alternative route for MSC-based therapies for IVD regeneration through ECM remodeling, thus opening new perspectives for MSC transplantation in IVD.

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Mesenchymal stem cell-derived microvesicles are actively involved in macrophage polarization and accelerate tissue repair in a mouse model of muscle injury

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INTRODUCTION: Activated Mesenchymal Stem Cells (MSCs) have been demonstrated to secrete immunomodulatory and trophic factors that can help the host system to repair damaged tissues, providing therapeutic potential for treating conditions characterized by the presence of an inflammatory component [1]. Increasing evidences show that besides the secretion of trophic factors, the release of extracellular microvesicles (MVs) constitutes an alternative paracrine mechanism adopted by MSCs [2]. In this study, we aimed to carry out a detailed characterization of MVs released by human adipose tissue derived-MSCs, and we studied their involvement in macrophage polarization from a pro-inflammatory (M1) to a pro-resolving (M2) phenotype and their subsequent therapeutic effect in enhancing tissue repair.

METHODS: The MV-isolation method was based on repeated ultracentrifugation steps of the medium conditioned by MSC- exposed to both normoxic and hypoxic conditions (MV-normo and MV-hypo, respectively), The resulting pellets enriched in MV were characterized through electron microscopy, flowcytometry, western blot for the expression of the proteins typically present in MVs, and microarray for the expression of key microRNAs. Macrophage polarization was evaluated *in vitro*, culturing bone marrow-derived macrophages with MV-normo and MV-hypo previously stained with the fluorescent lypophilic dye PKH67, and *in vivo*, in a mouse model of cardiotoxin-induced muscle injury.

RESULTS: Culturing macrophages with MVs (normo and hypo) for 72 hours resulted in the effective uptake of MVs by macrophages (Fig. 1), in an enhanced macrophage proliferation and, more importantly, in a functional switch to a pro-resolving (M2) phenotype. The flowcytometric analysis showed that when macrophages were cultured in standard medium, they did not express the typical M2 markers, such as CD206, CD51 or CD36. Interestingly, the percentage of M2 macrophages significantly increased after the treatment with MVs from cells cultured in both normoxic and hypoxic conditions. The increase was even higher after treatment with MVs obtained in hypoxic conditions. These results were confirmed in an *in vivo* setting of cardiotoxin-induced muscle injury. The injection of both MV-normo and MV-hypo in the damaged *Tibialis Anterior* muscles induced a significant Modulation of the inflammatory response and an accelerated tissue repair, as indicated by the enhanced expression of the embryonic myosin heavy chain (eMyhc).

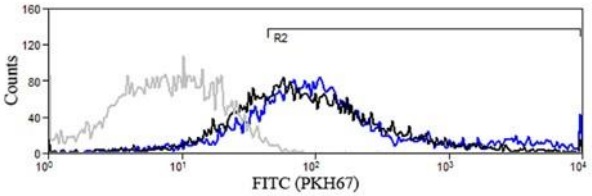

Fig. 1: Representative flow cytometric analysis of macrophages cultured for 72 hours in their standard medium (grey line), in the presence of MV-normo (black line) and in the presence of MV-hypo (blu line). The endogenous expression of the fluorescent dye PKH67 used to stain MVs is represented to evaluate MV-uptake by target cells.

DISCUSSION & CONCLUSIONS: We showed that MSC-MVs are biologically active mediators of paracrine effect. They can stimulate cell proliferation in the short term, reduce the inflammatory milieu in vitro and in vivo, and enhance muscle tissue repair. The treatment with MVs may represent a novel translational approach based on the exploitation of the paracrine potential of the MSC secretome for future therapy.

[2]

3D bioprinting of cell-laden ECM-functionalised biological implants for meniscus tissue regeneration

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INTRODUCTION: Injuries to the meniscus of the knee commonly lead to osteoarthritis. Current therapies for meniscus regeneration, including meniscetomies and cell-free scaffold implantation, fail to result in complete functional regeneration of the tissue [1]. For this reason, many researchers are currently investigating alternative methods, such as cell and gene therapies and tissue engineering approaches to meniscus regeneration. The implantation of a biomimetic implant, incorporating cells, growth factors and extracellular matrix (ECM) derived proteins, represents a promising approach to functional meniscus regeneration [2]. The goal of this project is to use 3D bioprinting to engineer a biological implant suitable for meniscal regeneration.

METHODS: ECM from different regions of porcine meniscus tissue was extracted through pepsin digestion. Subsequently, the extracted ECM was combined with alginate in order to produce a hydrogel bioink including porcine adult stem cells ($10x10^6$ cells/mL). Cell-laden ECM/Alginate bioinks were stimulated either with 10 ng/mL TGFβ3 (to induce chondrogenic differentiation) or 100 ng/mL CTGF (to direct cells towards a fibrogenic phenotype). Outer region ECM/Alginate bioinks and inner region ECM/Alginate bioinks (hereafter referred as OUT_ECM and IN_ECM, respectively) were compared to plane alginate bioinks. Finally, these bioinks were reinforced with 3D printed polycaprolactone (PCL) scaffolds to increase the mechanical property of the implants.

RESULTS: ECM extracted from the outer meniscus stained more intensely for Collagen I (Col. I), while ECM from the inner meniscus stained more intensely for Collagen II (Col. II, Fig. 1 A). Stem cells maintained in the OUT_ECM bioinks and treated with CTGF for 21 days showed an elongated morphology typical of fibroblast-like cells (Fig. 1B, bottom) and an upregulation of fibrochondrogenic genes (compared to unmodified alginate hydrogels). In contrast, stem cells cultured within IN_ECM bioinks and treated with TGFβ3 showed a chondrocyte-like rounded morphology (Fig. 1B, up). No difference in stem cell morphology was observed in unmodified alginate hydrogels.

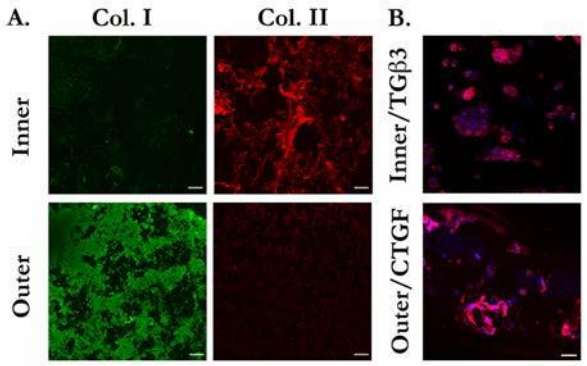

Fig. 1: (A) Immunofluorescence staining of inner and outer region ECM gels for Col. I (green) and Col. II (red). (B) To visualize the cell shape, F-actin was decorated by Phalloidin-TRITC (red). Nuclei were counterstained in Blue (DAPI). Scale bars:100 μm.

The solution containing cells, ECM and alginate, was then integrated into 3D printed PCL scaffolds and maintained in culture for 14 days. The PCL did not negatively impact on the capacity of the ECM-alginate bioinks to direct stem cell fate.

DISCUSSION & CONCLUSIONS: The results of this study demonstrate that OUT_ECM and IN_ECM functionalized alginate hydrogels are promising bioinks for meniscal tissue engineering. In addition, the combination of PCL and these cell laden bioinks can be used for developing bioprinted implants with appropriate mechanical properties for meniscus regeneration. As a next step, 3D bioprinting will be used to generate a biological implants mimicking the anatomic shape of the meniscus, with both the mechanical strength and biochemical composition to support meniscal tissue regeneration.

Composite hydrogel made of silated HPMC and chitosan for cartilage tissue engineering

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INTRODUCTION: It was believed that biomaterial selection was performed based on its macroporosity, biocompatibility and degradability properties but recently its ability to withstand biomechanical stress has also been considered. Discher et al. have indeed shown that material stiffness induces critical effects on cell behaviour and differentiation. Therefore, we have recently embarked on the development of mechanically-competent hydrogels for bone and cartilage tissue engineering. We prepared a cellulose based polymer capable of self cross-linkage (Si-HPMC) to avoid any toxicity issues resulting from using cross-linking chemicals or photo cross-linking. Therefore, LIOAD has been working on the development of hydrogels as ECM for bone and cartilage regeneration. The objectives of the present work are to modulate the physico-chemical and biological properties of our hydrogels by mixing two silated polysaccharides Si-HPMC and Si-Chitosan.

EXPERIMENTAL METHODS: The self cross-linkable polymer (Si-HPMC) was prepared according to an already published method developed in LIOAD. Silanol groups were linked to amine moieties of the chitosan via thio-urea bond.

Hydrogel characterizations: Hydrogels and reinforced hydrogels were characterized physico chemically to determine their elastic moduli using MARS rheometer (G'), Dynamic Mechanical Analysis (E') and microscopy.

Biological investigations have been done with human nasal chondrocytes by determining the cell viability in 2D using MTS, 3D by confocal microscopy after Live&Dead staining.

In vivo investigations have been performed in subcutaneous sockets in nude mice and in focal defects in rabbit.

RESULTS AND DISCUSSION: The characterizations showed that adding Si-Chitosan within Si-HPMC hydrogels increases the elastic moduli without interfering with the biocompatibility. Indeed, all the constructs that we used in this study showed no toxicity both in 2D and 3D (as shown in *Fig. 1*). Culturing cells in 3D within our hydrogel containing Si-Chitosan also induces cell adhesion onto the matrice as I can be observed on confocal micrograph in *Fig. 2*.

It is worth noted that after 5 weeks of implantation, the chondrocytes showed a good viability and still express a chondrocyte phenotype. Moreover, during the surgery, due to the presence of chitosan, our constructs showed good adhesion behaviour onto the cartilage focal defect.

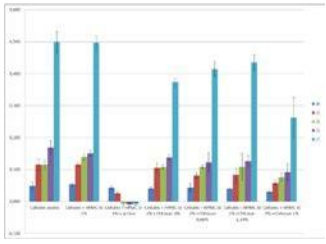

Fig. 1: 2D hASCs viability in contact with Si-HPMC/Si-Chitosan hydrogels (Positive control : cell alone, control for cell death : actinomycin).

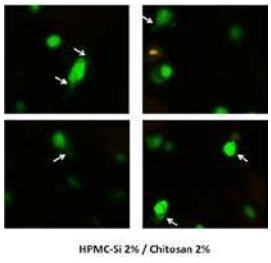

Fig. 2: Confocal micrographs showing cell adhesions after 7 days culture in Si-HPMC/Si-Chitosan hydrogel.

CONCLUSION: The study demonstrates the feasibility of our approach to modulate our self-setting hydrogel while keeping them biocompatible.

ACKNOWLEDGMENTS: We would like to acknowledge ANR (ANR-09-PIRI-0004-01) and Pays de la Loire council for their funding and technical support.

Biomaterials in cartilage regeneration - from research to clinical application

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Cartilage tissue is a anisotropic, multiphasic, and viscoelastic material with a very low coefficient of friction, which allows to withstand millions of cycles of joint loading over decades of wear. The unique composition and the highly organized structure of articular cartilage are key elements for its complex mechanical properties. However, upon damage, cartilage tissue has a low self-regenerative capacity due to its hypocellular, aneural, and avascular structure. Therefore, the healing of articular cartilage defects remains a significant clinical challenge.

The field of cartilage tissue engineering as a method of treatment for damaged tissue has developed over the last twenty years and a plethora of biomaterials have been proposed to deliver devices for cartilage regeneration. Biomaterials for cartilage regeneration have been developed for both, to improve the clinical outcome of existing therapies, like the autologous chondrocyte implantation, and to enable new therapies. But despite extensive efforts to develop novel biomaterials and biological solutions for cartilage regeneration, only few have been successfully translated into the clinical routine application.

The development of the most recent generation of biomaterials is strongly influenced by the increasing knowledge of the anatomical and structural complexity of articular cartilage. Combined with technological advances in the field of material sciences, biomaterials with many desired properties can now be created with unprecedented precision and versatility. The increasing capacity to design and synthesize materials with molecular resolution opens new path in biomaterial development. In addition to being biocompatible and biodegradable, biomaterials can now be bioactive, biomimetic, and bioresponsive, providing signaling with spatio-temporal control and response that is selective to defined stimuli. Such biomaterials, which can ideally be implanted using minimally invasive procedures are object of many studies.

The presentation will give an overview of biomaterials currently used in the clinic, and the technical and medical requirements for such biomaterials. Regulatory aspects will also be addressed as hurdles in the transition of biomaterials from the laboratory into the clinic. Current trends and some innovative strategies for the development of next generation biomaterials will be presented.

In the second part of the lecture the focus is on our own development of two biomaterials, which are already successfully used in clinical applications: a biphasic solid collagen scaffold and an injectable, in situ polymerizing hydrogel. Main considerations of our development strategy will be shown, and some pre-clinical and clinical results will be discussed.

Polymer surface mobility controls hMSC fate

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INTRODUCTION: Cells are highly responsive to their surrounding and desired effects e.g. better implant integration can be achieved by fine-tuning surface properties. Cellular receptors including integrins interact with surfaces via specific structural sites on extracellular matrix (ECM) proteins e.g. fibronectin, some of which can bind growth factors (GFs) thus causing synergistic downstream signalling. Here we show that surface mobility, a poorly understood material property significantly affects cell behaviour. We use a unique set of four polymers – Poly(methyl-, ethyl-, butyl- and hexyl- acrylate), i.e. PMA, PEA, PBA and PHA – with similar surface properties except for their increasing mobility, determined by the decreasing glass transition temperature as the length of the side group increases. Three of these surfaces have been shown to induce spontaneous formation of nanonetworks of fibronectin (FN) fibrils similar to those assembled in vivo.[1] We show that the mobility of our surfaces translates to the protein layer which in turn affects the presentation of important integrin binding sites producing a non-monotonic effect on cell differentiation.[2] We then utilize FN domains to bind bone morphogenetic protein-2 (BMP-2) on the polymer surfaces and present it to human mesenchymal stem cells (hMSCs). We evaluated the osteogenic induction by these surfaces, and to dissect intracellular signalling processes we analysed cell adhesion and differentiation in the presence of inhibitors.

METHODS: Polymer film production: Solutions of bulk polymers (polymerized with 1 wt% benzoin) in toluene were prepared, spin-coated onto glass cover slips and vacuum dried at 60°C.

Surface Characterization: Spin-coated films were analysed by AFM, WCA and ELISA to characterize surface roughness, stiffness and thickness, fibronectin adsorption, exposure of integrin binding sites and BMP-2 binding on adsorbed Fn.

Cell Culture: hMSCs were cultured on FN coated polymers for up to 4 weeks, in the presence and/or absence of GFs; BMP-2 and inhibitors of; Rho-associated coiled-coil containing protein kinase (ROCK), ERK1/2 phosphorylation and myosin II.

RESULTS: The AFM and WCA results confirmed the physicochemical similarity between the four surfaces while ELISA results indicated increased exposure of the cell binding (RGD) and synergy (PHSRN) sites on FN networks compared to the globular fibronectin found on the least mobile polymer. Osteogenic markers were most expressed on the surface with 2 methyl groups in the side chain (PEA), this surface was also least affected by contractility inhibitors.

We hypothesize that this surface promotes BMP-2 receptor/Integrin co-localisation.

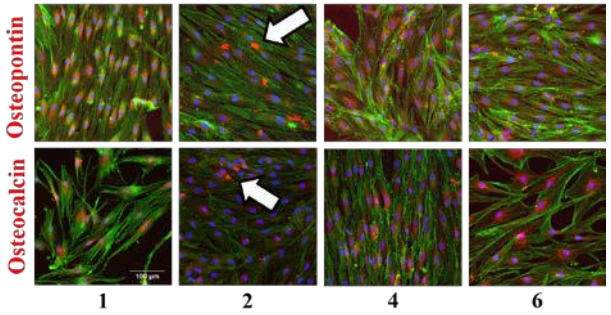

Fig. 1: hMSCs cultured on polymers for 21 showing bone markers; osteocalcin and osteopontin nodules (red). Numbers 1-6 represents the number of methyl groups in the polymer side chains (1:PMA, 2: PEA,4: PBA and 6: PHA).

DISCUSSION & CONCLUSIONS: Surface mobility influences the adsorption, conformation and mobility of adsorbed FN, affecting the exposure of ECM sites and GF presentation both of which are crucial for modulation of cell behaviour and differentiation. The strength of this study is that it highlights the potential to use polymer surface mobility as a tool to further modulate cell behaviour and more specifically PEA as a potential coating for biomaterials to enhance osteointegration.

The influence of defined immune cell subsets on the bone healing process

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INTRODUCTION: Bone healing remains a clinical challenge with up to 10% of delayed or disturbed fracture healing situations. Recently the interconnectivity of the skeletal and the immune system became a topic of interest and osteo-immunological research has proven that immune cells are essential for a successful bone healing process [1, 2]. However not all immune reactions are beneficial, as for example CD8+ T cells were found to delay bone regeneration [3]. Hypothesis was, that bone healing could be accelerated by distinctly altering the initial inflammatory reaction.

METHODS: Wildtype (WT) and Rag1$^{-/-}$ (mice without mature B and T cells) mice were used to investigate the relevance of immune cells for the bone healing process. A mouse osteotomy (0.7 mm gap) was stabilized with an external fixator (RISystem) on the left femur. Healing was monitored over 21 days. The initial shift from pro- to anti-inflammation in the healing cascade was consecutively altered through adaptive cell transfer, depletion, or cytokine treatment (using regulatory T cells, aCD25, IL-4/IL-13). Healing was monitored using µCT, biomechanical testing, FACS, histology and immune histology. Animal experiments were carried out according to the Animal Welfare Act after approval of the local legal representative (Landesamt für Gesundheit und Soziales Berlin).

RESULTS: Comparing WT and Rag1$^{-/-}$revealed differences in the bone quality with a stiffer bone less capable to withstand impact in the animals without B and T cells. This highlights the importance of immune cells for the bone forming process. Apparently, immune cells play different roles during bone regeneration and can either hinder or help bone formation. While CD8+ T cells hinder healing[3], regulatory T cells support the healing process, as we could show by adaptively transferring freshly isolated natural regulatory T cells prior to osteotomy (µCT: WT vs. Treg+: BV p=0.015; BV/TV p=0.002). Depleting activated T cells using a depletion antibody against CD25 did not enhance bone healing as both beneficial (Treg) and detrimental (CD8+ T cells) cells were missing. However, steering the initial immune reaction towards an M2/Th2 phenotype using cytokines and thus terminating the pro-inflammatory response early on, enhanced bone formation in our mouse model (µCT: WT vs IL4/IL13: CV p=0.009; BV p=0.03).

DISCUSSION & CONCLUSIONS: The distinct switch from pro- to anti-inflammation offers promising potentials for new treatment approaches in bone healing. However, the complexity of the interaction between the skeletal and immune system harbours the danger to alter bone homeostasis by addressing the immune system. Since the bone and immune system share progenitors and signalling molecules, this has to be carefully considered. This work shows that a distinct modulation of the M2/Th2 switch can be beneficial in bone healing in order to enhance the regenerative capacity of bone. A more in depth understanding of the interdependency seems essential at this point, particularly with respect of the changing immune cell composition during aging. Elderly people present a chronic more pro-inflammatory environment due to their aged immune system and also show a higher incidence for fractures and delayed healing. Stable approaches in modulating the inflammatory cascade to a fast anti-inflammation will help to stimulate healing even in compromised situations.

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A shape-controlled tunable microgel cell delivery platform for low-dose delivery of primed stem cells for in vivo therapeutic neovascularization

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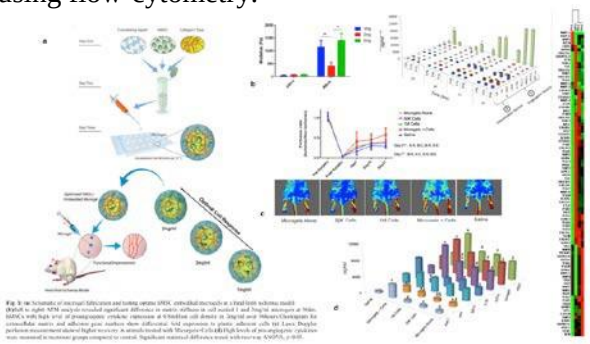
INTRODUCTION: Hydrogel-based cell delivery platforms fabricated from synthetic or natural polymers have evolved from a cytoprotective design to a functional biomaterial design. During natural tissue regeneration, cellular microenvironment and soluble cues influence reparative mesenchymal stem cells (MSCs). Prolonged ischemia and lack of blood supply drive cellular apoptosis and tissue death. However, delivery of primed MSCs via a tunable microenvironment triggers an 'angiocrine' response, driving therapeutic angiogenesis[1]. Hence, in this study, it is hypothesised that delivery of primed MSCs on a shape-controlled microgel platform at a low-cell dose promotes angiogenesis in a murine model of hind-limb ischemia (HLI). The specific objectives of the study were to investigate the cellular behaviour on tunable parameters such as matrix concentration, cross-linker concentration and cell densities and therapeutic efficacy of the optimized group in a hind-limb ischemia model.

METHODS: Collagen-based microgels of concentrations 1, 2 and 3mg/ml were fabricated by neutralizing type-I collagen and dispensing a mixture composed of cells and cross-linker 4S-Star-PEG Mw 10000 KDa (Jenkem Tech, U.S.A) on to a hydrophobic surface. hMSCs at an optimized cell density of $0.8x10^6$ were embedded within the microgels[2]. Cell embedded microgels were tested for changes in morphology by confocal microscopy; protein and gene expression quantitation by multiplex ELISA and gene arrays; and changes in matrix stiffness with atomic force microscopy and confocal microscopy. *In vivo* experiments were performed in a hind-limb ischemia mouse model. Animal groups (n=12/group) were PBS; microgels alone; microgels with 50,000 hMSCs; 50,000 and 1,000,000 cells alone. Laser-Doppler perfusion and clinical signs of disease severity were assessed along with molecular evaluation using; multiplex ELISA, gene expression arrays and MALDI-imaging mass spectrometry. Statistical analysis was performed using two-way ANOVA with $p<0.05$.

RESULTS & DISCUSSION: The use of 4S-StarPEG combined with the high surface hydrophobicity allowed gelation after 40 minutes, conferring a spherical shape to the microgels. Over 80% viability and low apoptosis was observed using flow-cytometry.



2mg/ml microgels at $0.8x10^6$ cell density showed up-regulation of paracrine factors involved in angiogenesis and distinguishing integrin expression profile (Fig.1) AFM analysis revealed insignificant changes in matrix stiffness and unique cell morphology in 2mg/ml microgels compared to 1 and 3mg/ml microgels. Results from the *in vivo* hind-limb ischemia studies showed increased blood perfusion, up-regulation of protein and gene markers related to angiogenesis, and changes in glycan environment between the treatment and control groups.

CONCLUSIONS: Tunable shape-controlled collagen based microgel platform can be used to deliver hMSCs at a low cell dose for angiogenesis *in vivo*.

ACKNOWLEDGEMENTS: Science Foundation Ireland (SFI) co-funded under the European Regional Development Fund under grant no 13/RC/2073. SFI under grant no.09/SRC/B1794.

The role of cell traction forces during tissue growth in microvoids

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INTRODUCTION: Tissue defect regeneration after traumatic injury can be induced by soft macroporous biomaterials that provide a guiding structure for the endogenously synthetized extracellular matrix (ECM). Such biomaterials represent promising candidates for tissue regeneration. However, the processes of endogenous ECM formation and organization inside such structures are poorly understood. Interestingly, we previously found *in vitro* an accumulation of fibronectin in the centre of the pores rather than on the pore walls where tissue growth had started [1]. Void filling is considered to be a mechanism of tissue formation inside the pores by layer on layer growth based on cell proliferation [2]. In this work we show that dynamic tissue movement resulting from cell traction forces plays a major role in microvoid filling and ECM organization.

METHODS: Primary human dermal fibroblasts (hDFs) were seeded in a 2½ D grid system and 3D porous structures (pore size: 100 to 550 µm) prepared from polydimethylsiloxane (PDMS). Cells were cultivated in DMEM (10% FBS, supplemented with ascorbic acid). Once the desired microvoid closure was achieved, 100 µM of blebbistatin were supplemented to inhibit cell cytoskeleton contractility. Consequent changes in open microvoid area and tissue geometry were captured by time lapse microscopy for 4 hours. Proliferative cells, actin cytoskeleton, secreted fibronectin and fibrillar collagen distribution in the tissue were also analysed by confocal and multiphoton microscopy. Finally, microtissue stress state distribution was characterized by AFM.

RESULTS: Tissue relaxation compared to the initial closed area after blebbistatin treatment was independent of the stage of microvoid closure but was more pronounced in higher curvature areas. Proliferating cells were found in regions distant from the tissue edge. AFM data showed significantly higher stiffness at the tissue edge indicating higher cell-induced tension compared to the bulk tissue. Finally, microtissue formation in the 3D porous structures revealed cells initially adhered to the pore wall, gathering towards the centre of the pore in later stages of tissue growth.

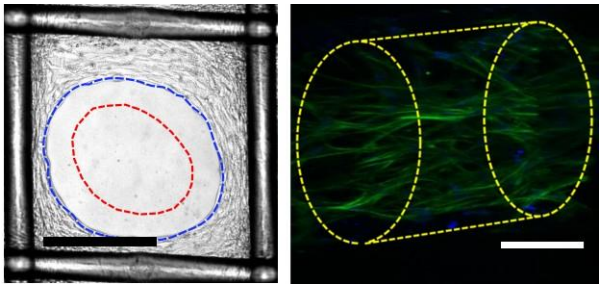

Fig. 1: Initial open area defined by the tissue edge (red dashed line) and open area after blebbistatin treatment (blue dashed line) in 2½ D (left). Cell organization inside 3D pores after 7 days. Pore edge (yellow dashed line), actin (green) and nuclei (blue). Scale bars 250 µm.

As a consequence of centripetal cell movement, collagen fibres are accumulated in the centre of the pore and aligned along the main axis.

DISCUSSION & CONCLUSIONS: The present work highlights the role of cell traction forces as a core element of microvoid closure and ECM organization. The ECM accumulation in the centre of the pores cannot be explained by layer on layer tissue formation processes but rather by a material transport driven by centripetal tissue contraction. Such contraction of the tissue is a result from individual cell traction forces. Similar observations were made *in vivo* in a bone defect model showing more mature ECM (fibrillar collagen) in the centre of scaffold pores rather than in the periphery. The understanding of the spatial and temporal organization of the ECM is of special relevance since it could be utilized to structurally guide and enhance endogenous tissue regeneration in critical sized defects.

ACKNOWLEDGEMENTS: This work was supported by the German Federal Ministry of Education and Research (grant no. 13N1215.).

Fibronectin-based biointerfaces to control stem cell fate: nanotopographies, protein mobility, self-assembly and growth factor binding – *looks matters*

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INTRODUCTION: Cells don't interact directly with synthetic materials but with a surface layer of proteins which provide anchoring points to establish a biological cell/material interaction. Within this context, material properties have been tuned seeking to control stem cell differentiation. From stiffness to nanotopography, materials have been engineered to control behaviour. Yet, cells will only feel these properties through this intermediate layer of proteins. Here we show material-based strategies to control fibronectin (FN) presentation, as a robust and translational way to develop tissue engineering constructs. We will show examples of the interaction of FN with nanopits topographies, protein surfaces which control the mobility of FN at the material interface as well as chemistries which promote FN-FN interactions and the organisation of FN at the material interface. This last strategy constitutes a translational way to incorporate highly efficiently growth factors at very low doses.

METHODS: Nanotopographic pits were imprinted on polycarbonate by injection moulding using electron beam lithography master substrates 150 nm diameter pits of 100 nm depth and 300 nm pitch. Polymer sheets were obtained by radical polymerization methyl (MA), ethyl (EA), butyl acrylate (BA) and hexyl acrylate. Fibronectin adsorption was characterised in terms of surface density (BCA) as well as organisation, distribution and conformation using AFM and ELISA with monoclonal antibodies. Labelled FITC-FN was used to quantify FN mobility at the material interface. FN was photo-bleached and then recovery measured as a function of time. BMP-2 binding to self-assembled FN on PEA was quantified and correlated with stem cell differentiation *in vitro* and bone regeneration *in vivo*, using a non-healing defect in the mouse radius. Human Mesenchymal Stem cells (Promocell) were characterised for osteogenic markers using immunofluorescence, qPCR and ALP expression.

RESULTS: It is known that nanopit topographies control stem cell differentiation. Simply changing order and disorder of nanopits leads to ostegenesis or maintenance of stem cell phenotypes [1]. Here we show that FN is indeed adsorbed into nanopits and that cells bend their membranes seeking to reach the botton of the peak, a phenomenon called nanoimprinting. Also, that this happens in a cell-contractile dependent manner (Fig. 1A).

We have engineered surfaces that promote the organisation of FN into fibrillar nanonetworks, in a conformation that promotes integrin binding and GF binding, to promote synergistic signalling. We have shown that BMP-2 can be bound FNIII12-14 and that this promotes efficient stem cell differentiation in vitro. When, the system was implanted in a non-healing bone defect in the bone radius, it led to full regeneration and bridging of the defect with minimal doses of BMP-2 (~ 15 ng)[2].

As mentioned, PEA organises FN into fibrillar nanonetworks. We have shown that by increasing the length of the polymer side group, moving from PEA to PBA and PHA, FN is still organised into fibrillar nanonetworks but with increased mobility, which facilitates protein reorganisation as well as cell differentiation [3] (Fig. 1B).

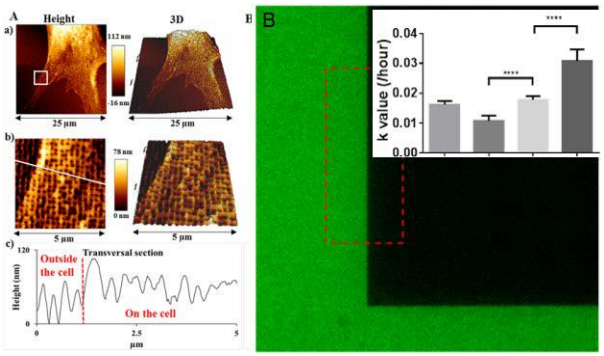

Fig. 1: A) Nanoimprinting of cells on nanopits reaching FN at the botton of the pit. B) Labeled FN was bleached and the rate of recovery of the interface followed to measure FN mobility

DISCUSSION & CONCLUSIONS: Materials control FN conformation and organisation to control stem cell differentiation and tissue repair.

ACKNOWLEDGEMENTS: Financial support from MRC (MR/L022710/1) is acknowledged.

Multilayers of poly-L-lysine and hyaluronic acid assembled on ordered nanostructures influence mesenchymal stem cell differentiation

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INTRODUCTION: Topographical and mechanical signals are important regulators of cell behaviour in body tissues. The presented work aims to design a unique system with variable viscoelastic and geometric parameters. We show here for the first time that nanostructures fabricated by laser interference lithography (LIL) subsequently modified with a multilayer system assembled by the Layer-by-layer (LBL) technique can be used to control stem cell differentiation by synergistic mechanical and topographical stimuli for potential applications in regenerative medicine.

METHODS: Hexagonally arranged gold nanostructures with different dimensions and periods were produced by LIL using different angles of incidence (14°, 20°, 36°). After passivation of the background surface with poly (ethylene glycol) (PEG), multilayers of poly-L-lysine (PLL) and hyaluronic acid (HA) were spray-coated onto the nanostructures to design a heterogenic system with specific properties. Multilayer viscoelasticity was adjusted by crosslinking using 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS). Thereby, the EDC concentration was varied to achieve different E-moduli. Native and modified nanostructured surfaces were analysed by atomic force (AFM) and scanning electron microscopy (SEM) as well as characterized by water contact angle (WCA) measurements. Finally, adhesion, growth and differentiation of human adipose-derived stem cells (hADSC) were studied in order to gain knowledge on the influence of nanostructures with distinct mechanical properties on the cellular response. Immunocytochemistry was used to visualize signal transduction markers as well as cellular structures applying confocal laser scanning microscopy (CLSM).

RESULTS & DISCUSSION: Pristine hydrophobic nanostructured surfaces became hydrophilic upon adsorption of PLL and HA forming multilayers. AFM studies using colloidal probes on planar surfaces revealed that the highest EDC concentration used for cross-linking resulted in the highest E-moduli. Further, we found that the nanostructures were still detectable with AFM after multilayer coating resulting in distinct surface features. Adhesion and proliferation of hADSC were clearly influenced by the size and spacing of nanostructures. A period of 518 nm turned out to be favoured by the cells with increased spreading. Further, the spacing of the structures had also a remarkable effect on the orientation of cells with more elongated cells on the smallest structures. Focal adhesion kinase (FAK) as an indicator of mechanical stress applied to the cytoskeleton as well as cell adhesion marker was found predominantly in the periphery of hADSC, but primarily linked to the nanostructures. The small GTPase RhoA was uniformly distributed within the hADSC without obvious dependence on the feature dimensions. Investigation of the differentiation potential of the modified nanostructures revealed that adipogenic differentiation was increased on larger structures, while osteogenic differentiation was improved on smaller structures, both on layers with lower cross-linking degree due to differences in spreading and cell cluster formation.

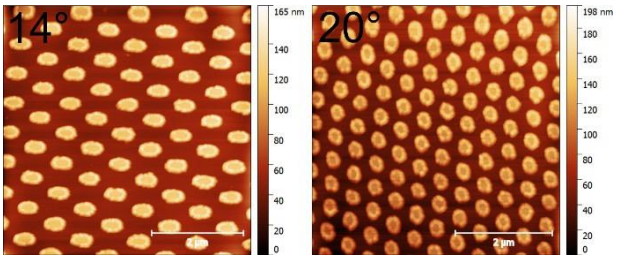

Fig. 1: AFM images of gold nanostructures on silicon obtained by LIL at an angle of 14° (left) and 20° (right). [Scale: 2 µm]

CONCLUSIONS: The LIL method presented here is a promising new technique for reproducibly generating nanostructures on relatively large areas. In combination with viscoelastic surface coatings it can be used in the future to control the differentiation of mesenchymal and other stem cells.

ACKNOWLEDGEMENTS: The authors grate-fully acknowledge the financial support partly by the Marie-Curie-Industry-Academia Partnerships and Pathways (FP7-PEOPLE-2012-IAPP) as well as the German Research Society (DFG) through Grant GR 1290/10-1.

Allogeneic platelet rich plasma prompts monocytes differentiation to M2 macrophages suppressing the inflammatory response

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INTRODUCTION: Platelet Rich Plasma (PRP) has been used as a "natural" biomaterial enhancing tissue regeneration by mimicking a physiological process represented by the blood clot formation which is the starting point of the wound healing cascade. In regenerative medicine the PRP is usually used to lead to a complete damage repair due to the physiological concentration of cytokines and growth factors which are responsible for the creation of an inflammatory burst, accelerating the healing. In some patients, such as diabetics, the use of an autologous PRP is not favourite. For this reason, we investigated the effects of allogeneic PRP on the immune response, focusing our attention on the differentiation of peripheral blood monocytes to dendritic cells (Mo-DC); these antigen presenting cells (APC) play an essential role starting the adaptive immune response.

METHODS: in vitro, standard Mo-DC were obtained from peripheral blood with GM-CSF and IL-4 [1]. The PRP effect was analyzed on the differentiation of the monocytes to immature DC, production of cytokines using MagPix array and skewing Th (T Helper) cells to regulatory T cells (T-Reg).

RESULTS: Mo-DC were characterized by the expression of CD1a and loss of CD14 and from the capacity to induce Th1 expansion in mixed lymphocyte reaction (MLR). By contrast, monocytes differentiated in presence of PRP (Mo-PRP) expressed features of type 2 macrophages (M2) both for the morphology that for the expression of specific markers such as CD206 and CD163, production and release of anti-inflammatory cytokines such as IL-10 and PGE2 (Fig 1) and the selective expansion of CD4+ T-Reg cells in MLR experiments. These T-Reg cells were characterized by the expression of high level of CD25 and FoxP3 (Fig 2).

1

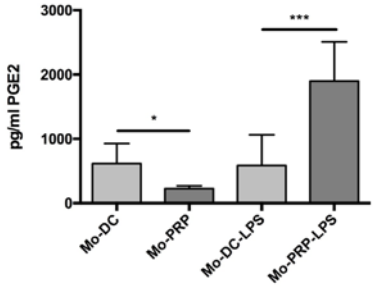

*Fig.1: Quantification of PGE2. In light grey Mo-DC and in dark grey Mo-PRP cells. Data represent means plus or minus SEM of 3 independent experiments. T-student statistical analysis *P <0.05; ***P <0.001.*

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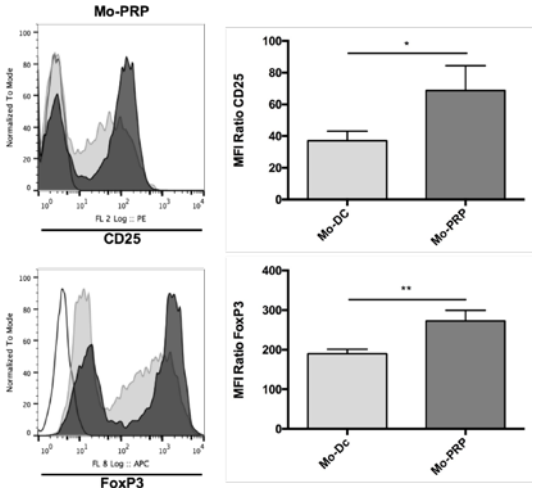

*Fig.2: CD4+ T naive Polarization to T-Reg Cells Expressing CD25 and FoxP3. Light grey Mo-DC, dark grey Mo-PRP cells. T-student statistical analysis *P < 0.05; **P <0 .01*

DISCUSSION & CONCLUSIONS: Allogeneic PRP fosters monocytes differentiation to M2 macrophages responsible of a strong immune-regulatory effect. This represents a possible explanation of the regenerative effects observed after PRP exposure due to its ability to modulate the immune response supporting the use of an allogeneic product in therapy.

Fabrication and characterisation of tailored ECM mimicking scaffolds based on elastin-like recombinamers to treat ischemic diseases

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INTRODUCTION: Ischemic conditions, related to decreased blood flow and inflammation [1], need to be faced by bioinspired smart systems favouring therapeutic angiogenesis. We developed an injectable elastin-like based hydrogel mimicking the extracellular matrix, praised with specific bioactivities, as cell adhesion and heparin binding as well as sensitiveness to matrix metalloproteinases (MMPs), ensuring a degradation rate of the platform guided by the cells colonizing it. A proangiogenic growth factor has been included in the system to stimulate angiogenesis, as a specific bioactivity for that purpose.

METHODS: Gene sequences of the elastin-like recombinamers (ELRs) forming the hydrogel were obtained by iterative-recursive recombinant methodology. The selected recombinamers were produced in *E. coli* and purified by inverse-transition cycling [2]. Characterization was performed by NMR, DSC, SDS-PAGE, MALDI-TOF and HPLC. The side chains of lysine residues of the recombinamers were chemically modified in order to bear an activated alkyne on one of the compounds, and an azide group on the other. Subsequently, the crosslinking of the hydrogel was performed in an aqueous ambient at room temperature by catalyst-free click chemistry [3] and the scaffold described by rheological measurements and SEM analysis. In vitro studies were carried out to evaluate bioactivity and cytocompatibility of the system.

RESULTS: The amino-acid sequence, MW, transition temperature and purity of the recombinamers were confirmed by various techniques. The modification rate of the single compounds was verified in detail by NMR. The mechanical features of the hydrogels were evaluated rheologically, showing a tuneable storage modulus in the 1-4 kPa range. SEM observation reveals high porosity of the scaffold. In vitro studies corroborate the expected bioactivity of the recombinamers and cell culture tests with HUVECs, HASMCs and HFF1s in 2D and 3D show adequate cytocompatibility and cell proliferation.

DISCUSSION & CONCLUSIONS: Catalyst-free click chemistry allows the development of a biocompatible crosslinking approach that permits the formation of a mechanically tuneable hydrogel, as a function of recombinamer concentration. The high porosity of the scaffold facilitates adequate supply of oxygen and nutrients to the cells colonizing it. The bioactivity of the overall structure is ensured by the presence of specific amino acid sequences in the backbone of the ELRs, which favour cell adhesion and heparin binding, indirectly supporting the formation of stable vascular networks through the recruitment and retention of endogenous proangiogenic factors. The ubiquitous release of MMPs guarantees an appropriate degradation rate making the designed hydrogels promising ECM mimicking platforms for cell colonization and eventual formation of a vascular network.

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Bio-acoustic levitational assembly of heterocellular multilayer constructs for tissue engineering

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INTRODUCTION: Tissues such as skin, brain or heart are comprised of multilayered structures for which spatial organization and inter-layers cellular communications are essential to sustain their biological functions. Generation of multilayered constructs is motivated by the need to better understand inter cellular interactions and build three-dimensional (3D) microenvironments for broad applications in tissue engineering and regenerative medicine. To model complex multilayers tissues, such as skin or cerebral cortex, bioengineering of multilayered constructs with tunable layers thickness and cell types is necessary. Current technologies to model 3D tissue organization such as bioprinting, multistep photolithography or layer-by-layer assembly are mostly based on generation of multiple 2D monolayer platforms and poorly represent the microscale native tissue spatial organization and functionality. However few of these techniques provide micrometer-scale controls over spatial organization of heterocellular layers such as interlayer spacing and intralayer cell proximity, which are significant to recapitulate microphysiological functions.

METHODS: Here, we present a novel strategy to bioengineer multilayered tissues with micrometer-scale control over biological and structural features of native layered tissues by combining bulk acoustic levitation and layer-by-layer assembly. Bulk acoustic levitation enables easy and rapid formation of closely-packing cellular layers, tunable interlayer spacing and layer thickness with micrometer scale resolution, while layer-by-layer assembly enables formation of heterogeneous layers of cells. This method can be carried out in a simple, rapid and biocompatible way in a fluidic environment and is potentially used as a widespread strategy for bottom-up tissue engineering and regenerative medicine.

RESULTS: To demonstrate the ability of the approach to bioengineer heterogeneous multilayers of cells in a single construct, three types of cells were assembled subsequently via layer-by-layer (Figure 1). We first generated a hydrogel sheet containing 3 layers of HeLa-mCherry cells by levitating 1 million cells in 167µl of fibrin pre-polymer solution. The two subsequent subunits were generated on top of the first one using regular Hela cells and HeLa-eGFP. The composite image clearly demonstrates the possibility of spatially distributing the different cell types in 3D to bioengineer heterocellular multilayer of cells in a single hydrogel construct.

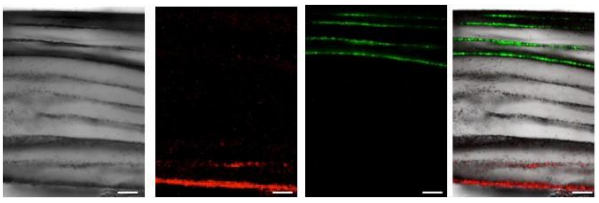

Fig. 1: Heterogeneous cell populations assembly into a single multilayered hydrogel construct. The multilayered construct was fabricated with layer-by-layer assembly, using live cells, constitutively expressing eGFP or mCherry. Scale bar is 250µm.

DISCUSSION & CONCLUSIONS: We have developed a bulk acoustic levitation technique to assemble, within minutes and with minimal acoustic pressure, multilayered constructs with multiple cell types in fibrin hydrogels [l]. This acoustical method is potentially useful for broad fields, where multilayered cell organization is required, to assemble cells alone in high densities to mimic the natural density and cell-cell interactions observed in the physiological complexity, such as skin, cardiac and breast tissue.

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Artificial nervous system tissues to model and repair the central nervous system

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INTRODUCTION: The combination of cells and biomaterials to construct living artificial nervous system tissues provides a powerful approach for both regenerative medicine and neuroscience research. Constructing CNS tissue presents specific challenges to the tissue engineer that demand new approaches beyond traditional combinations of cells and biomaterials. CNS tissue is mechanically compliant, contains a highly organised combination of multiple cell types, and function is dominated by complex cell-cell interactions. This talk will discuss some of the challenges associated with engineering CNS tissue and will report results of experiments exploring the use of 3D hydrogels containing organised CNS cells.

METHODS: Co-cultures of CNS neurons and glia have been constructed using soft collagen hydrogels that enable cells to adopt a natural phenotype in vitro [1]. These have been used to simulate damage and disease scenarios including spinal cord and traumatic brain injury, hypoxic/ischaemic damage, neurodegenerative disease and host cell responses to transplanted stem cells [2]. Production methodology for the reliable and scalable construction of engineered CNS tissue models has been developed [3]. The biomechanical properties of natural CNS tissues have been explored (using a powerful dynamic mechanical analysis approach) in order to inform the development of biomaterials that could integrate mechanically with the surrounding host tissues. Engineered tissues containing organised CNS glia have been constructed and tested for the support of neuronal regeneration [4].

RESULTS: Neurons, astrocytes, microglia, oligodendrocytes and endothelial cells have been used in various combinations within collagen hydrogels to create artificial CNS tissues. The response of neurons and glia to simulated damage and disease situations has been characterised in detail using a range of outcome measures. Robust production techniques have been developed to permit the scaling and widespread adoption of engineered CNS tissues within industry and the broader academic neuroscience research community. Biomechanical properties of natural spinal cord tissue have been investigated and methods for tuning the stiffness of cellular hydrogels have been developed.

DISCUSSION & CONCLUSIONS: Tissue engineering using CNS cells and hydrogel matrices provides a useful approach for constructing artificial CNS tissues. These can be used as models for neuroscience R&D, or developed for use in regenerative medicine.

ACKNOWLEDGEMENTS: This presentation includes work from numerous colleagues and collaborators and was funded by various organisations that will be acknowledged in the talk.

Construction of engineered neural tissue using clinically relevant materials and cells

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INTRODUCTION:

In an attempt to mimic the autograft, which is the current clinical gold standard treatment for repairing long gaps in damaged peripheral nerves, aligned cellular materials have been developed. By combining Schwann cell self-alignment in tethered rat tail collagen gels with stabilisation using plastic compression, sheets of engineered neural tissue (EngNT) can be made [1]. These have been shown to support regeneration of host neurons across critical-sized nerve gaps in a rat model. This talk will describe subsequent attempts to progress the technology towards clinical translation through the use of clinically more relevant cells and materials.

METHODS:

To replace the rat Schwann cells in the EngNT, various sources of therapeutically relevant cells were identified and tested. Potential autologous and allogeneic stem cells were differentiated towards a Schwann cell phenotype. As an alternative to rat tail collagen, other sources of collagen or alternative hydrogel materials were explored. Following characterisation, cells and materials were used to construct EngNT and tested for the ability to support neuronal regeneration. In vitro assays were followed by preclinical testing of constructs in a rat sciatic nerve injury model.

RESULTS:

EngNT was constructed using differentiated stem cells from adipose tissue or dental pulp [2,3], as well as cells derived from clinical grade neural stem cells. Support and guidance of neuronal regeneration was quantified in vitro then the ability of EngNT constructs to repair nerve gaps was tested in vivo.

DISCUSSION & CONCLUSIONS:

Engineered neural tissue was constructed with therapeutically relevant stem cell-derived cells instead of rat Schwann cells, and using alternative materials to rat tail collagen. This is an important step towards the clinical translation of tissue engineered alternatives to the use of the autograft.

ACKNOWLEDGEMENTS: This presentation includes work from numerous colleagues and collaborators and was funded by various organisations that will be acknowledged in the talk.

Designs of three-dimensional printed scaffolds promote formation of vascularised engineered bone.

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INTRODUCTION: Engineering bone constructs for osteoarthritis applications poses multiple challenges. The key parameters include three-dimensional (3D) scaffolds, stem cells and 3D culture systems [1]. The ultimate goal of BTE is to create a living scaffold that could survive after implantation, where this engraftment sought a rapid formation of a stable and functional vascular network [2]. During *in vitro* culture, nutrients can be supplied to the larger tissue-engineered scaffolds using the advanced technology of bioreactors and pre-designed aligned channel scaffolds. However, after implantation cells can only survive with a limited distance of about 200 μm from the next capillary network for the supply of nutrients and oxygen and for the removal of waste products. One popular approach to create pre-vascularized tissue-engineered scaffold involves the paracrine communication between endothelial cells (i.e. HUVECs) and human bone marrow-derived mesenchymal stem cells (hMSCs) [2, 3]. The main aim of this study was to investigate the effect of different structural and functional designs of 3DP hybrid scaffolds coated with a novel SiCHA powders in promoting osteogenesis as well as angiogenesis.

METHODS: Mesh, two- (2C) and four channel (4C) scaffolds were fabricated using PLA by Fused Deposition Modelling (FDM) technique followed by coating with 5-bilayers of a novel coating materials assembly consists of SiCHA powders in hyaluronic acid and collagen type I solutions. Rotary Cell Culture Systems (RCCS) Synthecon® was used to study the growth of the cellular scaffolds in dynamic condition as compared to static condition in the well plate. hMSCs were cultured on the scaffolds in osteogenic (OM) and proliferation media (PM) for 21 days in both conditions. Osteogenesis was then characterized by determining the early osteogenic differentiation, formation of mineralized matrix and production of total protein after 7, 14 and 21 days culture. The best scaffold design was then used for co-culture study, where HUVECs incorporated in the Matrigel were directly seeded in the channels and subsequently hMSCs were added on the surface of the scaffolds. The production of PDGF and VEGF were then quantified, as these are the two most important pro-angiogenic growth factors.

RESULTS: Among the investigated scaffolds, 4C scaffolds showed the highest level of ALP/DNA and total protein production as compared to mesh and 2C scaffolds. It was more apparent that more dense areas were associated with 4C scaffolds in OM for both culture static and dynamic conditions compared to those in PM (Fig. 1). Culturing 4C scaffolds in co-culture model resulted in the secretion of both PDGF and VEGF expressions.

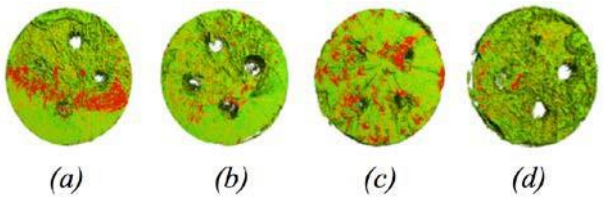

Fig. 1: Density maps of 4C scaffolds under different culture conditions; (a) SOM, (b) SPM, (c) DOM and (d) DPM after 21 days.

DISCUSSION & CONCLUSIONS: The structural design of the scaffolds, dynamic culture condition and culture media have a pronounced impact in determining cells fate. As conclusion, the overall best culture conditions for this study was 4C scaffolds cultured under Dynamic/OM. Positive expressions of both pro-angiogenic markers proved that the concept for pre-vascularization of engineered bone construct can be tested by co-culture model developed in this study.

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ECM-capturing peptides permit dynamic synthetic hydrogel remodelling by encapsulated human mesenchymal stem cells

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INTRODUCTION:

The role of the extracellular matrix (ECM) in governing (stem) cell fate is well established[1]. ECM proteins not only provide support to cells but also display critical cell-instructive cues[1]. *In vivo*, the ECM is a highly dynamic microenvironment that is constantly being remodelled by the cells themselves[2]. Here we present a hydrogel system designed to specifically capture cell-secreted ECM proteins to closer mimic the matrix remodelling dynamics of *in vivo* environments for encapsulated cells. We propose that this approach enables us to recapitulate cell-mediated ECM remodelling and therefore facilitates the generation of self-inductive biomaterials capable of directing (stem) cell fate.

METHODS: Binding affinities of the protein binding peptides (PBPs) for specific ECM proteins were validated using quartz crystal microbalance with dissipation (QCM-D). A Norbonene functionalized 8 arm PEG was synthesized and during crosslinking with 4 arm Thiol-functionalised PEGs, the ECM-specific thiol-modified PBPs were incorporated. Immunofluorescence staining was used to investigate the 3D binding of proteins in functionalized PEG gels. The ability of gels to recruit secreted protein from encapsulated mesenchymal stem cells (MSCs) was investigated.

RESULTS: QCM results show that the PBPs can bind the targeted ECM proteins with high specificity and minimal non-specific binding to other ECM molecules (Fig.1). Minimal background binding was observed. The gels possess a wide range of mechanical properties that can be tailored by varying UV exposure time, monomer and cross-linker concentrations. The observed degradation (via hydrolysis of an included ester bond in the PEG arms) confirms that the gels are useful for a range of cell culture purposes. The results from 3D affinity investigations and cell secreted ECM capturing experiments confirmed that the PBPs are able to bind specific ECM proteins in 3D (Fig 2).

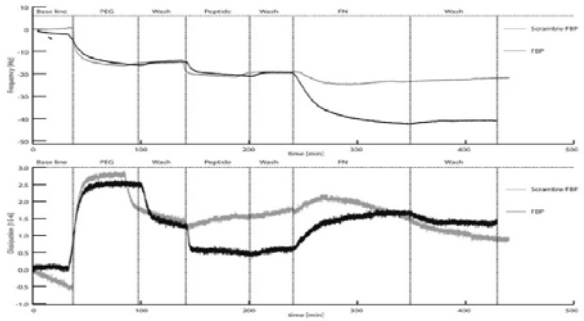

Fig.1: surface binding affinity of fibronectin binding peptide to fibronectin.

The viability of encapsulated hMSCs into the PBP-PEG hydrogels confirmed their ability to support these cells. Immunofluorescence staining for specific ECM proteins in these cell-loaded gels revealed increased staining intensity in the regions surrounding the encapsulated cells in PBP-PEG conjugated gels compared to PEG only hydrogels, confirming the presented PBPs were able to recruit ECM proteins locally secreted from cells.

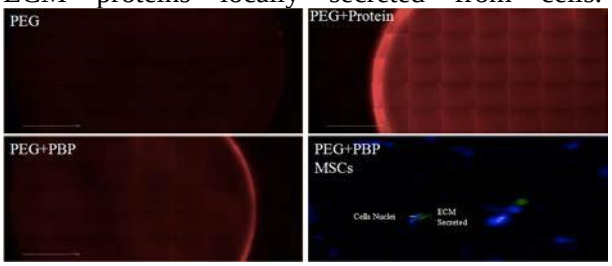

Fig.2: binding affinity of Protein binding peptide to fibronectin in 3D PBP-PEG gels

DISCUSSION & CONCLUSIONS: The 2D and 3D assessment of PBP affinity to specific ECM proteins when presented on PEG chains (on a QCM substrate or as a 3D hydrogel) confirmed the ability of these ECM targeting peptides to bind ECM proteins regardless of substrate format. The PBP-PEG hydrogels were able to encapsulate MSCs and recruit ECM proteins secreted from MSCs (Fig 2).

REFERENCES:[1]P.Singh,J.E.Schwarzbauer

Endothelialisation of a reconstructed 3D tissue-engineered vaginal mucosae via the self-assembly technique for implantation in vivo

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INTRODUCTION: A major challenge in tissue engineering is the vascularization of reconstructed tissues prior to implantation as graft survival and success highly depends on it. In order to one day, use reconstructed autologous vaginal mucosae for vaginal reconstruction, we must first develop a model that closely mimics the vaginal tissue and that is suited for surgery. We have developed a unique 3D in vitro model of the human vaginal mucosae using the self-assembly technique. In this study, we aim at pre-vascularizing our reconstructed model prior to implantation in vivo by the incorporation of endothelial cells

METHODS:
Vaginal stromal and epithelial cells were isolated from vaginal mucosae biopsies from healthy female donors. Stromal cells produced their own extracellular matrix, thereby bypassing the need to use any exogenous biomaterials. We co-seeded vaginal stromal cells with human umbilical cord vein cells (HUVEC). After four weeks of culture, vaginal epithelial cells were seeded on top of the stromal layer. Epithelial cells proliferated for a week under submerged conditions, after which the endotheliallized stromal sheets were stacked and allowed to fuse in an air/liquid interface. We have used a culture medium with growth factors and various oestrogen concentrations.

RESULTS

Characterization of our in vitro model confirms the morphological similarity with the original biopsy. We assessed the formation of a 3D micro-capillary network within the reconstructed tissues using specific markers such as CD31 and NG2 by immunofluorescence. Furthermore, we tested different cell seeding techniques to determine which one provides the greatest mechanical resistance compatible with surgical implantation.

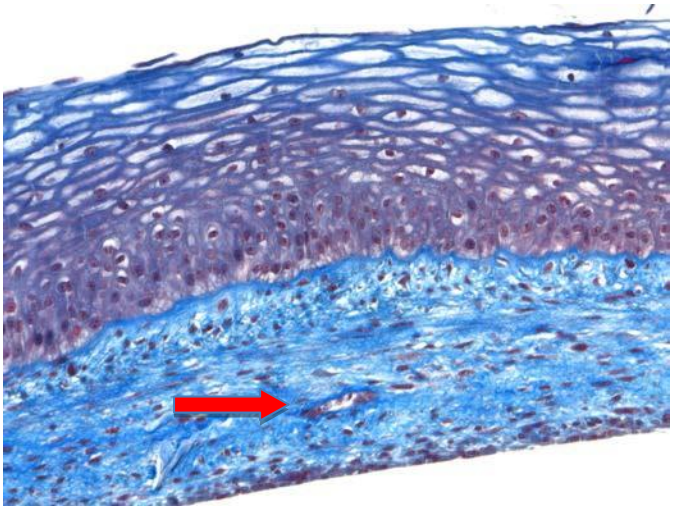

Fig.1: Masson's Trichrome stain of our endothelialized reconstructed model of the vaginal mucosae produced with the self-assembly technique.

DISCUSSION & CONCLUSIONS:

Our experimental model generated reconstructed vaginal 3D tissues with well-developed endothelial network able to withstand sutures during implantation. Albeit its surgical reconstruction purpose, such a model is of valuable importance to conduct fundamental research.

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Enhancing bone regeneration by matrix-assisted MSC recruitment

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INTRODUCTION: Regeneration of large bone defects caused by trauma or disease requires, particularly in elderly patients, surgical intervention, with transplantation of autologous bone being the gold standard. Although, transplantation of a patient's own bone provides best clinical outcome, it is accompanied with a number of obvious disadvantages such as donor site morbidity and limited supply. Hence, great efforts were put into the development of off-the-shelf materials. An intriguing approach is based on the delivery of recombinantly produced osteoinductive factors, such as bone morphogenetic protein (BMP) [1]. Although promising results were obtained in preclinical studies, with quantities needed that exceed the physiological concentrations by magnitudes, its clinical outcome did not meet expectations. One of the reasons for this seems to be the limiting number of available endogenous mesenchymal stem cells (MSCs) at the fracture site that can give rise to bone cells. Here, we aim at engineering a biomimetic implant that augments homing of endogenous MSCs to bone defects by controlled presentation of biomolecules and thereby allows for fully exploiting the osteoinductive potential of BMP delivery.

METHODS: A cell-permissive biomimetic hydrogel was implanted into calvarial critical-sized defects in mice to serve as cell trap for cells participating in bone regeneration [2]. Traps were explanted after 8 days of bone regeneration and trapped cell populations were enzymatically recovered. Potential MSCs were identified and prospectively isolated by FACS according to a specific marker profile [3]. Multilineage differentiation potential of trapped MSCs was assessed *in vitro* as well as *in vivo*. An *in vitro* three-dimensional (3D) screen was performed to identify migration-inducing biomolecules [4]. Best candidate biomolecule was incorporated into hydrogel cell traps in combination with BMP-2 and biomolecule delivering cell traps were implanted into critical-sized calvarial defects. The homing of MSCs to cell traps and induced bone formation were assessed by immunohistochemistry 8 days post-implantation and by micro-CT 4 weeks post-implantation, respectively.

RESULTS: We report on a biomaterial-based approach to entrap and prospectively isolate MSCs participating in healing of a large bone defect. We show that non-hematopoietic and non-endothelial Sca-1$^+$ cells are highly enriched in bone wounds and that they exhibit current progenitor cell definitions *in vitro*. In BMP-2 induced heterotopic bone organs these cells turn into subendothelial reticular cells as well differentiate into cells of the osteogenic lineage. To identify growth factors and cytokines that potentially enhance the *in vivo* fracture homing of these progenitor cells we perform an *in vitro* microtissue-based 3D migration screen. Best candidate growth factor, PDGF-BB, is engineered for controlled *in vivo* delivery and is shown to be capable of promoting homing of Sca-1$^+$ MSCs to bone defects as well as augmenting the osteoinductive effect of BMP-2 resulting in enhanced bone regeneration.

DISCUSSION & CONCLUSIONS: We show a bio-inspired engineering approach to create potential next generation bone implants that aim at mimicking and augmenting the natural signaling cascade responsible for MSC recruitment.

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rhCollagen scaffold combined with PRP enhances healing in tendinopathy

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INTRODUCTION: Platelet Rich Plasma (PRP) is proposed for the treatment of soft tissue injuries by providing growth factors (GFs) that are essential for the healing process. PRP injection in chronic inflammatory indications, such as tendonitis, is a common practice, believed to increase the GFs concentration at the injured site and accelerate the healing process. However the fast degradation of the fibrin-clot and the elimination of the GFs limit the clinical benefit. To boost the potential healing effect of PRP *in situ*, we developed a Collagen scaffold (Vergenix™STR) based on a novel recombinant human type I collagen (rhCollagen) purified from transgenic Tobacco. The new product, in combination with PRP provides sustained release of GFs at the injured tissue for several weeks. The new product has been evaluated in preclinical models and already entered a clinical phase.

METHODS: *Preclinical studies:* two studies in rat models were undertaken to demonstrate the degradation profile and the performance of the Vergenix™-STR-PRP compared to PRP alone. STR-PRP and PRP alone were injected into a subcutaneous pocket and the remaining clots were assessed histologically and for PDGF and VEGF content at several time points. In the second study Vergenix™-STR-PRP was compared to PRP alone in a rat model for Common Calcaneal tendon (Achilles tendon) tendinopathy. Tendinopathy was first induced by collagenase injection followed one week later by treatment injection into the same tendon. Tendons were histopathological evaluated at several time points. *Clinical study:* safety and performance were evaluated in a prospective, open label, single arm clinical study in patients suffering from lateral epycondilitis (tennis elbow). The protocol included a single injection of Vergenix™-STR-PRP followed by clinical evaluations (up to 6 months). Product performance was assessed by measuring reduction in pain and recovery of motion, as by the specific Patient Related Tennis Elbow Evaluation questionnaire ("PRTEE").

RESULTS: *Preclinical studies:* evaluation of the injection sites in the subcutaneous rat model showed that upon Vergenix™-STR-PRP injection, PDGF and VEGF are localized in the injection site for about 30-40 days and the final decrease in the GFs content is concomitant with collagen/PRP composite degradation. On the contrary upon injection of only PRP no GF were detected and no clots were observed already 24 hours after the application. Histopathological analysis of Achilles tendon samples showed that rats treated with Vergenix™-STR-PRP had markedly lower level of inflammatory mononuclear cells 3 and 14 days after treatment when compared with animals treated with PRP alone (Figure 1). In addition, at day 14, STR-PRP group displayed lower level of immature granulation as compared with the PRP alone group (Figure 1).

Clinical study: At 3 months, patients (N=20) reported an average PRTEE score improvement of 55.8%. At 6 months, patients (N=20) reported a mean PRTEE score improvement of 58.1%. The performance of Vergenix™STR compared very favourably to published results of standard-of-care tennis elbow therapies (PRP and steroids).

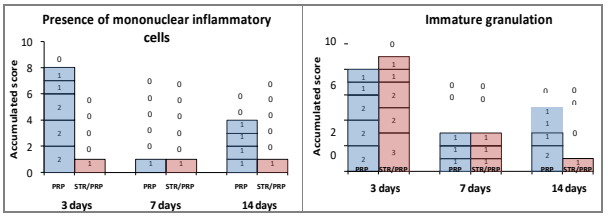

Fig. 1: histological scoring upon STR-PRP and PRP injections in a rat model for Common Calcaneal tendinopathy.

DISCUSSION & CONCLUSIONS:

We propose here a collagen scaffold (Vergenix™-STR) that localizes PRP at the target application to ensure sustained release of GFs while serving as a scaffold for tissue regeneration. As evidenced in the studies provided here, the combination of PRP with Vergenix™-STR improves the retention of GFs at the site of application and shows favourable histopathological results when compared to PRP alone in a rat model of tendinopathy. Furthermore, the outcome of the clinical study confirmed the significant performance of Vergenix™STR in patients with lateral epycondilitis.

Tissue engineering a bio-functionalised off-the-shelf heart valve based on lessons from human valvulogenesis

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INTRODUCTION: A major limitation of currently available heart valve replacements is their incapacity to grow or remodel post-implantation[1]. A tissue engineered alternative should consider both the populating cells of the heart valve and its supporting extracellular matrix (ECM). Cells contributing to semilunar valve formation originate from the second heart field (SHF) and the neural crest and during endocardial cushion formation, endocardial cells transition to a mesenchymal phenotype and migrate into the cardiac jelly [2]. Afterwards, the thick endocardial cushions, which act as primitive valves, elongate into thin fibrous leaflets that exhibit a typical trilaminar ECM structure. Semilunar valve leaflets have a well-described trilaminar histoarchitecture, with a sophisticated fibrous network composed of structural proteins such as collagens, elastic fibres and microfibrils as well as signalling molecules like water-storing proteoglycans (PGs), glycosaminoglycans (GAGs) and growth factors. This keynote presentation describes our work in mapping human semi-lunar heart valve development with respect to cells that colonise the developing valve and their supporting extracellular matrix structure. Based on the ECM development patterns we observed, we sought to incorporate our findings within the construction of an electrospun biomaterial heart valve to closely recapitulate valvulogenesis in heart valve engineering design.

METHODS: Here, we systematically analysed tissues from human foetal first (4-12 weeks) and second (13-18 weeks) trimester, adolescent (14-19 years) and adult (50-55 years) hearts to monitor the temporal and spatial distribution of extracellular matrix and cell colonisation, focusing on semilunar valves. Atomic force microscopy (AFM) was employed to obtain layer-specific measurement of the Young's modulus in porcine valves. As a proof of principle, key proteins that exhibited spatiotemporal expression profiles during development were introduced to a hybrid electrospun scaffold, demonstrating the ability to bio-functionalize the hybrid valve based on nature's blueprint.

RESULTS & DISCUSSION: Tropoelastin/ elastin was first detectable in 7-week leaflets. We revealed that immature elastic fibres are organized in early human cardiovascular development and that mature elastin-containing fibres first evolve in semilunar valves when blood pressure and heartbeat accelerate. AFM revealed that spongiosa

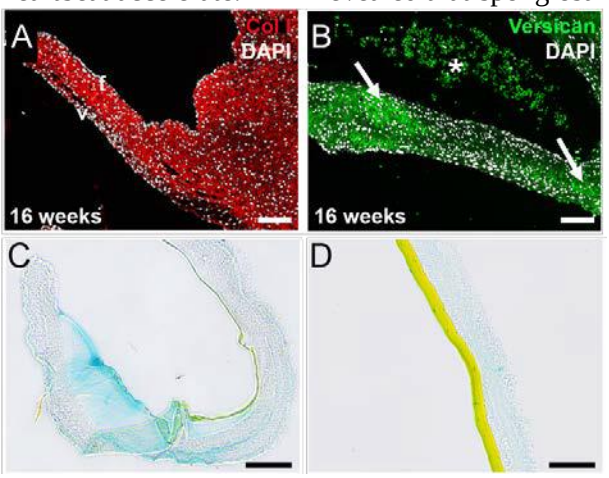

*Fig. 1: Immunohistological analyses reveal protein expression patterns in developing human valves. (A): collagen type I (red): and (C), versican (green) at 16 weeks. * indicate erythrocytes, f=fibrosa; v=ventricularis. Scale bars equal 50 µm. (C, D): Alcian blue and saffron staining of a tissue-engineered valve leaflet. Collagen is stained in yellow and proteoglycans (versican) are visualized in blue. Scale bar equals 200 µm.*

stiffness was much lower compared with the fibrosa and ventricularis. Moreover, investigations into human foetal heart valve development identified collagen type I and versican as important structural proteins. These results highlight the importance of developmental pathways in tissue engineering design strategies.

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A novel pulsatile platform for the *ex-vivo* evaluation of early remodelling events in human saphenous veins

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INTRODUCTION: After coronary artery bypass grafting (CABG), structural modifications of the human saphenous vein (hSV) wall lead to the lumen narrowing in response to the different hemodynamic conditions experienced: pulsatile pressure of 80-120 mmHg, pulsatile flow with mean flow rate of 250 ml/min, and elevated shear stress (1-7 Pa) [1]. Here we present a novel pulsatile platform conceived for mimicking the CABG surgery hemodynamic conditions and studying the hSV early remodeling events.

METHODS: A culture system (Fig.1A) was developed to stimulate vessel samples with CABG-like pressure/flow patterns, *i.e.* a sphygmoid-like pulsatile pressure in counter-phase with a pulsatile flow rate (Figure 1B). Six hSV samples were subjected to pulsatile stimulation for 7 days, and native hSV segments served as control. Dextran (3.5% w/v) was added to DMEM, to reach 3.07±0.07 cP viscosity. At the end of the conditioning period, hSVs were fixed and stained with Masson's trichrome and Weigert van Gieson stainings for morphological/morphometric evaluation. Immunofluorescence investigations (αSMA for labeling smooth muscle cells and CD31 and vWF for endothelial cells) were also performed.

RESULTS: *Ex vivo* experiments with hSVs revealed the ability of the CPD to mimic the complexity of the coronary hemodynamic environment with good fidelity in comparison with state-of-the-art devices. Morphometric analysis on Masson's trichrome stained sections (Fig.1C) revealed that both intima and media thickness were significantly decreased after 7 days of conditioning, resulting in significantly reduced wall thickness, but without affecting lumen perimeter. The media consisted mainly of SMCs, and on the luminal lining, most cells expressed CD31/vWF. There was a partial EC denudation.

DISCUSSION & CONCLUSIONS: Our novel platform enables prolonged ex vivo stimulation of hSVs segments with CABG-like hemodynamic conditions. Seven day conditioning resulted in thinning of both intima and media of the veins. Studies are currently ongoing in our Laboratories to correlate the hemodynamic changes to the activation of cellular and molecular pro-pathologic pathways involved in the SV graft disease.

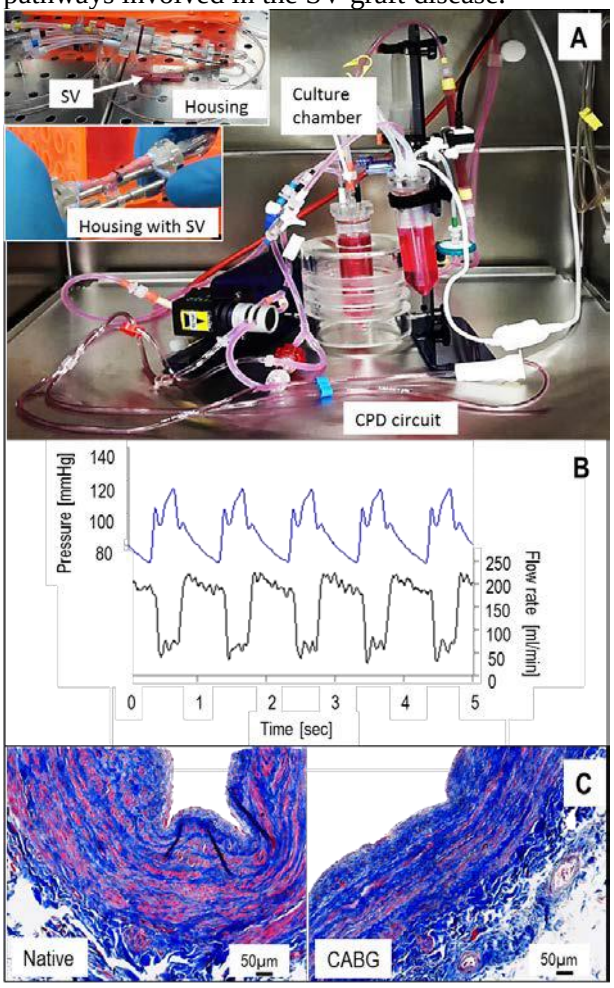

Fig. 1: Image of the CPD platform (A). Pressure and flow rate traces acquired during the experiments (B). Masson's trichrome sections before (Native) and after conditioning (CABG).

ACKNOWLEDGEMENTS: This work is partially supported by the Italian Ministry of Health (RF-2011-02346867).

Designing an integrated hybrid cryogel-based bioreactor with potential application as a bioartificial liver support

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INTRODUCTION: An ideal extracorporeal liver device combines the detoxification capacity of artificial liver devices and the metabolic activity of bioartificial liver (BAL) devices to bridge patients to liver transplantation and alleviate conditions of liver failure. Conventionally, BAL devices constitute separately of a plasmapheresis unit- to separate plasma from circulating blood, an adsorbent column- to remove water soluble toxins from circulating plasma and a hepatocytes-seeded bioreactor unit. In our integrated hybrid bioreactor design we have combined the plasmapheresis and adsorbent column modules along with a hepatocyte-laden cryogel bioreactor in one compact multi-chambered unit to improve the efficiency of the bioartificial liver

METHODS: A 3-chambered bioreactor was designed wherein a plasma separation membrane and an activated carbon cloth are placed in the top-most chamber. HepG2-seeded poly (N-isopropylacrylamide)-chitosan (pNC) cryogel is placed in the middle chamber over a 0.22µm cellulose membrane in the bottom chamber. Rat models of acute liver failure were developed using 70% partial hepatectomy (PHx) with ischemic conditions. The designed bioreactor was connected to these models and biochemical parameters monitored over a period of three hours. These were compared with those of rat models connected to a HepG2-seeded cryogel bioreactor in the conventional setup of BAL devices.

RESULTS: There was a 20-50% decrease in the metabolic parameters bilirubin and aspartate transaminase as compared to a 5-15% decrease in the conventional setup. Additionally a significant amount of human albumin was detected in the rat plasma, indicating functionality of the cells in the bioreactor while connected to the rat model. The total volume of blood circulated through the bioreactor was 4-5 mL as compared to 6-8 mL in the conventional setup.

DISCUSSION & CONCLUSIONS: A larger bioreactor calls for more volume of blood withdrawn from the patient.[1] However, only a limited amount of blood/plasma may be withdrawn from a patient at a time. Our bioreactor design reduces the total volume of blood in extracorporeal circulation by a significant amount and maintains most of the metabolic functions of the liver.

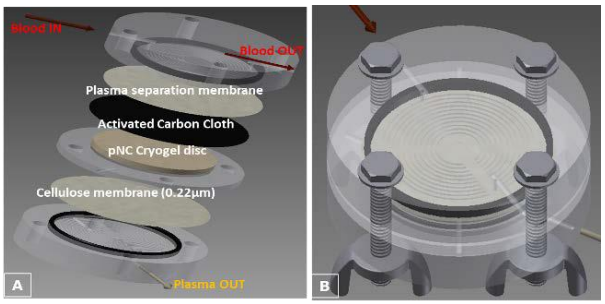

Fig. 1: Integrated hybrid cryogel-based bioreactor for application as bioartificial liver support. (A) Components of the bioreactor system (B) Design of the bioreactor setup

Table 1: Percentage change in biochemical parameters in rat plasma after three hours of extracorporeal circulation.

Biochemical parameter	Integrated design	Conventional setup
	% change	% change
Bilirubin	-54	-15
Urea	+38	-9.5
AST	-18	-4.5
Rat Albumin	+8	+5

ACKNOWLEDGEMENTS: This work has been carried out in collaboration with Institute of Liver and Biliary Sciences (ILBS), New Delhi and Kyushu University, Fukuoka, Japan. The authors would like to thank DBT and DST-JSPS, Govt. of India for financially supporting this work. AD acknowledges IIT Kanpur for financial support.

4D bioprinting of developmentally inspired templates for whole organ engineering

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INTRODUCTION: The ability to 3D bioprint patterns of cellular and extracellular components in three dimensions provides tissue engineers with tools to recapitulate the complexity of native tissues [1]. However recapitulating the structural and compositional complexity of an entire organ is beyond the capabilities of current bioprinting technology. A potential alternative 4D approach could involve the patterning of an organ rudiment at an earlier, less complex stage of development, which could then mature over time, providing a template for subsequent organogenesis.

During skeletogenesis the long bones of the body are formed by endochondral ossification, whereby chondrocytes within the developing limb bud undergo a coordinated sequence of proliferation and hypertrophy, providing a growing template for bone formation [2]. Cartilage canals then act as conduits for vascular invasion as the cartilaginous template is replaced by bone. Here we propose a 4D bioprinting strategy involving the biofabrication of developmentally inspired hypertrophic cartilage templates for whole bone organ tissue engineering.

METHODS: First the vertebrae of a human skeleton model was scanned and next using a dual-bioprinting approach polycaprolactone (PCL) and MSC laden bioink filaments (RGD modified alginate) were co-deposited building a composite structure mimicking the geometry of the vertebrae. The compressive modulus was 400 fold higher with the incorporation of PCL. The vertebrae were next chondrogenically primed *in vitro* and implanted subcutaneously in nude mice for 12 weeks to explore whether they could mature along an endochondral pathway *in vivo*. As a control we implanted PCL free hydrogels to explore whether the addition of PCL would influence endochondral bone formation *in vivo (bioink).* In addition we explored if introducing a network of interconnected bioink free micro canals, achieved by controlling the placement of the bioink within the composite, could accelerate vascularization *in vivo (bioink/PCL + channels).*

RESULTS: Post-implantation the chondrogenically primed vertebrae were extensively vascularized and mineralized with μCT analysis demonstrating a bone volume of ~24% (1b, c). Collagen X staining surrounded networks of hypertrophic chondrocytes confirming bone formed via an endochondral pathway (1e, f). Bone marrow like structures surrounded by bony trabeculae confirmed the development of a mature bone organ (1c, d). Interestingly the incorporation of PCL accelerated vascularization *in vivo* (1g).

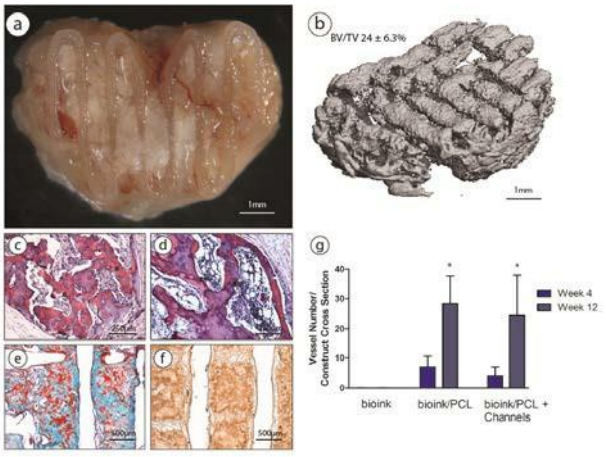

Fig. 1: (a) Vertebrae post-implantation (b) μCT reconstruction, (c, d) H&E & (e) Goldners trichrome, & (f) Collagen X staining post implantation, (g) Vascularisation in (bioink, bioink/PCL, bioink/PCL + channel) groups.

DISCUSSION & CONCLUSIONS: Using a 4D bioprinting strategy it was possible to engineer developmentally inspired cartilage rudiments, which over time matured along an endochondral pathway into more complex bone organs *in vivo*. PCL incorporation provided the added mechanical functionality necessary to implant such developmentally immature tissues into a load bearing location. In conclusion this developmental approach adds a fourth dimension to the traditional bioprinting paradigm.

Biological bases of therapeutic vascularization

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INTRODUCTION: Therapeutic angiogenesis, i.e. the generation of new vessels by delivery of specific factors, is required both for rapid vascularization of tissue engineered constructs and to treat ischemic conditions. Vascular endothelial growth factor (VEGF) is the master regulator of angiogenesis. However, uncontrolled expression leads to vascular tumors (angiomas). Major challenges to fully exploit VEGF potency for therapy include the need to precisely control in vivo distribution of growth factor dose and duration of expression. We previously found that the therapeutic window of VEGF delivery depends on the VEGF amount in the microenvironment around each producing cell rather than on the total dose, since VEGF remains tightly bound to extracellular matrix and a few "hotspots" of high expression are sufficient to cause angioma growth even if the total dose is rather low [l]. On the other hand, short-term expression of less than about 4 weeks leads to unstable vessels, which promptly regress following cessation of the angiogenic stimulus [2].

METHODS, RESULTS, DISCUSSION & CONCLUSIONS: Here I will present recent work aimed at: 1) translating fundamental principles of VEGF function into clinically applicable approaches to induce controlled angiogenesis, through the use of genetically modified progenitors or extracellular matrix engineering with recombinant factors [3]; and 2) investigating the mechanisms that regulate acquisition of VEGF independence in vivo, to identify novel and more specific molecular targets to achieve rapid stabilization of induced angiogenesis despite transient factor delivery [4] (Fig. 1).

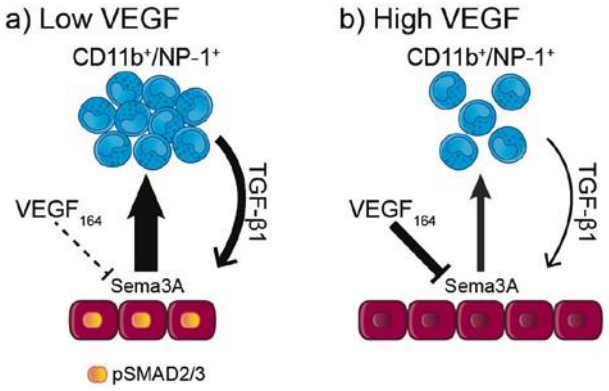

Fig. 1: VEGF dose regulates vascular stabilization through Semaphorin3A and the recruitment of Neuropilin-1+ monocytes (NEM). High VEGF doses inhibit endothelial Semaphorin3A expression, thereby impairing recruitment of NEM, TGF-β1 production, SMAD2/3 activation and endothelial survival. Conversely, Semaphorin3A treatment promotes NEM recruitment and accelerates stabilization of VEGF-induced angiogenesis, enabling vessel persistence despite transient delivery [4].

ACKNOWLEDGEMENTS: These studies were supported in part by the European Union FP7 grants ANGIOSCAFF (CP-IP 214402) and AngioMatTrain (CP-IP 317304) and by the Swiss National Science Foundation (grants 127426 and 143898).

A combined advanced therapy medicinal product for spinal cord injury repair

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INTRODUCTION: While clinical impairments upon a spinal cord injury (SCI) persist untreated, new therapeutic approaches advance to pre-clinical testing aiming functional recovery[1]. Herein, a new combined advanced therapy medicinal product (ATMP) has been designed, consisting of an *in vitro* cultured graft based on a collagen conduit[2] and pre-differentiated mesenchymal stem cells (MSC). Delivery of such MSC, differentiated into neuronal/glial lineages, are intended to aid function restoration at the injured tissue while the tubular conduit shall retain and guide such cells, providing structural support within the injury site.

METHODS: A collagen-based tubular conduit was produced at human scale (12mm∅) and rat scale (3mm∅) for testing (Vornia Biomaterials). Pore size, porosity and pore morphology were determined by µCT and SEM. Human adipose-derived MSC (hASC) were manufactured cGMP (Stemmatters), fully characterized for standard stem cell markers by flow cytometry (CD105+90+73+34-). hASC were seeded into conduit, assessed for viability by live/dead assay and differentiated for 2 weeks into the neural lineage (Gibco CTS). Differentiation was assessed by immunocytochemistry (ICC) and gene expression of GFAP, ßIII tubulin, MBP and NEFL. Electrical activity of differentiated cells was determined by patch clamp at controlled current and controlled voltage.

RESULTS: hASC *in vitro* cultured within collagen conduit, aligned within its pores (Fig.1a) and maintained high viability up to 2 weeks of neural differentiation (Fig.1b). Conduit device maintained its integrity allowing subsequent *in vivo* implantation in a rat SCI (study ongoing). The structure displayed 80±5% porosity, 90±7% interconnectivity and an average pore size of 167±18µm, evaluated by µCT (Fig.1c). The heterogeneous population of cells expressed GFAP and ßIII tubulin demonstrated by ICC. MBP and NEFL were overexpressed by 8x, while a 13x gene overexpression was detected for ßIII tubulin by qRT-PCR. Cell electrical activity was identified by presence of voltage-gated K+ and Na+ channels.

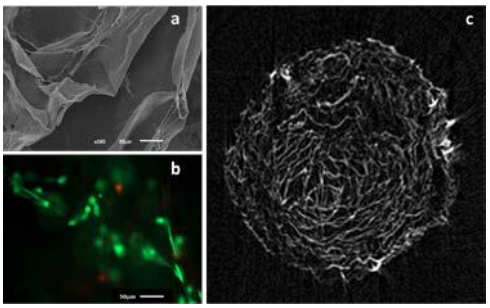

Fig. 1: a) SEM imaging of hASC cultured in conduit; b) Live/dead imaging of cells aligned in conduit walls (green: live, red: dead); c) µCT scan of conduit displaying its porous structure.

DISCUSSION & CONCLUSIONS: Restoration of function after SCI has been postulated to occur via plasticity of damaged or spared projections[3]. The delivery of electrically active, connected cells, among a heterogeneous population of neural/glial-lineage committed cells, to the lesion site, might play a role on activation or connection of novel circuits. The obtained protein, gene expression and electrical activity of cultured hASC provide promising evidence towards these achievements. Delivery of such cells within the three-dimensional conduit structure, with aligned pores, could aid guidance of such circuits through the ascending / descending pathways, while retaining therapeutic cells at the lesion site. The biodegradable character of the conduit shall allow its replacement by native tissue throughout remodelling process *in vivo*.

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Therapeutic angiogenesis by a VEGF and PDGF-BB gene-activated matrix

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INTRODUCTION: Therapeutic angiogenesis aims at promoting vascularization of ischemic tissues by delivery of exogenous growth factors. Vascular Endothelial Growth Factor (VEGF) is the master regulator of vascular growth. However, uncontrolled VEGF delivery can cause the growth of angioma-like tumors or edema [1]. We previously found that co-delivery of PDGF-BB can prevent the toxic effects of VEGF and induce only normal angiogenesis despite uncontrolled VEGF levels [2]. For clinical gene delivery, integrating viral vectors raise safety concerns, due to the risk of oncogenic transformation, while plasmid DNA vectors have an excellent safety profile, but suffer from limited transfection efficiency *in vivo*. In order to overcome these limitations and achieve safe and effective angiogenesis, here we sought to develop a gene-activated matrix (to ensure prolonged and efficient delivery of a plasmid vector) in combination with balanced co-expression of VEGF and PDGF-BB (to prevent the side-effects of uncontrolled VEGF expression *in vivo*). The gene-activated matrix was based on an Elastin-like polymer (ELP) biomaterial [3], because: 1) it is an injectable liquid that self-assembles into a hydrogel at body temperature; 2) it is fully biocompatible; 3) after functionalization with RGD peptides it sustains cell adhesion and provides a favorable environment for endothelial migration and vascular assembly.

METHODS: Homology-based recombination was used to clone the bicistronic VEGF-IRES-PDGF-BB (VIP) cassette into the pVIVO2 plasmid that is optimized for *in vivo* expression. The plasmid DNA was complexed with a novel Dendron-based transfection agent (FFG3K) generated by the University of Brighton. The ability to complex the plasmid was assessed by gel retardation assay. Cytotoxicity of the polyplexes on myoblasts was measured *in vitro* by FACS quantification of DAPI exclusion. RGD-ELP was injected in mouse skeletal muscles to evaluate material degradation and muscle damage by histological analysis.

RESULTS: The pVIVO2-VIP plasmid, carrying the VEGF and PDGF-BB genes in a single bicistronic expression cassette (named VIP for **V**egf-**I**RES-**P**dgfb), ensured co-expression of both factors in every transfected cell at a fixed ratio regardless of absolute level. FFG3K is a third generation Dendron with 16 exposed NH2 groups allowing efficient complexation with the pDNA. FFG3K did not show any relevant cytotoxicity at the condensing concentrations. Preliminary data showed that 7 days after injection in hind-limb skeletal muscle of SCID mice the RGD-ELP hydrogel was still present with no signs of necrosis in the muscle tissue. Interestingly, robust infiltration of the RGD-ELP by $CD11b^+$ monocytes was observed, as well as efficient ingrowth of microvascular structures. These *in vivo* data confirm the biocompatibility of the RGD-ELP biomaterial and its ability to sustain colonization by host cells and vascularization.

DISCUSSION & CONCLUSION: Balanced co-expression of VEGF and PDGF-BB from a gene-activated matrix is an attractive approach to restore perfusion of ischemic tissue. The efficacy and duration of gene expression after plasmid delivery from the ELP is being assessed *in vitro* and *in vivo*. Functionality and morphology of induced angiogenesis will be analysed in normal muscles and, lastly, therapeutic potential will be tested in a pre-clinical model of mouse hind-limb ischemia.

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Messenger RNA therapeutics for regenerative medicine

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INTRODUCTION: During recent years, messenger RNA therapeutics have enjoyed increasing attention as a viable alternative to gene and protein therapies. However, the strong potential of exogenously applied mRNA to activate the innate immune system has been a major obstacle for its use as a therapeutic entity beyond tumor vaccines. This has been overcome by manufacturing mRNA with chemically modified nucleotides, partially replacing standard nucleotides which has paved the way for potentially wide-spread use of mRNA therapeutics for treating metabolic, hereditary, infectious and degenerative diseases. In contrast to gene therapy, mRNA transcript therapy does not harbour the risk of insertional mutagenesis while like gene therapy having the advantage that the therapeutic protein of interest is produced in the patient's body by the patient's own cells. We have developed *transcript-activated matrices* (TAMs) with chemically modified mRNA formulations encoding BMPs and examined their potential to induce osteogenic differentiation of cells in vitro and an improved healing of a non-critical bone defect in vivo.

METHODS: Chemically modified mRNAs (cmRNAs) were produced by in vitro transcription, partially replacing cytidines and uridines with modified analogues. cmRNAs were formulated as lipid nanoparticles comprising a proprietary oligo-alkylene amine-based cationic lipidoid. The aqueous formulations were used to impregnate collagen sponges or combined with fibrin glue to ultimately fill bone defects. TAMs were first developed using cmRNAs encoding reporter proteins such as eGFP or luciferase. Various cell types including mesenchymal stem cells were cultivated on/in TAMs to examine transfection efficiency and kinetics as well as dose-response profiles by FACS analysis or bioluminescence measurements. These characteristics were compared with those of standard transfection in 2D format. Finally, optimized TAMs were prepared with BMP-encoding cmRNAs and used to fill non-critical 2 mm drill hole defects in rat femora. Empty carrier biomaterial (fibrin or collagen) was used to fill contralateral defects as controls. Osteogenic differentiation in vitro and bone regeneration in vivo was evaluated by histological analysis, qPCR for marker protein expression and µCT.

RESULTS: cmRNA used in this project turned out to be substantially less immunogenic than unmodified mRNA. In contrast to transfection in 2D format which results in only short-term translation of cmRNA, TAMs yielded sustained production of cmRNA-encoded proteins over at least one week. Cell viability on TAMs was excellent. In both 2D and 3D transfection formats, osteogenic differentiation was demonstrated when cmRNA encoded BMP-2 or BMP-7. In TAMs, up to 100% of cells became transfected, and the system turned out robust with respect to the density of cells cultivated on TAMs. When TAMs encoding BMP-2 or BMP-7 were implanted in non-critical bone defects in rats, significantly more new bone was produced within 2 weeks as compared with contralateral defects filled with empty fibrin or collagen which were the carrier materials for cmRNA formulations in TAMs.

DISCUSSION & CONCLUSIONS: mRNA transcript therapies are emerging powerful alternatives to gene and protein therapies and in certain cases may become the only safe option to treat or even cure so far undruggable diseases. While systemic administration of mRNA still poses numerous challenges with respect to delivery systems, target tissue selectivity and sustainability of mRNA translation, localized delivery is well established and safe. With TAMs, microgram quantities of mRNA can yield a sustained therapeutic effect. This is of particular interest in regenerative medicine when endogenous healing potential is impaired. For healing tissue defects, a localized and short-term translation of minor quantities of mRNAs into desired growth factors can kick-start self-sustained regenerative processes.

ACKNOWLEDGEMENT: Support by German Federal Ministry of Education and Research Go-Bio grant 0315986 is gratefully acknowledged.

DISCLOSURES: Ethris GmbH has commercial interest in cmRNA transcript therapy.

Establishment of *Xenopus laevis* as a model to study cellular and molecular facilitators of spinal cord regeneration

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INTRODUCTION *Xenopus laevis* offers the chance to compare spinal cord (SC) regenerative success in the tadpole to regenerative failure in the froglet. However neither a detailed procedure to induce SC transection nor a standardised method to quantify functional recovery have been described. We hypothesise that establishing such protocols and employing them to compare the tadpole and froglet response to injury will reveal key cellular and molecular mediators of regeneration.

METHODS: SC transection was performed in tadpole and froglet. Motor function was tested using a novel device of vibrating motors to stimulate the animals, tracking these afterwards with the Daniovision system. Timeline of repair was studied immunohistochemically using markers of neurons (acetylated anti-tubulin), radial glia (anti-BLBP) and growth (anti-GAP43). Proteomic and glycomic analysis was performed as in Fig. 2.

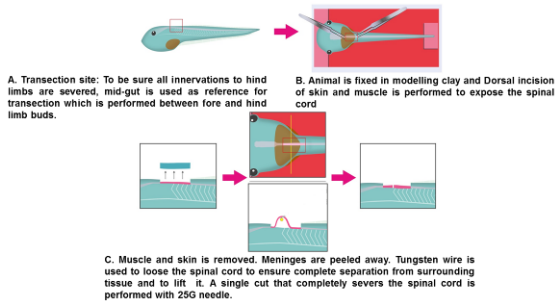

Fig.1: Schematic for spinal cord transection procedure.

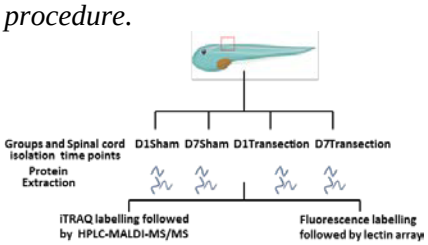

Fig. 2: Design and workflow of proteomic and glycomic profiling

RESULTS: Complete, reproducible SC transection was achieved (Fig.3) Significant motor recovery was seen at D7 (Fig.4). This was accompanied by the appearance of a neuronal bridge across the lesion (D7-14) (Fig.4). The axons bridging the damaged region travelled on an ordered cellular scaffold consisting of BLBP+ cells (Fig.5) and showed maximal growth activity at D5-7. Further, iTRAQ analysis at early repair stages revealed proteins involved in inflammation and regeneration. Changes in glycosylation status were also revealed.

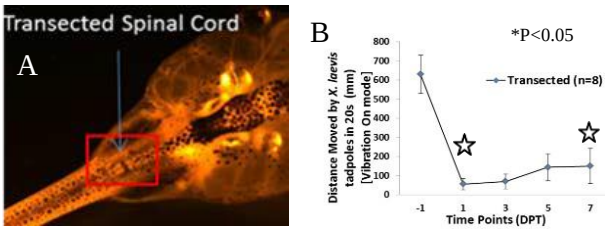

Fig. 3(A) SC transection of tadpole (B) Mean distance of a 20s swimpath following SC injury.

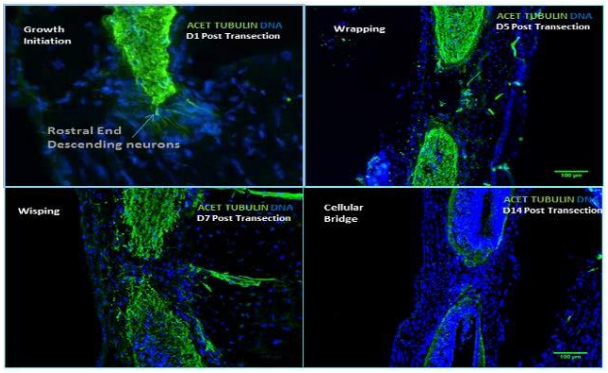

Fig. 4 Stages of spinal cord regeneration in Xenopus laevis tadpoles

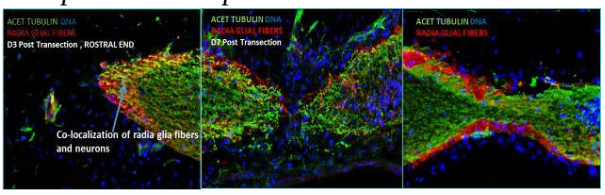

Fig.5 BLBP+ radial glia cells associate with regenerating axons at D3 and D7

DISCUSSION & CONCLUSIONS: A reproducible spinal cord transection procedure and quantitative behavioural test were established for *Xenopus laevis* tadpole. These were utilised to establish the mechanisms of SC repair.

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Exploring the therapeutic potential of Mesenchymal Stem Cells Secretome for Parkinson's Disease Regenerative Medicine Applications

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INTRODUCTION: The use of mesenchymal stem cells (MSCs) has been proposed as a promising therapeutic option for central nervous system (CNS) neurodegenerative disorders, including Parkinson's disease (PD). Their secretome has been suggested as the main responsible for their therapeutical effects, possessing a broad range of neuroregulatory factors that could promote an increase in neurogenesis, immunomodulation, neuronal and glial cell survival, as well as relevant neuroprotective actions into different pathophysiological contexts [1]. Indeed, we have previously shown that the secretome *per se* was able to revert the parkinsonian phenotype from both the histological (increasing densities of dopaminergic neurons) and motor outcomes. Having this in mind the objective of the present work was to assess the feasibility and possible advantages of using a secretome based therapy for PD, when compared to traditional cell transplantation approaches.

METHODS: Human MSCs derived from bone marrow (hBMCs) were expanded in tissue culture flasks, and their secretome collected and characterized as previously described [1]. Subsequently human progenitor cells (hNPCs) were incubated with MSCs secretome. Additionally MSCs and its secretome were also injected intracranially in the substantia nigra (SNc) and striatum (STR) in a 6-OHDA PD rat model. Effects on functional motor outcomes were assessed through the staircase and rotarod tests [2].

RESULTS: In vitro experiments revealed that MSCs secretome was able to differentiate hNPCs towards a neuronal phenotype (MAP-2$^+$ and DCX$^+$ positive cells) of hNPCs *in vitro*, fact that was related with the presence of neuroregulatory molecules present in their secretome and identified through MS (Galactin-1, PEDF, VEGF, BDNF, IL-6, GDNF). Moreover it was observed that when injected into a PD rat model, the secretome of MSCs induced increased functional motor outcomes, when compared to control (untreated PD animals) (Figure 1). Additionally it was also observed that the group injected with hBMSCs secretome disclosed similar levels to those obtained by the hBMSCs cell transplanted group (Figure 1). Similar findings were observed in the number of TH+ cells and fibers in the SNc and STR, respectively.

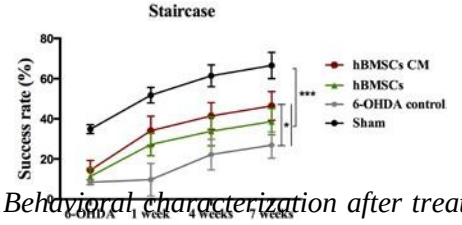

Fig.1: *Behavioral characterization after treatments. Performance of rats in staircase test is expressed as success rate of eaten pellets. *p< 0.05,***p <*

DISCUSSION & CONCLUSIONS: The secretome of MSCs potentiates neuronal differentiation, of human neural progenitors. When applied into a PD model, the secretome of MSCs lead to the recovery of motor performance of PD animals, demonstrating similar outcomes than those transplanted with cells. This indicates that the secretome of MSCs withholds promising characteristics for the development of future strategies for PD regenerative medicine, namely those dealing with the sole use of the secretome, without the need of cell transplantation.

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DISCLOSURE: The authors declare no conflict of interest

A mathematical model with the capacity to direct and accelerate the design of cellular peripheral nerve repair conduits

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INTRODUCTION: Implantable, bioengineered tissues are increasingly showing promise as clinical solutions to traditional treatments for diseased or damaged tissue. Currently the design of such devices is driven by the outputs of experimental research, without an overarching consensus on how to combine and spatially arrange biomaterials, cells and chemical factors to achieve a defined and tissue-specific outcome. Mathematical modelling has the potential to accelerate and refine this design process, reducing financial and time costs, whilst minimizing the required number of animal-based experiments. Here we present case studies on the design of tissue-engineered constructs for peripheral nerve repair, where tissue-engineered solutions currently fall short of the gold-standard autograft.

METHODS: We present two case studies based on the following questions:

(1) How should we arrange materials within a nerve repair construct to provide sufficient mechanical guidance for directed axon growth, whilst leaving enough space for regeneration?

(2) How should we distribute cells in a construct to ensure adequate oxygenation for cellular function, whilst stimulating angiogenesis *in vivo*?

In each case study, mathematical models are presented that describe the spatial distributions of materials and factors, and the cellular response to these mechanical and chemical cues. The models rely on parameter values, which are tissue specific and quantify important behaviours (for example, the rate of metabolism of oxygen by therapeutic cells). Outputs of the mathematical models are compared against experimental data in order to inform these parameter choices and validate the model predictivity. Finally, design proposals are made for peripheral nerve repair constructs.

RESULTS: (1) The mathematical models predicts a sensitive competition between increased mechanical guidance offered by increased material content, whilst leaving sufficient space for elongating neurites to grow. Fig. 1 shows the proportion of regenerating neurites that successfully transverse a construct, as a ratio of those generated at the proximal stump (hit ratio), for varying material content, and distance down the construct. A consistent optimum fluid volume fraction around 0.7 is identified. In a recent nerve repair experiment[1], 1500 phosphate glass fibres were arranged in a 1.8mm-diameter construct; the current analysis indicates increasing the fibre number to 3000 will improve regeneration.

(2) The mathematical model predicts oxygen, vascular growth factor and cell density levels in a construct as a function of position. Gradients in each are established due to the balance between solute diffusion and metabolism/ production, and cell proliferation and death. The model predicts the correlation between the minimum oxygen level in a construct, proportion of cell death, and production of growth factors required to induce angiogenesis and thus repair. Seeded cell distributions are presented that capitalise on this sensitive balance.

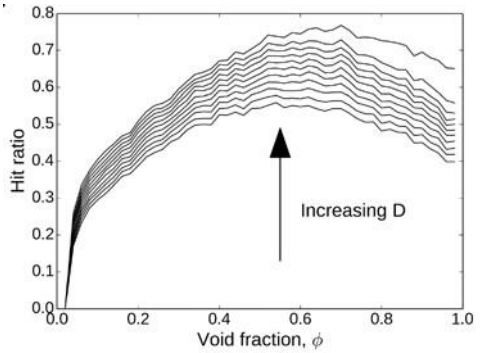

Fig. 1: Simulated hit ratios for a range of longitudinal distances down the construct, D, as a function of void fraction ϕ (volume fraction of fluid space in the construct).

DISCUSSION & CONCLUSIONS:

To develop the next generation of bioengineered tissue substitutes, it is essential to understand how to organise therapeutic cells and materials within constructs to support tissue regeneration and/ or function. Mathematical modelling has the potential to direct this design process, streamlining a field that currently relies on costly experimentation.

A novel method for sorting equine MSC subpopulations: isolation and characterization of pericytes and adventitial cells

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INTRODUCTION: The use of Mesenchymal Stem/Stromal cells in equine regenerative medicine has expanded dramatically in the last decade. Equine MSCs are typically derived from bone marrow or adipose stromal fractions, and are characterized according to International Society for Cellular Therapy guidelines. Clinical equine MSC preparations are heterogeneous in nature which results in variability in immunophenotype and therapeutic effects reported. Therefore, better defined cell populations that can be robustly isolated and expanded in culture are needed, particularly when considering the allogeneic use of MSCs. The aim of the present study was to isolate and characterize equine adipose stromal cell populations with regenerative capacity, identified in humans as pericytes (CD146+) and adventitial cells (CD34+ cells) [1].

METHODS: Equine adipose tissue (AT) was harvested post-mortem (n=6 horses; 2-20 years old) at the School of Veterinary Studies of the University of Edinburgh. Stromal vascular fractions (SVFs) were obtained [1] and cells sorted by Flow-Cytometry using a BD FACSAria Fusion (New Jersey, USA) and grown in DMEM 20% FBS (Thermo Fisher Scientific, Paisley, UK). QPCR and Flow Cytometry analysis were performed at passages 2 or 3. Adipogenic, osteogenic and chondrogenic differentiation were performed using StemPro kits (Thermo Fisher). Results were analysed by one-way ANOVA followed by post hoc Tukey's test and significance was set at $p<0.05$.

RESULTS: SVFs were processed by Flow-Cytometry Cell Sorting to isolate pericyte (CD146+) and adventitial cell (AC; CD34+) fractions, after elimination of CD45+ and CD144+ fractions, corresponding to hematopoietic and endothelial cells, respectively. Standard MSC cultures were also derived from direct culture of unsorted SVFs. All cultured cell types (Pericytes, ACs and MSCs) had an elongated shape and produced alkaline phosphatase positive colonies when seeded at low density. Pericytes, ACs and MSCs were able to differentiate into adipocytes, chondrocytes and osteocytes as evidenced by positive staining with Oil red O, Alcian blue and Alizarin red, respectively. QPCR analysis showed significantly higher expression of the pericyte marker, CD146, in cultured pericytes than in MSCs ($P<0.05$). ACs expressed variable levels of CD34, a gene which expression is lost in culture. Pericytes, ACs and MSCs expressed typical MSC markers (CD29, CD44, CD90, CD105) at similar levels, in agreement with flow cytometry analysis. All three cell types also expressed the angiogenic genes, VEGF and ANGPT1, although pericytes had significantly higher levels of VEGFA than ACs or MSCs ($P<0.005$ and $p<0.05$, respectively); and ACs expressed lower levels of ANGPT1 than MSCs and pericytes ($P<0.05$). These findings suggest a relatively higher capacity of pericytes to promote angiogenesis and experiments are currently underway to assess their potential in vitro and in vivo.

DISCUSSION & CONCLUSIONS: In this study we have successfully established a method to isolate equine pericytes (CD146+) and ACs (CD34+) and to expand and maintain these cells in culture. We have shown that both pericytes and ACs display an MSC-like phenotype, including expression of MSC markers and capacity for tri-lineage differentiation. These findings are in agreement with our previous results showing co-localization of MSC and perivascular markers in equine tissues [2].

Through their pro-angiogenic effects pericytes may provide a potentially superior regenerative subpopulation of MSCs.

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Determining scaffold optimal scaffold stiffness for *in vivo* models: a novel *in sillico* method

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INTRODUCTION: It has long been known that deformation of the soft tissue/haematoma is a driving force for tissue differentiation as bone regenerates in vivo. An optimal amount of deformation promotes bone formation, while overloading or under loading lead to increased levels of cartilaginous tissue formation or reduced levels of bone formation respectively, both can potentially lead to non-unions in extreme cases. Previously through using time-lapsed *in vivo* micro-computed tomography (microCT) in combination with micro finite element analysis (microFE), we have demonstrated that up to 88% of bone formation during the reparative stage of fracture healing occurs in tissue with a certain amount of strain energy density (SED) (Betts et al. 2015). We hypothesised that the material properties of a scaffold inserted into a healing bone defect will influence the amount of deformation in the tissue and consequently the size of the viable volume in which bone can regenerate. In this study we virtually implant scaffolds and perform a parameter study assessing the effect scaffold stiffness on potential bone formation.

METHODS:

A femoral defect of 1.24 [SD=0.13] mm was created in five female mice (C57BL/6) by first stabilizing the femur with an external fixator (MouseExFix, RISystem, Switzerland). Mice were scanned weekly using micro-CT imaging (vivaCT 40, Scanco Medical, Switzerland) over a period of 6 weeks, resulting in a series of time-lapsed images. The osteogenic strain range for bone formation in this empty defect was determined in a previous study (Betts et al. 2015). A scaffold of porosity 0.75 was virtually implanted (fig 1.) into the defect; the scaffold was 100 µm shorter than the osteotomy and matched the profile of the periosteal surface extending 50 µm radially further away from the surface. Material properties were assigned to the scaffold leading to elastic moduli between 2.5 MPa and 65 MPa of the bulk scaffold in the wet state. These models were then solved using ParOsol and the strain energy density (SED) calculated, the ratio of soft tissue voxels within the osteogenic SED range in the scaffold model compared to the empty defect was calculated, we term this the "viable volume fraction" (VVF).

RESULTS:

We found that a range of material properties exist (3-6 MPa) which increased the VVF above 100%, however that values both higher and lower result in decreased VVF as seen in fig. 1B.

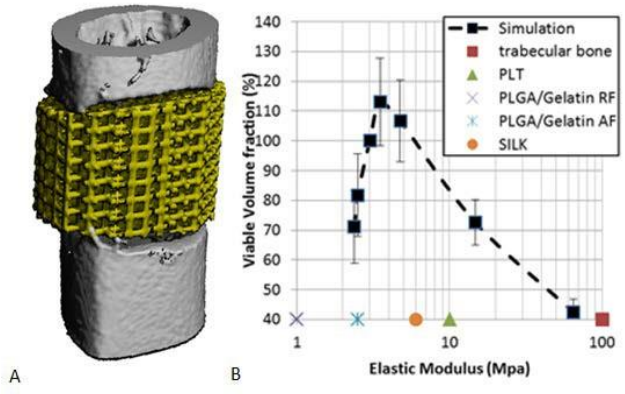

Fig. 1:A) Visualisation of the virtually implanted scaffold (yellow) in microCT scan of defect. B) The viable volume fraction of the different scaffold materials, as well as several materials commonly used for scaffold production.

DISCUSSION & CONCLUSIONS: This study neglects the (bio)chemical influence from the material instead focusing purely on mechanic effect. We have demonstrated the role scaffold material properties play in influence the local mechanical environment.

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"Functionalised polyurethane scaffolds mimicking cardiac primitive cell niche microenvironment by additive manufacturing"

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INTRODUCTION: Human cardiac fibroblasts (CFs) have been found to deposit *in vitro* a 2D "biomatrix" (BM) with similar composition to the natural cardiac extracellular matrix (ECM) [1]. The study of the behaviour of human cardiac primitive cells (CPCs) in contact with BM showed that laminin-1 (LN1) promotes the adhesion, viability, proliferation and differentiation of CPCs [1]. Recently, a polyurethane (PU) with elastomeric-like properties was synthesised and scaffolds were prepared by melt-extrusion additive manufacturing (AM) [2]. In this work, PU scaffolds were surface functionalised with LN1 and BM with the aim to reproduce CPC-niche microenvironment. Gelatin (G) was also grafted as a control.

METHODS: PU was synthesized from poly(ε-caprolactone) diol (Mn = 2000 Da), 1,4-budandiisocyanate and L-lysine ethyl ester dihydrochloride [2]. Bi-layered scaffolds with 0°/90° lay-down pattern were prepared by additive-manufacturing technique [2]. Functionalisation was performed by two steps: 1) acrylic acid grafting/polymerization following Argon Plasma treatment; 2) carbodiimide-mediated grafting of LN1, G or solubilised BM (produced by *in vitro* culture of CFs, followed by decellularisation and further BM solubilisation in a pepsin solution). Physicochemical characterisation of the surface coating was performed by XPS, FTIR-ATR, colorimetric tests, static contact angle measurements and ELISA assay. In vitro cell tests were performed using CPCs.

RESULTS: PU scaffolds showed a mean fibre diameter of 152±5 μm and mean spacing of 505±5 μm. FITR-ATR analysis of coated scaffolds showed higher intensity of the absorption bands at 3370 cm-1 (-OH and –NH stretching) and 1650 cm-1 (amide I). Contact angle decreased from 90° for PU to 60-70° for LN1, G and BM coated PU. XPS analysis confirmed the successful surface functionalisation. CPC proliferation on PU-LN1 scaffolds was higher than on PU scaffolds, increasing from 8.2 % on day 7 to 11.8% on day 14. LN1-functionalization also stimulated CPC differentiation into cardiomyocytes, smooth muscle cells and endothelial cells (RT-PCR analysis) (Fig.1). Scaffolds were found to slowly degrade *in vitro* by hydrolytic mechanism, whereas using an enzyme (lipase) degradation occurred in 3 weeks. Subcutaneous implantation of scaffolds in mice showed their tissue compatibility, low inflammatory response and low degradation rate (they were stable after 1 month).

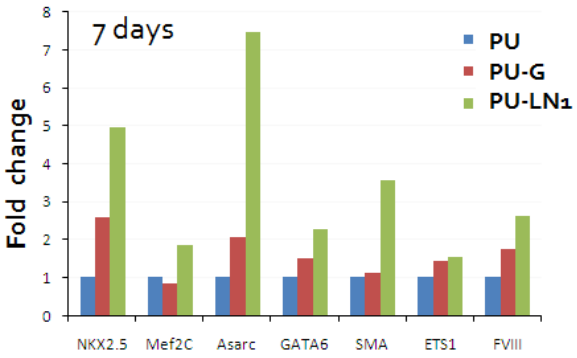

Fig. 1: RT-PCR analysis on CPCs cultures on PU, PU-LN1 and PU-G scaffolds.

DISCUSSION & CONCLUSIONS: PU scaffolds fabricated by AM and surface grafted with LN1 or BM were developed as 3D substrates mimicking CPC niche microenvironment. They could be used as cellularised patches for *in vivo* implantation in myocardial tissue engineering or as *in vitro* models of CPC niches to study CPC behaviour in both normal and pathological conditions.

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Dynamic hydrostatic pressure – a translatable osteogenic stimulus for mesenchymal stem cells in 3D hydrogel culture

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INTRODUCTION: Hydrogel scaffolds provide an ideal culture environment for many cell types, being able to approximate some of the biochemical and structural elements of tissues, yet it can be challenging to provide an appropriate mechanical stimulus to cells in such a soft matrix. Nevertheless, these physiological forces are known to be crucial in mediating critical aspects of tissue formation, function, growth and repair.

METHODS: Dynamic hydrostatic pressure is an important mechanical stimulus for cells throughout the body, transducing the compressive loading of tissues into a fluctuating change of pressure in the interstitial fluid which can then be detected by cells. In this study, a range of hydrostatic pressure regimes were applied to human mesenchymal stem cells in 3D hydrogel cultures using a bioreactor (*fig. 1*).

RESULTS: Stimulation of the constructs with 280kPa cyclic hydrostatic pressure at 1Hz (matched to normal physiological exercise) resulted in up to 75% mineralisation in the hydrogel, whilst standard incubator culture, constant high pressure or either low-frequency or low-magnitude stimulation had no effect (<2% mineralisation; *fig. 2*). These results suggest that hydrostatic pressure is a potent stimulus in stimulating MSC differentiation into actively secreting osteoblasts, and MSCs may be primed to respond to pressures within a specific physiological range.

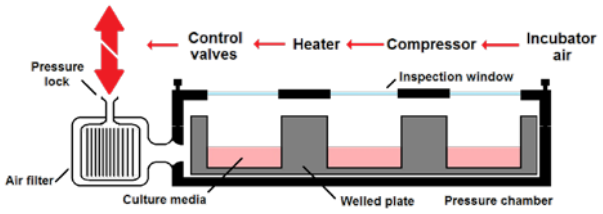

Fig. 1: Incubator air is compressed and directed into a chamber containing the cell-seeded hydrogels in a standard welled culture plate. Dynamic (cyclic) pressures are applied for one hour per day over 28 days.

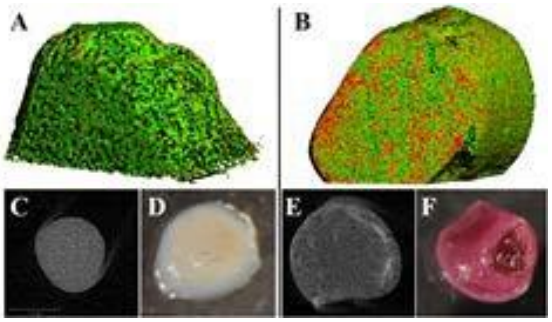

Fig. 2: Comparison of cell-seeded hydrogels after 28 days in either static culture (left) or stimulated (right). µCT reconstructions (A&B) show bone formation in red within the low-density green hydrogel. Mineralisation is also visible in 2D X-ray (C&E) and alizarin red staining (D&F).

DISCUSSION & CONCLUSIONS: As tissue engineering strategies are now entering clinical translation, understanding how physiological loading interacts with, drives and directs tissue growth will be fundamental to optimising treatments. Mechanical signals are therefore an essential component in recreating tissue environments and translating *in vitro* regenerative medicine into effective clinical treatments.

ACKNOWLEDGEMENTS: This work was supported by Biotechnology and Biological Sciences Research Council (BBSRC) Grants BB/G010560/1 (to Keele University). We also thank our collaborators in groups led by Prof. Kevin Shakesheff (University of Nottingham), Prof. Richard Oreffo (University of Southampton) and Prof. Molly Stevens (Imperial College London).

Epithelial-mesenchymal hair follicle model to study hair regeneration

AP Marques

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INTRODUCTION: Unlike to what might be commonly thought, hair follicle (HF) is a central skin appendage that in addition to adjust body temperature and facilitate evaporation of perspiration, regulates skin dendritic cells trafficking and acts as repository of multipotent cells capable of giving rise to all skin lineages. Thus, bioengineered HFs represent a major opportunity to advance the discovery of new therapeutic targets for hair loss disorders as well as to bring cell-based therapies for hair-follicle neogenesis closer to a therapeutic reality.

METHODS: Whether for the purpose of establishing realistic in vitro models or designing analogues for HF regeneration, reconstituting functional HF cell–cell interactions ex vivo, and stable human HF remains an important challenge. These cell-cell interactions are greatly influenced by the anatomical relationship between the epithelial and mesenchymal components as well as by the surrounding environment. Hydrogel's resemblance, in terms of high water content and physical properties, with the extracellular environment of natural soft tissues has been highly explored in the recreation of those niches. Gellan gum (GG), a bacterial exopolysaccharide[1,2], was chemical modified[3] rendering a versatile material into which can be coupled virtually any signalling molecule to design bio-instructive hydrogel-based matrices and generate the different relevant microenvironments. The generation of an epithelial mesenchymal model followed the rational that epithelial components with increased responsiveness to dermal/mesenchymal signals are determinant for the phenotype and efficiency of HF inductive structures containing human dermal papilla (DP) cells, the gold-standard mesenchymal origin cells with hair-inductive potential. From the perspective of generating a model that better reproduce the signalling network involved in HF formation, growth and cycling activities, epithelial-mesenchymal and intradermal adipose tissue interactions should be integrated.

RESULTS: Following a rationale based on the different extracellular matrix (ECM) compositions and the specificities of the targeted microenvironments, while the ECM glicosaminoglycans (GAGs) is simulated by the GG, the functionalities of the basement membrane that separates the two components, and of the DP ECM, are supported by peptide-functionalized GG. The selection of matrices that depict the properties capable to support the phenotype of the different cells and lead to a characteristic secretome is the ultimate goal to avoid compromising the reliability of the model.

DISCUSSION & CONCLUSIONS: To date, efforts to recreate a HF come down to few epithelial-mesenchymal models combining dissociated adult human HF cell populations, as microspheric clusters or within more or less organized hydrogels-based layers whose structural and biological resemblance to HFs remains disappointing. Additionally, from the perspective of inducing hair-follicle neogenesis, although these models have been proved sufficient to initiate it, efficient organogenesis is still to be demonstrated. This fact raises the question whether the key cell-cell and cell-ECM interactions and consequently the microenvironment signalling is being accurately reproduced and paves the way for the development of new models.

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3D Bioprinting of iPSC and chondrocytes with novel nanocellulosebioinks

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INTRODUCTION: Today, several hundred million people are suffering from joint disorders; like traumatic cartilage lesions that can develop into degenerative cartilage destruction leading to osteoarthritis (OA). The hypothesis is for such lesions to create cartilage from 3D bioprinted induced pluripotent stem cells (iPSC) for OA disease modelling or ultimately regenerative medicine transplantation. The advantages by using an established iPSC line [1] are unlimited, immortal characterized cell source, however, designing protocols that generates hyaline cartilage from pluripotent cells *in vitro* is still a challenge, due to that joint formation are late in development and far from the pluripotent state. There are recent protocols for hyaline-like cartilage generation from iPSCs [1]. The objective of this study is to develop a bioink for 3D bioprinting of human derived iPSCs and direct to cartilage.

METHODS: Two gel-forming Bioink components were tested (e.g. alginate or hyaluronic acid). Different bioink ratios of nanocellulose and alginate were prepared to encapsulate patient derived iPSCs and characterized as previously described [1]. After printing the mixture of bioink and iPSCs, cell proliferation and extracellular production was examined with label-free nonlinear microscopy (2-photon autofluorescence and second harmonic generation (SHG)) and histology for up to 4 weeks of differentiation using protocols with or without co-culture [1].

RESULTS: Bioprinting demonstrates homogenous distribution of cells with rounded morphology in 60/40 and more irregular shaped for 80/20. Using a lower concentration of nanocellulose yielded a higher number of viable cells. After 21days hyaline-like cartilaginous tissue appeared and label- free microscopy showed higher cell density in the more cartilage like area (GAGs, darker blue and cells green in Fig.1).

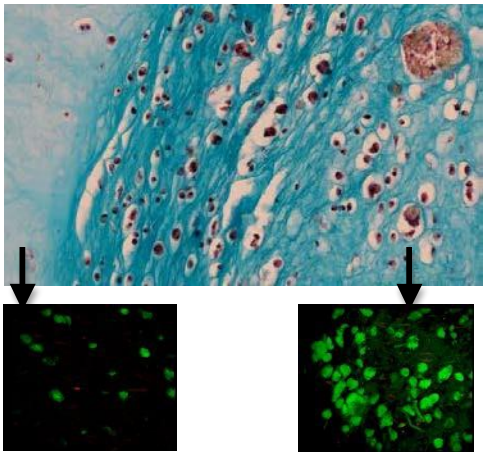

Fig. 1: Histology section of 3D printed constructs at 21 days of differentiation stained with Alcian Blue van Gieson for proteoglycans (GAGs) – (blue) and nuclei (brown) with corresponding nonlinear images to light and dark blue regions (fluorescence image cell-green, collagen-red).

DISCUSSION & CONCLUSIONS: The bioink of nanocellulose and alginate at 60/40 fractions demonstrated the best 3D support and environment for printing, survival and differentiation. Hylaline- like cartilaginous tissue was observed in the 3D prints after 3 weeks particular when using the co-culture protocol. We conclude that the developed nanocellulose bioink is well suited for bioprinting with iPSCs, sustaining cell viability over time, and support cartilage production.

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The influence of curvature on extracellular matrix components in 3D tissue engineered bone constructs cultured under static and perfused conditions

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INTRODUCTION: The adult skeleton constantly adapts its structure to changes in long-term mechanical loading. This continuous process of bone resorption and formation is called bone-remodelling [l]. The refilling of resorption sites *in vivo* has been shown to be most probably curvature driven [2]. *In vitro*, essential work on the influence of curvature on non-mineralized tissue in static cultures has been performed. Local curvature increased tissue growth formed by osteoblasts [3]. Cytoskeletal changes have been observed when comparing endothelial cells cultured on curved or flat surfaces. The changes were additionally enhanced when cells were exposed to perfusion [4]. The effects of curvature and perfusion on mineralized extracellular matrix (ECM) formation have, however, never been observed in 3D. Hence, the aim of this study was to investigate the effects of curvature on different ECM components in a 3D tissue engineered bone construct under static or perfused conditions.

METHODS: Porous silk fibroin scaffolds were produced by introducing three channels of different sizes using biopsy punches. The relative curvatures of the three channels were set to -50% in curvature (S channel, d=1mm) or +50% in curvature (L channel, d=3mm) with respect to the M channel (d=1.5mm). Scaffolds were seeded with $5x10^6$ human mesenchymal stem cells each, supplied with osteogenic medium and cultured in either static or perfused bioreactors (N=10 per group). Micro-computed tomography (µCT) was performed weekly to monitor mineralized ECM formation. Mineralized ECM was evaluated in three different volumes: (i) the 'full scaffold volume', (ii) the 'void volume' of the channel (90% of channel diameter), and (iii) the 'full volume' of the channel (channel diameter plus 1mm). Histology was performed to visualize cell nuclei and non-mineralized ECM stained with Haematoxylin&Eosin (H&E) and collagen stained with Sirius Red (SR).

RESULTS: µCT monitoring showed curvature dependent ingrowth of mineralized ECM into the 'void volume' of the channels (Fig. 1). The coverage of the channels increased with curvature and was additionally increased by perfusion (Fig. 1A-C, right). The morphology of mineralized ECM was more cortical-like in static samples (Fig. 1A-C, left) compared to the more trabecular-like structure in perfused samples (Fig. 1A-C, right).

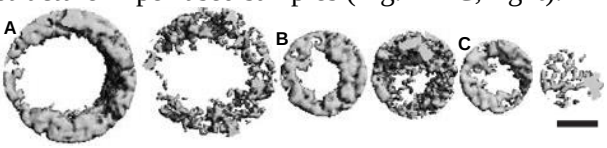

Fig. 1: 3D mineralized ECM in (A) L, (B) M, and (C) S channels (top view). A-C left: static samples. A-C right: perfused samples. Scale bar: 1mm.

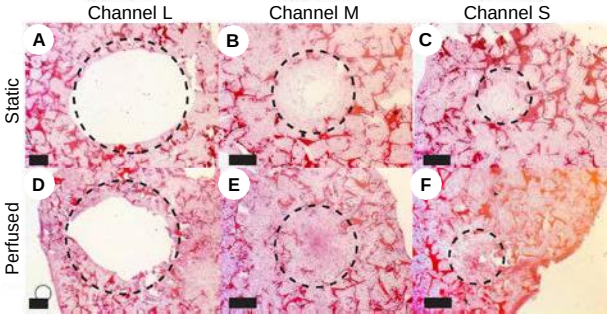

Fig. 2: H&E staining of: (A-C) static and (D-F) perfused samples. Scale bar: 1mm.

Histology images confirmed the results observed by µCT. H&E staining showed less coverage of channels with cells and non-mineralized ECM when cultured under static (Fig. 2A-C) compared to perfused (Fig. 2D-F) conditions. SR staining showed the same patterns for collagen.

DISCUSSION & CONCLUSIONS: This study showed a clear dependence of different ECM components like mineralization or collagen on local curvature. Perfusion showed to enhance the effects of curvature. Additionally, the results observed suggest a possible application of the scaffold model to investigate circular critical size defects in trabecular or cortical bone *in vitro.*

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Extracorporeal Shockwave Treatment accelerates peripheral nerve regeneration by altering Schwann cell phenotype

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**These authors contributed equally*

INTRODUCTION: The gold standard in peripheral nerve regeneration is the autologous nerve graft, where a sensory nerve is transplanted to a motor defect site. This treatment however shows inferior results for motor nerves, as the transplanted sensory Schwann cells and nerve channels differ in behaviour and structure. In this study we show how Extracorporeal Shockwave treatment (ESWT) improves peripheral nerve regeneration by altering Schwann cell phenotype.

METHODS:

In vivo model: A femoral nerve defect model was established in the rat. The effects of ESWT on motor fibers regenerating through a sensory environment have been evaluated using automated gait analysis, electrophysiology, histology and qPCR.

***In vitro* model**: Schwann cells have been isolated from motor, sensory and mixed nerves, respectively. Dissected nerves have been treated with ESWT prior to isolation. Cultured SCs were evaluated concerning expression of Schwann cell specific markers (S100b, P75, MAG, P0-flow cytometry) and proliferation (BrdU) in pro-proliferation (PPM)/pro-myelination (PMM) medium.

RESULTS: *In vivo* data indicate inferior regeneration of motor axons through a sensory nerve graft compared to a phenotypically matched graft. ESWT can ameliorate this effect and accelerate nerve regeneration. Cultured for 10 weeks in PPM all ESWT SCs showed no change in SC marker expression (P75, S100) or proliferation (BrdU), while marker expression and proliferation of control group steadily decreased. Motor SC cultured in PMM were able to switch to myelination quicker and to a higher extent than sensory SC (MAG, P0), with ESWT both phenotypes showed even stronger myelin-associated protein expression.

DISCUSSION & CONCLUSIONS:
Summarizing, significant differences in all parameters were observed only between the motor/sensory control group, and ESW treated groups showed improved results in vitro and in vivo. This study indicates that ESWT is able to accelerate peripheral nerve regeneration by altering SC phenotype in a model which reflects the clinical reality after autologous nerve transplantation.

In vivo micro-CT based approach for discrimination of physiological and impaired healing patterns in mouse femur defect models

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INTRODUCTION: Longitudinal non-invasive monitoring of the healing process is crucial in preclinical fracture healing studies. We recently described an in vivo micro-computed tomography (micro-CT) protocol, which was found to show no significant radiation-associated effects on callus properties [1]. In a second step, we now want to assess, whether the protocol is suitable for healing phase-specific detection of physiological vs. impaired fracture healing. For this purpose we monitored the healing process in femoral defect models with different gap sizes.

METHODS: Female 20 week-old C57BL/6J mice received a femur defect of either 1.2mm±0.1 (group 1, n=5) or 2mm±0.1 (group2, n=7), stabilised with an external fixator (MouseExFix, RISystem, Davos, Switzerland). During the healing period the animals received weekly scans of the osteotomy area (vivaCT 40, ScancoMedical, Brüttisellen, Switzerland, for detailed protocol see [1]). In order to assess dynamic callus parameters the scans from later weeks were registered onto earlier scans from the same animal. Statistics: Data were tested for normal distribution (Shapiro-Wilk-Test) and homogeneity of variance (Levene-Test). Comparisons of the two groups were done by Student's t-test or Mann-Whitney U-test. The level of significance was set at $p \leq 0.05$.

RESULTS: In the 1.2 mm defect group a physiological healing pattern was observed with distinct characteristics of the different healing phases (Figs. 1+2): In postoperative week 2 a significant 26x increase in bone formation was detected, indicating progression from the inflammation to the reparative phase. This led to a significant gain in bone volume in the defect with maximum osseous callus volumes being detected by week 3 (BV: $1mm^3$±0.4) and complete cortical bridging in 80% of animals at this time point. Subsequently, decreasing osseous callus dimensions in combination with increasing resorptive activities indicated the onset of the remodelling phase in postoperative week 4.

In contrast, in the 2mm defect group, both bone formation (week 3) and resorption rates (week 4) were significantly reduced by >50% with steady levels from weeks 2-4 and weeks 3-5, respectively (Fig. 2). Lack of characteristic peak values and only moderate onset of callus mineralization (BV_{week3}: $0.5mm^3$±0.2) indicated an impaired and delayed healing pattern.

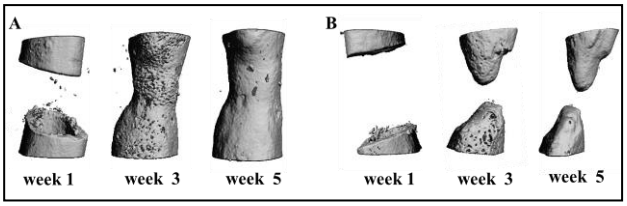

Fig. 1: Monitoring of the healing process by micro-CT. Reconstructions of the defect site and fracture callus for representative animals from the 1.2mm group (A) and 2mm group (B).

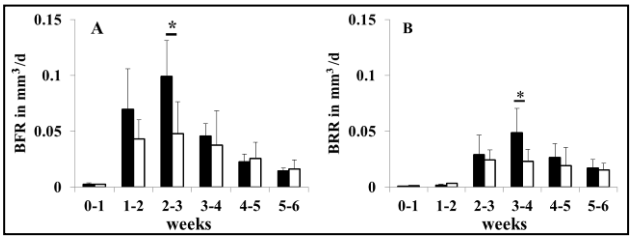

Fig. 2: Bone formation (A) and resorption rates (B) during defect healing of the 1.2mm group (black) and 2mm group (white).

DISCUSSION & CONCLUSIONS: Using the previously described scanning protocol with subsequent analyses of structural and dynamic callus parameters, we were able to detect callus properties indicative of the different fracture healing phases under physiological conditions. This method also allowed longitudinal characterization of the impaired and delayed healing process associated with increased defect size. In future studies this micro-CT-based approach will also be applicable to assess the healing potential of biomaterials in this preclinical femur defect model in mice.

ACKNOWLEDGEMENTS: The authors acknowledge financial support from the EU (BIODESIGN FP7-NMP-2012-262948).

Repair processes at the protein level – an exploratory whole-proteome profiling approach to follow up tendon and cartilage healing

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INTRODUCTION: Injured tendon and cartilage tissue does not regenerate but only repairs with scar tissue with significantly inferior biomechanical properties resulting in high re-injury rates and chronic disease. Therefore tendon and cartilage injuries are often reasons for reduced performance, early retirement from athletic use and chronic disability. Better understanding of the mechanisms behind tendon and cartilage repair would provide pivotal information required on the way to tailor regenerative therapies which induce regeneration rather than scar formation. Modern omics techniques offer a possibility to gain new insight into inflammatory and regenerative processes after cartilage or tendon injuries.

METHODS: The study was carried out in an ovine model. Standardized cartilage and tendon lesions were surgically induced in 2-4 year old sheep. Full thickness 7mm diameter cartilage lesions were drilled into the cartilage of the medial femoral condyle using a custom made hand drill. The lateral femoral condyle served as sham operated control. After debridement of the "injured" area, microfracture of the subchondral bone plate was performed using an awl. The tendon lesions were inflicted to the SDFT at the level of the Achilles tendon using a 3mm biopsy punch. Tendon and cartilage samples were harvested surgically according to the three stages of healing (inflammatory phase, reparative phase, remodeling phase) after 3 days, three weeks and 5 months. Samples plus sham operated and non-injured controls were collected and compared using three biological and three technical replicates per investigated time point. Exploratory whole-proteome profiling was chosen to identify key factors involved in adult healing. For mass spectrometry 30-100mg of tendon tissue were put into a non binding Eppendorf tube containing 5µL of PBS plus peptidic protease inhibitors. Tissue interstitial fluid was obtained by centrifugation. After centrifugation protease inhibitor was added and the sample frozen at -80° until further processing. The obtained protein fractions were digested with Lys-C and trypsin. The derived peptides were seperated by Nano Flow Liquid Chromatography (Dionex 3000 UHPLC) and analysed by mass spectrometry (Thermo QEXACTIVE orbitrap). Data were evaluated using Proteome Discover (Thermo) and MaxQuant software (free software by M.Mann). For accurate quantification of selected proteins, reaction monitoring using triple quadrupole mass spectrometry (Agilent 6490) was employed. Peptides were separated using nano-flow Chip-HPLC (Agilent) and quantified referring to isotope labeled internal standards.

RESULTS: It was shown that the chosen ovine cartilage and tendon defect models yield standardized and repeatable results. Both tendon and cartilage samples yielded sufficient tissue interstitial fluid for whole-proteome profiling. More than 3000 proteins could be identified and assembled from more than 30,000 distinct peptide identifications. The main factors identified were extracellular matrix proteins, growth factors and inflammatory mediators such as cytokines and chemokines. When comparing secretomes from injured samples with normal tissue samples, fold-control values greater than 2^{11} and p-values smaller than 10^{-11} were achieved, indicating robust and sensitive methodology and allowing analysis of the changes in the inflammatory response, growth factor expression and extracellular matrix compared to healthy cartilage and tendon tissue over the three healing phases.

DISCUSSION & CONCLUSIONS: The obtained data offering a broad overview of proteins expressed at different healing stages allows for a close insight into the processes of inflammation and healing. This may not only contribute to a better understanding of the interrelations during inflammation and healing but also help understand at which stage of healing to set which therapy measures to achieve full regeneration rather than scarring repair in the future.

Are encapsulated hepatic hetero-spheroids relevant for bioartificial liver?

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INTRODUCTION: To overcome organ shortage in liver transplantation, several types of bioartificial livers have been developed as temporary support but none of them managed to prove clinical efficiency yet [1]. These devices may suffer from a lack of efficiency in mass transport and hepatocyte functions, leading to limited clinical outcomes. As an alternative the potential of co-cultured hepatic cells encapsulated in alginate beads, to be placed in the fluidized bed bioreactor developed at UTC [2].

METHODS: Four different primary cell populations were isolated from a single rat liver: hepatocytes **(**HEP), stellate cells (SC), liver sinusoidal endothelial cells (LSEC), and Kupffer cells (KC). They were tightly packed in a spheroidal configuration according to pre-established cellular proportions and encapsulated in 800 µm alginate beads. Homo-spheroids (containing pure HEP population) represented the negative control. Culture in beads was performed over 7 days. Cell viability (Hoechst/IP) was assessed by fluorescence microscopy. Several hepatic functions, such as albumin and urea synthesis, ammonia detoxification, UGT and EROD activities, as well as cell polarization, were also analysed. Results were presented as mean ± SD of three independent experiments (n=3). Statistical significance was determined using nonparametric Mann-Whitney test by means of Prism - GraphPad tool (considering two-tailed test and confidence interval of 95%). P-values <0.01 were considered to be significant.

RESULTS: After 3 days of orbital oscillation culture, hetero-spheroids, formed by HEP and non-parenchymal cells (NPCs), were successfully encapsulated in alginate beads, as well as homo-spheroids for the control. Cell viability of spheroids was greatly preserved following encapsulation procedure (Figure 1). The addition of NPC-fraction determined the enhancement of detoxification activity (ammonia removal and consequent urea and glutamine secretion) and a slight improvement of synthetic functions (albumin and urea production) that was likely associated with a superior expression of connexin 32 and 43, responsible for homo- and heterotypic interactions but also for apical polarization of the HEPs. Fibronectin deposition may have also contributed to sustain the hepatic functions in these culture conditions. By contrast, non-parenchymal cells did not seem to beneficially affect hepatocyte phase I/phase II biotransformation activities.

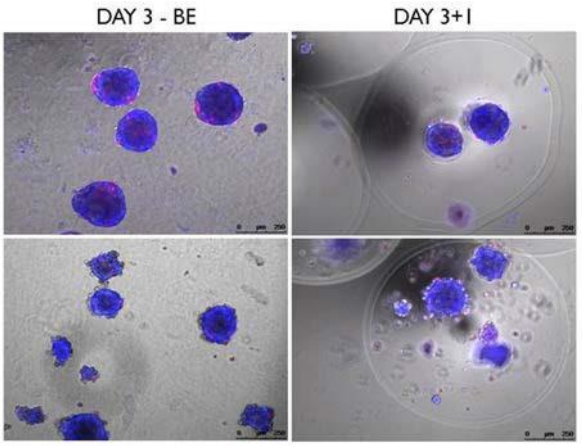

Fig. 1: Homo- (top) and hetero-spheroids (bottom) before (left) and after (right) the encapsulation step in alginate beads. (Blue staining indicates viable cells, pink one represents dead cells).

DISCUSSION & CONCLUSIONS: The very promising results regarding the cooperation between NPCs and HEPs organized as spheroids for the maintenance of liver functions *in vitro* need now to be translated to human primary sources and implemented directly after liver resection or non-transplantable liver collection *Proc IEEE Eng Med Biol Soc*, pp. 1335-8.

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BIOART/KIDNEY-LIVER

Glucose Delivery System Based-Hydrogel Composite Scaffold for enhancing MSC survival

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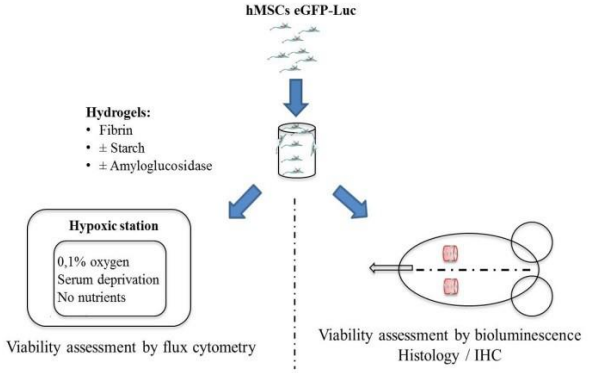
INTRODUCTION: In the context of cell-based regenerative medicine, exogenously administered mesenchymal stromal cells (MSCs) exhibited a poor survival rate. A possible explanation for this limited cell survival is that, upon implantation, MSCs encounter a considerable bioenergetic challenge in a harsh ischemic microenvironment characterized by low oxygen tension and nutrient deprivation. This issue can be overcome by in situ supplying glucose that acts as the main metabolic fuel for MSCs in severe hypoxia and significantly enhances their survival [1,2].
The objective of the present study is to engineer a tissue-construct providing glucose to MSCs in order to enhance their survival when transplanted *in vivo*. To achieve this goal, fibrin/starch (a polymer of glucose) hydrogels supplemented with alpha-amyloglucosidase (AMG) were prepared. The rationale is that enzymatic hydrolysis of starch by AMG will provide continuous production of glucose *in situ*.

METHODS: Hydrogels containing hMSCs, fibrin, starch and AMG were engineered and a follow up of cell viability was assessed both *in vitro* and *in vivo* as described thereafter.



RESULTS: Hydrogels were self-supported and released glucose amounts in accordance with that required by hMSCs for their survival. *In vitro*, under near anoxia, MSCs loaded in hydrogels containing starch and AMG exhibited a survival rate 115 times higher than the one loaded in fibrin hydrogels, after 14 days.

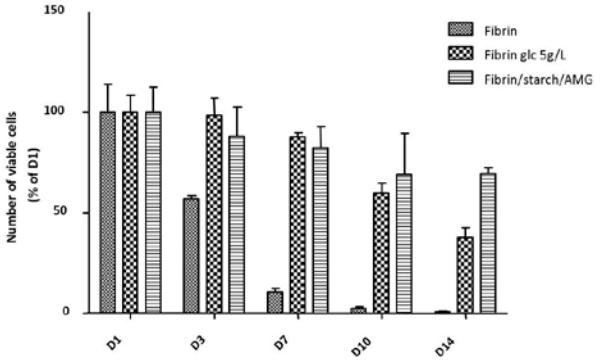


Figure 1: In vitro assessment of hMSCs viability

Moreover, when ectopically implanted in nude mice, luciferase-labelled hMSCs loaded in hydrogels containing starch and AMG exhibited a significant improvement of their viability (x10 after 14 days) in comparison to hMSCs loaded in fibrin gels as demonstrated by the follow-up of the luciferase activity by bioluminescence imaging. These data were further substantiated by immunohistochemically monitoring the number of human MSCs remaining in the hydrogels implanted ectopically in mice. At day 14 days, fibrin / AMG / starch scaffolds contained 7.5 times more viable hMSCs than fibrin hydrogels.

DISCUSSION & CONCLUSIONS: This work establishes for the first time the proof of concept that an innovative-engineered construct based on a starch hydrogel containing α-amyloglucosidase and starch is able to deliver glucose over time. Looking forward, and given the current wide interest in the use of cell for regenerative medicine applications, this new knowledge may be applied to develop improved regenerative medicine methodologies with enhanced cell survival. Further investigations are now needed to assess whether glucose-delivering hydrogel scaffolds can promote not only hMSC survival but also hMSC functionality.

Successes and hurdles in the translation of tissue engineered products for cartilage repair in clinical trials: focus on regulatory perspectives

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INTRODUCTION: Spontaneous healing of cartilage injuries is poor. Untreated cartilage defects are associated with pain and disability, and predispose to osteoarthritis. Current therapies, including innovative autologous cell-based treatments (exclusively based on articular chondrocytes), cannot predictably and reproducibly restore cartilage structure and function[1]. Based on several years of basic and pre-clinical research supporting the use of nasal chondrocytes as a suitable cell source for the regeneration of native cartilage, different clinical trials have been initiated by our group.

METHODS & RESULTS: Autologous nasal chondrocytes are isolated from a small nasal septum cartilage biopsy harvested in minimally invasive out-patient procedure. Nasal chondrocytes are expanded in vitro and then cultured onto a collagen membrane in chondrogenic medium to promote the deposition of abundant extracellular matrix (Fig. 1).

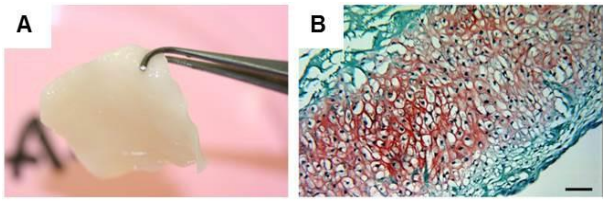

Fig. 1: Macroscopic (A) and histological (B, Safranin-O) picture of tissue engineered cartilage graft based on nasal chondrocytes.

In a Phase I clinical trial, tissue-engineered cartilage grafts were used for the reconstruction of nasal alar lobules, leading to a durable and stable repair with restoration of function and aesthetically satisfactory outcomes[2]. Based on subsequent *in vitro* and pre-clinical studies, a further Phase I clinical trial was conducted in which autologous nasal cartilage tissues were engineered for the treatment of full-thickness cartilage defects in the knee joint, providing evidence of engraftment of the graft within the defect site[3]. While the Phase I trials have demonstrated the safety and feasibility of the approach, a multicenter Phase II clinical trial is being initiated to finally prove efficacy of the tissue-engineered nasal cartilage grafts for the treatment of cartilage lesions in the knee [www.biochip-h2020.eu]. In parallel, innovative bioreactor-based platforms that automate, standardize, and scale up the production process are developed with the aim to foster exploitation of tissue-engineered products for widespread clinical use[4] [www.biocomet.eu].

DISCUSSION & CONCLUSIONS:
The translation of research scale production models into clinically applicable manufacturing designs that are compliant with regulatory requirements (e.g., GMP, GDP, GCP) and economically sustainable poses major challenges to bring tissue-engineered products into routine clinical practice. Successes, struggles and setbacks have characterized our way and will be discussed: from the bench to clinical trials, from single to multiple centres in Europe with centralized GMP production, from conventional to automated manufacturing. Close collaboration among clinicians, academic institutions, industrial partners, and regulatory agencies is being crucial to expedite the route to successful clinical translation of engineered tissue products.

ACKNOWLEDGEMENTS: Funding for this research has been received from Deutsche-Arthrose-Hilfe, from the European Union's Seventh Programme for research, technological development and demonstration under grant agreement No. 278807, BIO-COMET and from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 681103, BIO-CHIP.

Angiogenesis study in agarose-collagen type I hydrogels

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INTRODUCTION: Tissue Engineering is focusing evermore on angiogenesis to supply desired tissues with nutrients and oxygen. Planar and avascular tissues like cartilage and skin have already been engineered successfully [1-2]. However, adequate fabricate of scaffolds for thicker, more complex, and particularly tubular constructs, is still unavailable. Therefor 3D printing is a promising technique to combine rapid bio fabrication of scaffold materials with homogenous cell distribution for pro-angiogenic effects. Printable and more importantly angiogenesis –supportive hydrogel scaffolds have not been investigated yet. This study reveals hydrogel combinations showing desired characteristics in printing and angiogenesis evaluation.

METHODS: Experiments (n=3) were performed with endothelial cells from umbilical cord vein (HUVECs) and dermal fibroblasts from foreskin (HDFFs) with $3x10^6$ cells/mL hydrogel each. Hydrogel moulding was performed with fibrin gel scaffolds through the addition of $CaCl_2$ (4.75 mM) and thrombin (3 U/mL) to a fibrinogen solution (5 mg/mL). Fibrin gel was used subsequently as a control hydrogel. Hydrogel combinations of agarose and collagen type I were moulded as follows: Agarose solutions were diluted to final concentrations of 2 or 5 mg/mL, respectively. Collagen solution was diluted with sodium hydroxide (1 M), 10x DMEM Glutamax and DMEM medium to final concentrations of 2 and 5 mg/mL. After a cultivation time of 14 days samples were fixed and stained for CD31/Alexa Fluor 594 and HE staining. Evaluation was done by fluorescence, inverted light and two-photon laser scanning microscopy (TPLSM). For evaluation of structure length, volume, and area, Image-Pro software was used.

RESULTS: The study showed significant results in the comparison of structure length, mean volume of blood vessel-like structures, and vessel area, in tested hydrogel combinations. The most promising hydrogel was a mixture of 5 mg/mL of agarose and of collagen type I showing the formation of capillary structures with a mean length of 43.56 µm, a mean volume of 11724,65 μm^3 and 3995.25 μm^2 of area covered by capillaries.

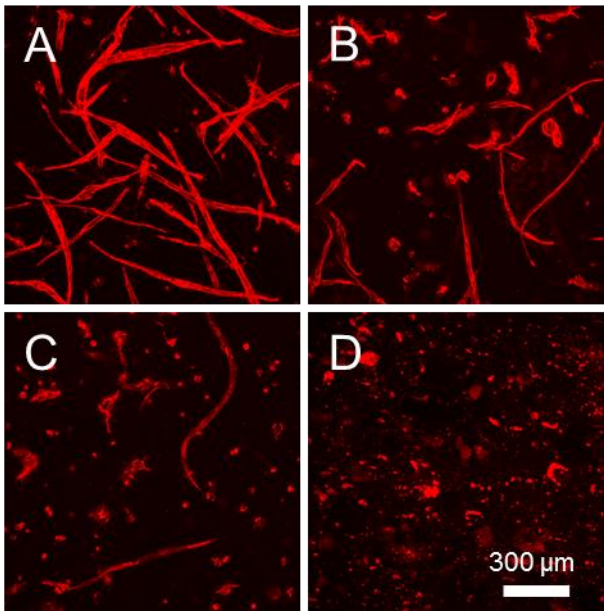

Fig. 1: TPLSM images for fibrin hydrogel (A) as control, printable hydrogel compositions of agarose- collagen in different concentrations (B; 0.5%-0.5%), (C; 0.5%-0.2%), (D; 0.2%-0.5%) stained for CD31/Alexa Fluor 594. Scale: 300 µm.

DISCUSSION & CONCLUSIONS: This study revealed the complexity and challenge of finding a balance between printable, and angiogenesis compatible, hydrogel compositions coping with an intense iterative process. Based on these initial results it is now possible to focus on 3D fabrication, and angiogenesis-supported supply, to highly complex tubular constructs such as the trachea, oesophagus and ureter.

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A joint theoretical-experimental approach to investigate the effects of low oxygen environments upon therapeutic cell viability and VEGF production

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INTRODUCTION: The speed and efficiency of progress in tissue engineering is hindered by the need to investigate experimentally a huge number of different parameters, such as initial seeded cell types and densities, which can impact the clinical efficacy of designs. A careful balance, dependent on oxygen levels, must be achieved to obtain sufficient levels of VEGF for vascularisation of engineered tissue, whilst maintaining a population of viable therapeutic cells. Mathematical modelling, a powerful tool able to make predictions based upon *in vitro* data which can be used alongside experimental work, has the potential to accelerate and direct tissue engineering design. The aim of this work was to investigate the effect of oxygen conditions upon the production of VEGF and the viability of stem-cell derived therapeutic cells, via a symbiotic theoretical-experimental method. The results of the work can be used to inform the design of nerve regeneration constructs and demonstrate the benefits of this interdisciplinary approach.

METHODS: Differentiated adipose-derived stem cells (dASCs) were maintained in plastic compressed collagen gels with various initial seeding densities and oxygen levels. Viability was assessed using CellTiter-Glo (Promega) and VEGF release measured using ELISA after 24h. A mathematical model able to simulate the interactions between oxygen and VEGF concentrations and cell density within both the *in vitro* gel and peripheral nerve construct scenarios was created. The theoretical framework consists of three coupled differential equations, including terms representing the processes of diffusion, cell proliferation and death, VEGF production and oxygen consumption. Parameters within the model were assigned using values from the literature and parameter fitting based upon the experimental results [1-3].

RESULTS: Initial cell density and atmospheric oxygen concentration both appear to impact viability over time (Figure 1). Model simulations show that initial conditions have a large effect upon subsequent conditions within a construct.

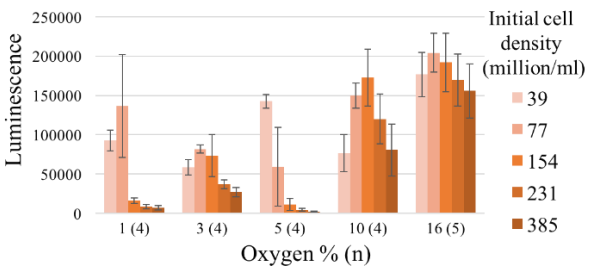

Fig. 1: dADSC viability in collagen gels after 24h incubation with different concentrations of controlled atmospheric oxygen (mean ± SEM).

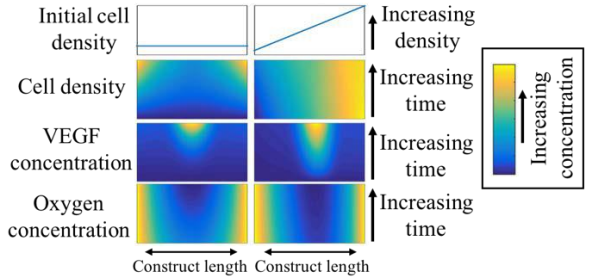

Fig. 2: Results of model simulation. Initial cell density has an effect upon spatial distributions of solutes within a peripheral nerve construct.

DISCUSSION & CONCLUSIONS: The experimental results suggest that there is an optimal initial seeding cell density that will ensure good continued cell viability within engineered tissue, under low oxygen conditions. The theoretical framework can be used to investigate in more detail which values and spatial distributions of cell density and oxygen provide a good balance between levels of cell viability and VEGF.

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3D manufactured islet micro-tissues for the treatment of type-1 diabetes

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INTRODUCTION: Type-1 *diabetes mellitus* is defined by the inability to control systemic glucose levels, due to an autoimmune destruction of the insulin-producing β-cells. Treatment involves routine glucose monitoring and insulin infusion. However, some patients with hypoglycaemia unawareness may require pancreas or islet transplantation [1]. Islet transplantation is a minimally invasive 'micro-tissue' therapy aimed at reducing hyperglycaemia and eliminating severe hypoglycaemia. This is achieved by infusing islets of Langerhans into the portal vein; where they engraft in the liver. Major limitations of islet transplantation include the need for life-long immunosuppression and a high tissue demand [2]. This work aims to overcome these restrictions and improve engraftment by applying 3D bioprinting to standardise islet size and ECM composition with the goal to reduce tissue loss immediately post transplantation and improve engraftment. A tri-component 'bioink' hydrogel was developed and investigated using collagen, alginate and fibrin (CAF), at three collagen concentrations (0.5%, 1% and 2.5%), for β-cell replacement therapy to treat type-1 diabetes.

METHODS: CAF hydrogels were produced +/- MIN6 β-cells. Morphology was characterised using SEM and TEM, while chemical composition was evaluated using XRD and FTIR. Mechanical properties, viscosity and biodegradation of the hydrogel material was also analysed. CAF hydrogels seeded with MIN6 β-cells were cultured for 7 days, to assess biocompatibility. Micro-tissues <150 μm were manufactured using an *nScrypt* 3Dn printer and evaluated for viability, metabolic activity, and functionality via immunostaining for insulin and E-cadherin.

RESULTS: CAF hydrogels with 0.5%, 1% and 2.5% collagen demonstrated pore size in the 40-200 μm range (Fig. 1A). 0.5% and 1% CAF hydrogels had highly inter-connected porosity, which was not present in 2.5% gels. TEM showed collagen fibre formation in hydrogels made with 1 % collagen, but not in 0.5 and 2.5% gels (Fig. 1B). Rheological testing demonstrated that all CAF hydrogels had a G' modulus of ~1000 Pa, similar to human pancreatic tissue [3]. 1 and 2.5% CAF hydrogels were stable for >15 days of biodegradation, while 0.5% hydrogels disintegrated after 5 days. Biocompatibility assessed by cell viability and metabolic activity was inversely proportional to collagen concentration, with the best results found for 0.5% collagen. Furthermore, bioink extrusion became more difficult as collagen concentration increased and 2.5% CAF hydrogels could not be accurately extruded. Insulin and E-cadherin immunostaining demonstrated similar functionality of MIN6 β-cells before and after bioprinting.

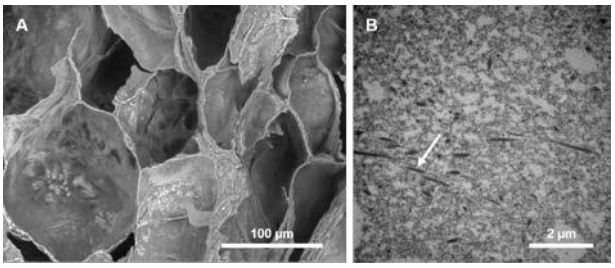

Fig. 1: SEM (A) of hydrogel architecture and TEM (B) of collagen fibre formation (indicated by arrow), in CAF hydrogels made with 1% collagen.

DISCUSSION & CONCLUSIONS: A novel collagen, fibrin and alginate hydrogel (CAF) was developed for use as a bioink substrate in the manufacture of islet micro-tissues. A general enhancement in mechanical properties, porosity and resistance to biodegradation was observed with increasing collagen content. This was offset by reduced biocompatibility and an increased difficulty to extrude the bioinks, which may make printing of CAF hydrogels impossible. 7 day culture of micro-tissues highlighted the influence of hydrogel structure and composition on cell compatibility in 3D – demonstrating the necessity for appropriate bioink formulation as a substitute for native ECM in manufactured micro-tissues.

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Recellularization of skeletal muscle extracellular matrix for tissue engineering approaches

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INTRODUCTION: Recently we characterized the extracellular matrix (ECM) obtained from decellularized mouse diaphragmatic muscle [1]. Given the important cues that this scaffold displayed, such as biomechanical features similar to the fresh tissue, immunological properties and angiogenic activity, we focused our research on the in vitro creation of a complete graft using mouse decellularized diaphragm-derived ECM and human muscle precursor cells (hMPCs) isolated from paediatric biopsies.

METHODS: Skeletal muscle ECM were obtained with 3 detergent-enzymatic treatments as previously described [1]. Human MPCs were isolated from paediatric biopsies following the protocol described for adult samples [2].

After 5-6 passages in culture, hMPCs were characterized (phenotypic profile, myogenic markers expression, fusion ability) and about 500.000 cells/cm2 were injected into diaphragm ECM. The recellularized grafts were cultured for 4, 7 and 15 days. At each time point patches were analysed for the expression of myogenic markers both at protein and molecular level.

RESULTS: Diaphragm ECM was able to sustain the expansion and differentiation of hMPCs. In all the analysed samples, cells covered between 55 and 65% of the scaffold, remodelled ECM and increased its thickness. After 4 days in culture inside the matrix, 10% of cells were Ki-67 positive, and at the same time a good percentage of cells expressing myogenic transcription factor such as MYOD and MYF5 was found. After induction with proper stimuli, in the last time points there was an increase in the expression of structural protein like Sarcomeric Actin and Myosin heavy chain.

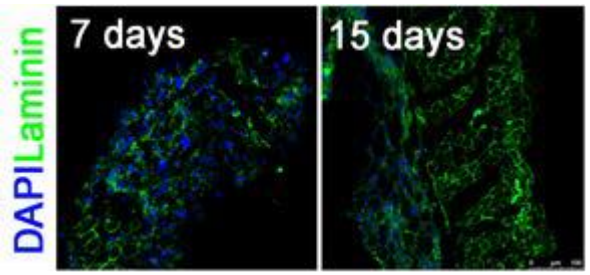

Fig. 1: Images of recellularized patches after 7 and 15 days in culture. Cells were stained with DAPI, scaffold was stained with Laminin.Table 1. Relative allocation and amount of resources in research.

DISCUSSION & CONCLUSIONS: With the aim to develop a xenograft model using mouse ECM and human cells as proof of principle for future clinical application, we developed and characterized in vitro a recellularized skeletal muscle patch.

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Development of an advanced 3D-engineered neuronal cell culture as a screening tool for drugs promoting peripheral nerve regeneration.

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INTRODUCTION: Injuries associated with peripheral nerve damage effect ~1 million patients per year. Damage to the peripheral nervous system can be extremely debilitating and result in loss of end organ function, coupled with poor neuron regeneration capacity [1]. Currently the treatment option for injuries are microsurgical and there are no drug therapies available to improve recovery. Pharmacological treatments could potentially be used to maintain neuronal viability, encourage axonal growth, improve axonal specificity to end-organ targets and reduce neuropathic pain. Some drugs and targets have been identified but there are challenges associated with understanding mechanisms of action and moving therapies towards clinical translation. For successful regeneration following injury Schwann cells need to support regeneration and myelination of neurons [2]. This 3D cell-cell interaction is a key feature that needs to be recreated in order to regenerate effective in vitro models. Engineered neural tissue (EngNT) supports the regeneration of neurons within an aligned 3D Schwann cell-seeded collagen gel environment and has the potential to be used as an effective co-culture model. The aim of this study therefore was to develop an assay based on EngNT to analyse the effect of drugs on neurite growth. Initial studies tested the effect of ibuprofen, a drug that has shown some positive effects on nerve regeneration in animal models [3].

METHODS: Anisotropic 3D co-culture gels were prepared by tethering 1ml of solution containing; 80% Type I rat tail collagen, 10% 10x MEM, 5.8% neutralising solution and 4.2% Schwann cell suspension, within rectangular moulds to facilitate cellular self-alignment. After 24h incubation at 37°C, the aligned cellular gels were stabilised using plastic compression and PC12 neurons were added to the surface. Co-cultures were subjected to drug treatments for 72h before fixing. Neurites were visualised using βIII-Tubulin immunoreactivity and fluorescence microscopy. Neurite growth was quantified by measuring neurite length using ImageJ software.

RESULTS: Treatment with 100µM ibuprofen elicited an increase in neurite length of 54% compared to the control (Fig 1).

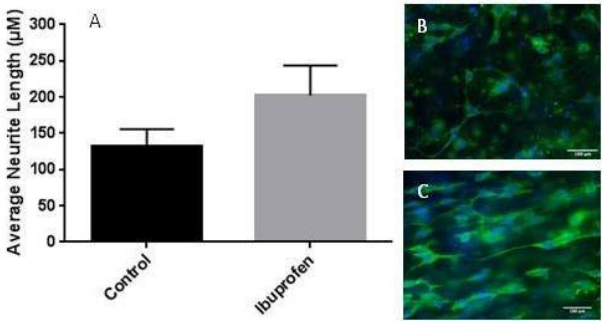

Fig. 1: A -Neurite length after 72 h in the presence or absence of 100µM ibuprofen. Representative fluorescence, micrographs show βIII-Tubulin immunoreactivity (green) and DAPI (Blue) in control (B) and ibuprofen treated co-cultures (C).

DISCUSSION & CONCLUSIONS*:* The results demonstrate that this engineered tissue model containing neurons and Schwann cells aligned within a collagen hydrogel mimics the pro-regenerating effects of ibuprofen seen previously *in-vivo*. This indicates that the 3D engineered co-culture gels can be used as an effective assay for screening drugs that have the potential to improve nerve regeneration.

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Strontium-doped bioactive glasses: bone repair and cellular global responses

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INTRODUCTION: Bioactive glasses (BG) have shown enhanced repair of small bone defects through stimulation of both angiogenesis and osteogenesis. The glass compositions are highly tailorable, which not only allows the modulation of their biological performance, through modification of the ionic release profile, but also plays a critical role in their formulation as 3D porous materials [l]. Incorporation of strontium ions within BG (SrBG) has been shown to positively influence osteoblast activity *in vitro* [2], and favour osteointegration *in vivo* [3], although the mechanism involved in these events has yet to be elucidated. Here, we hypothesised that SrBG with an optimised composition and 3D architecture could provide an attractive solution for the challenging regeneration of critical-sized bone defects. We further aimed to gain knowledge on the effects of strontium incorporation within BG on the global responses of human mesenchymal stem cells (hMSC).

METHODS: A novel porous SrBG was developed and implanted in a critical-sized cancellous bone defect in the medial femoral condyle of adult sheep. Bone formation was assessed after 6 and 12 weeks of implantation. For *in vitro* experiments, BG with 0, 10 or 100 mol% strontium substitution (for calcium) were incubated in cell culture medium, which allowed a dose-dependent release of strontium. Following exposure of hMSC to the conditioned-media, gene expression levels were determined using whole genome Affymetrix ST 1.0 gene arrays and the most relevant changes were identified by functional annotation clustering analysis. Further investigations included TIRF microscopy and Raman spectral imaging.

RESULTS: We developed a novel SrBG that possesses an optimised 3D porous architecture for the healing of substantial bone defects. Its implantation in critical-sized bone defects for 6 to 12 weeks showed improved bone-to-material contact, when compared to 45S5 BG. To determine whether this was a result of the demonstrated Sr ion release from the scaffold, we then aimed at uncovering how Sr incorporation within BG affects the global responses of hMSC. Our objective whole genome microarray analysis revealed exposure to SrBG-conditioned medium had a significant impact of hMSC gene expression [4]. In particular, SrBG treatment strongly up-regulated metabolic pathways that are critical to the biosynthesis of sterol and steroid metabolites, and protein prenylation. These further translated to changes in cell cholesterol content and membrane composition and, importantly, were accompanied by an increase in actin/myosin activity, a key regulator of hMSC commitment.

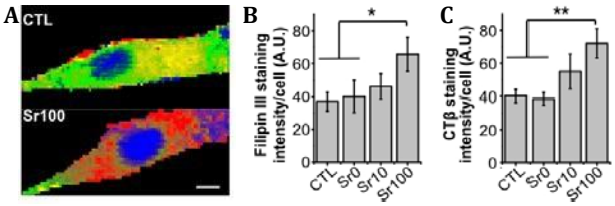

Figure 1: *A) Raman spectral maps of hMSC cultured in basal (CTL) and Sr100-conditioned medium (red and yellow = cholesterol/lipid rich areas. Bar = 10µm). B) & C) Quantification of Filipin III (cholesterol) and cholera toxin β (lipid raft content) abundance from TIRF images (mean +/- SD, n = 3, * p< 0.05 using 1-way ANOVA).*

DISCUSSION & CONCLUSIONS: This study highlighted that SrBG, optimised in structure and composition, supported high quality critical-sized bone repair. *In vitro* studies further demonstrated that the incorporation of strontium within BG triggers important unexpected cellular responses through modification of ionic environment.

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Disclosure: M.M.S. is co-inventor on IP on SrBG.

The manufacture of synthetic stem cell niches for regenerative medicine.

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INTRODUCTION: Over the past decade the interest in understanding the mechanisms that govern 'the stem cell niche' has grown considerably. The ability of controlling and comprehending the complexity of the stem cell microenvironment opens the door to a broad range of applications in the healthcare sector, not only based on the possibility of creating innovative analytical tools and drug screening models, but in the field of regenerative medicine with the design of the future generation of smart biomaterial devices. Although the native stem cell niche is characterised by its intricate nature, mimicking certain aspects of the niche is a plausible approach that has recently become a challenging and cutting-edge area of research. Dr. Ortega and coworkers have established a new research line at The School of Dentistry in Sheffield with the aim to create a platform technology for the development of synthetic niche-equipped smart microfabricated devices and *in vitro* models for ultimately control and direct stem cell behaviour for a range of tissue regeneration applications including skin, cornea, brain and musculoskeletal tissues.

METHODS: Prototype microfabricated membranes have been manufactured using a patented combination of novel 3D-printing techniques and conventional electrospinning [1]. Computer-designed templates fabricated using stereolithography and selective laser melting were used as electropinning collectors. The additive manufacturing part of the fabrication allows the creation of intricate structures which can simulate to a certain degree the morphology and size of the native stem cell niche; on the other hand, the use of electrospinning techniques allows the creation of biodegradable membranes and the use of FDA approved polymers (polycaprolactone and poly lactic-co-glycolic acid have been used in this work). The microfabricated membranes have been evaluated using a range of cell lines and tissue models including limbal cells, hypothalamic cells and primary mesenchymal stromal cells (MSCs).

RESULTS: We have developed and optimised a method for the fabrication of versatile devices and models containing well-defined synthetic microenvironments (Fig.1) which can be tested using a broad range of cell lines and 3D tissue models. Using cornea, brain and MSCs models, we have demonstrated that the presence of tailored microfeatures has a direct effect on different aspects of cell behaviour including migration, cell morphology and stemness.

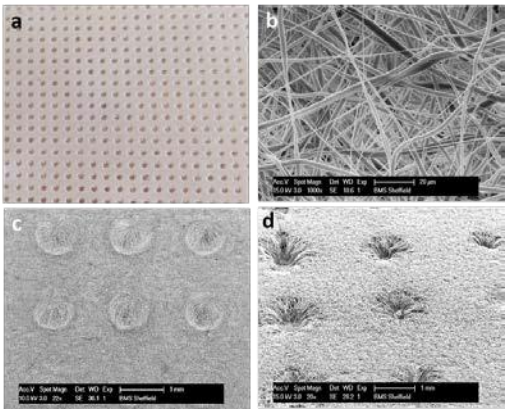

Fig. 1: Example of microfabricated membrane with circular artificial microenvironments (a); Scanning electron microscopy (SEM) image of a PCL membrane (b); SEM images of different types of microenvironments made using SLM and Electrospinning (c, d).

DISCUSSION & CONCLUSIONS: The development of this platform technology sets the basis for the creation of artificial 3D microfabricated constructs with the ability of controlling stem cell fate; this can be ultimately translated to the design of innovative *in vitro* models and to the manufacture of biomaterial devices presenting enhanced regenerative capability with potential use in the regeneration of hard and/or soft tissues.

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Non viral collagen-nanohydroxyapatite microRNA-activated scaffolds rapidly enhance human mesenchymal stem cells bone forming capacity

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INTRODUCTION: microRNAs (miRNAs) are short non-coding RNA molecules which have recently emerged as promising therapeutics to stimulate bone repair, owing to their ability to intercept entire gene cohorts [1], in addition to the availability of enhancers and inhibitors (miR-mimics and antagomiRs). However, the development of a safe and efficient localised delivery system is required for successful clinical translation of miRNA therapeutics to bone tissue engineering. The overall goal of this study was to determine the potential of non-aggregating nano-sized hydroxyapatite particles (nHA; [2]) and collagen (coll)-nHA scaffolds [3] as non viral 3D platforms for the delivery of a series of miRNAs to human mesenchymal stem cells (hMSCs), maintaining functional silencing activities, and to determine the miRNA candidate leading to optimal osteogenesis while coupling with angiogenesis.

METHODS: nHA-miRNA complexes were formed in situ [4] and coll-nHA scaffolds synthesised using a freeze-drying technique [2]. nHA-miRs were physic-chemically characterised and used to activate the scaffolds. Picogreen and MTS cytotoxicity assays, flow cytometry for uptake efficiency and qPCR for silencing functionality -reporter targets: GAPDH (mimic), miR-16 (antagomiR)- were performed. Therapeutic candidates: miR-210 mimic, antagomiR-16, -133a and dual combinations of these, targeting key orchestrators of osteogenesis (BMP-TGFβ, Runx2) and angiogenesis (Ephrin-VEGF). Osteogenesis: osteogenic gene expression and calcium deposition were analysed by qPCR, alizarin red staining and a Calcium Liquicolor kit. Angiogenesis coupling: VEGF Enzyme-Linked Immunosorbent Assay (ELISA) and Matrigel tubulogenesis assays were carried out. Statistics: two-way ANOVA plus Tukey post-hoc test, $p<0.05$ (*) and $p<0.001$ (**).

RESULTS: nHA particles combined with low miRNA doses (20 nM) demonstrated significant uptake and highly pronounced silencing activities in hMSCs, to a level comparable to viral and lipid-based vectors, while resulting minimally cytotoxic. Following this, miRNA activated coll-nHA scaffolds demonstrated silencing functionality over 2 weeks, shown by the significant decrease in the % target expression (Fig. 1A). Subsequently, all nHA-miR therapeutic candidates enhanced hMSC osteogenesis while the combinatorial delivery facilitated VEGF secretion and tubulogenesis. From this study antagomiR-133a emerged as the optimal osteo-therapeutic and when incorporated into the scaffolds upregulated Runx2 and orchestrated an enhanced calcium deposition by just 14 days in culture (Fig.1B), thus denoting a promising bone repair potential. To further demonstrate the therapeutic potential of this novel strategy for bone grafting, *in vivo* bone healing in rat calvarial defects is currently under assessment.

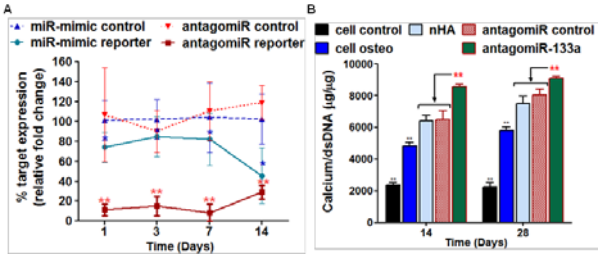

*Figure 1. miRNA-activated coll-nHA scaffolds **A)** silencing functionality & **B)** bone repair potential when seeded with hMSCs. Mean + std (n≥3).*

DISCUSSION & CONCLUSIONS: Collectively, this study demonstrated the superior ability of nHA particles to deliver miRNAs to hMSCs compared to that reported for other non-viral systems. Additionally, this study demonstrated successfully enhanced osteogenesis while coupling with angiogenesis using combinatorial miRNA delivery. When applied in 3D, a miRNA-activated scaffold with significantly enhanced therapeutic potential was achieved, as evidenced by the increased calcium deposition. This underlines the immense potential of extending this platform to different fields of tissue engineering beyond bone repair.

ACKNOWLEDGEMENTS:

Mechanical stimulation of stem cell seeded scaffolds to enhance angiogenesis and identify new therapeutic targets for bone repair

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INTRODUCTION: A limitation in the repair of large bone defects using tissue engineering (TE) scaffolds is insufficient cell penetration and vascularization following implantation. One way to overcome this is to use bioreactor culture to improve cell distribution and to prime the construct for enhanced osteogenesis and angiogenesis following subsequent in vivo implantation. In our laboratory, we adopt such an approach for therapeutic tissue engineering applications but we also use these in-vitro culture conditions as model systems to identify mechanically augmented genes as novel therapeutic targets that can be used to facilitate enhanced angiogenesis and osteogenesis in vivo. This presentation will outline some of our work in the area.

METHODS: Mechanical stimulation was applied to cell-seeded porous collagen scaffolds by steady state flow-perfusion in an in house designed bioreactor (1mL/min for 1h followed by 7h at 0.05mL/min) for 48h as previously described[1]. The results from two separate studies will be presented. In the first study, a gene expression microarray (~24,000 gene identifiers) was used to discover mechanically augmented genes during the osteogenic differentiation of Mesenchymal Stem Cells (MSC) seeded on collagen-based scaffolds undergoing flow perfusion. Mining of this data identified a number of genes that were significantly up-regulated in response to mechanical stimuli with potential targets selected for assessment as novel new therapeutics to enhance angiogenesis and osteogenesis. The aim of the second study was to investigate if pre-vascularisation of the scaffolds can be enhanced and stabilised through mechanical stimulation. This was achieved by initially using a co-culture of stem cells and endothelial cells to engineer an *in vitro* vascular network within the scaffolds[2,3] and then subjecting this to flow perfusion to identify whether the stability and distribution of the micro-vessels could be enhanced within the scaffolds and the cell network primed to facilitate enhanced vascularization post-implantation.

RESULTS: Study 1: Of the genes considered to be osteogenically significant (>2 fold increase for osteogenic media compared to growth media static controls), the microarray analysis yielded 13 genes that had a further >2 fold increase with the application of mechanical stimulation. Of these, placental growth factor (PGF), which has not previously been identified to be mechanosensitive, was selected for further analysis. The functional role of PGF in modulating MSC osteogenic differentiation was interrogated, and a concentration-dependent response seen whereby low concentrations exhibited the strongest pro-osteogenic effect. Furthermore, pre-osteoclast migration and differentiation, as well as endothelial cell tubule formation also showed concentration-dependent responses to PGF thus suggesting a potential role for PGF in both and angiogenesis and bone regeneration. Study 2: Subjecting the pre-vascularised scaffolds to mechanical stimulation resulted in improved cell distribution and microvessel stability. In addition, there was a significant increase in the level of pro-angiogenic vascular endothelial growth factor (VEGF) production immediately after mechanical stimulation thus demonstrating the potential of using flow perfusion to enhance vascularisation.

DISCUSSION & CONCLUSIONS: The results described show the potential of using mechanical stimulation both to enhance the therapeutic potential of existing bone tissue engineering approaches as well as to study the processes of stem cell mechanoregulation with a view to identifying new therapeutic targets to facilitate enhanced angiogenesis and bone formation.

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Nanofibrillar cellulose hydrogel for 3D cell culture and advanced analytics

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INTRODUCTION: Standard 2D *in vitro* cell culture systems do not mimic the complexity found in the native tissue. The cells within tissues are in continuous interaction with other cells and surrounding extracellular matrix. One approach to mimic cells' natural microenvironment in a dish is to use biomaterials such as hydrogels. However, the shifting trend from the 2D cultures to more advanced 3D systems challenges the basic analysis techniques. Especially, stabilizing the architecture of 3D spheroids for detailed morphological examination has proven to be challenging. Our aim was to identify a simple 3D cell culture setup from which the formed spheroids could be easily transferred into various analysis procedures including high-resolution imaging.

METHODS: We studied two hydrogels, plant-derived nanofibrillar cellulose (NFC) hydrogel (GrowDex®) and hyaluronic acid-gelatin (HG) hydrogel (HyStem®-C), to construct functional liver 3D spheroids. Human liver progenitor HepaRG cells and hepatoma HepG2 cells were embedded into 3D hydrogels according to earlier published protocol [l] and they were examined by SEM, qPCR, albumin ELISA, immunostaining, and P450-Glo™ CYP3A4 assay. The formed spheroids were analyzed within the hydrogels or after releasing them with cellulase enzyme as earlier described [2]. Spheroid structure was stabilized for SEM imaging by silica bioreplication (SBR).

RESULTS: Both of the studied hydrogels supported 3D spheroid organization of HepaRG cells and their functional polarization [3]. The 3D hydrogel cultures promoted faster hepatic differentiation compared to standard 2D culture. The generated spheroids in NFC hydrogel were metabolically more active than in the HG hydrogel. We were able to combine NFC hydrogel culture with high-resolution imaging since it was possible to recover the intact spheroids from the matrix. This could not be done with the HG hydrogel. SBR stabilized the 3D spheroid structure and enabled detailed morphological analysis. Dense microvilli-like structures and extracellular materials were observed on HepG2 cell membrane[4]. Cellular antigens were partially remained during the SBR.

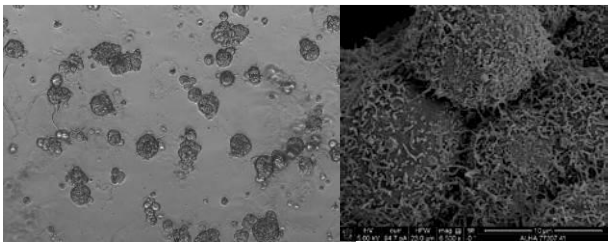

Fig. 1: Liver cell spheroids in NFC hydrogel. Intact 3D spheroids can be transferred for high-resolution imaging after enzyme treatment (left). SBR preserved fine cellular structures such as microvilli in HepG2 cells (right).

DISCUSSION & CONCLUSIONS: Even though there are numerous of biomaterials described for cell culturing only a few of them are optimal for analysis of 3D spheroids. Especially, imaging of the specimen can be hampered or even prevented by the surrounding matrix. Compared to other commercial hydrogels, the most significant advantage of GrowDex is that it can be degraded with specific enzyme to release viable spheroids. The intact spheroids can be flexibly used for many downstream applications and analyses. The generated HepaRG spheroids could serve a useful liver model in drug development for substance testing. In addition, the described SBR is a powerful technique to maintain the intact spheroid architecture to study morphogenesis or create new biomimetic materials.

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Enhancing diffusion in stem cell niches through 3D printed channels

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INTRODUCTION: Hydrogels are extensively used in 3D cell culture due to their biomimetic nature. However the diffusion of oxygen and nutrients poses a great challenge for tissue engineering.[1]

Despite stem cell niche being characterized by low oxygen content, anoxia at the core of cellularized scaffolds has long been associated with poor in vivo results of transplanted constructs. The diffusion of nutrients and oxygen is of outmost importance, since it influences cell metabolism and has a great impact on stem cell fate. Tissue vascularization by 3D printing is a research area field and various methods have been used to fabricate channels in constructs.[2] We aim to build perfusable complex structures through co-printing, with various cell densities and hydrogel concentrations based on their diffusion coefficients.

METHODS: Si-HPMC basic solution was mixed with acidic buffer to a final concentration of 2% and final pH=7.4 to promote crosslinking. 6% porcine gelatin concentration was prepared in PBS. Co-printing of the two materials was performed by extrusion printing using a contact dispensing 3DDiscovery® (RegenHU, Switzerland). Perfusion was made with Nile red in methanol. 1x106 ihMSC were seeded in Si-HPMC 2% prior to co-printing with gelatin at 6%. Cell viability was assessed with LIVE/DEAD® Cell viability assay (Thermo Fisher).

RESULTS: We were able to co-print perfusable structures using a sacrificial ink which dissolves in water at 37°C. A perfusion test was carried out showing that all the printed channels at different planes are interconnected. From the cross sections, we could confirm that we had a perfusable 3D printed cellular construct. At day 7 we observed that cell viability was higher for constructs with channels and channels with perfusion when compared to a bulk construct.

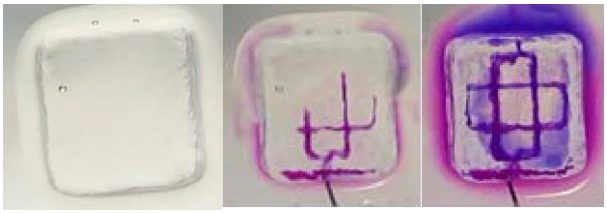

Fig. 1: Perfusion of the co-printed construct after removal of the sacrificial ink (8.4x8.4x6mm). Images present different time points during the perfusion.

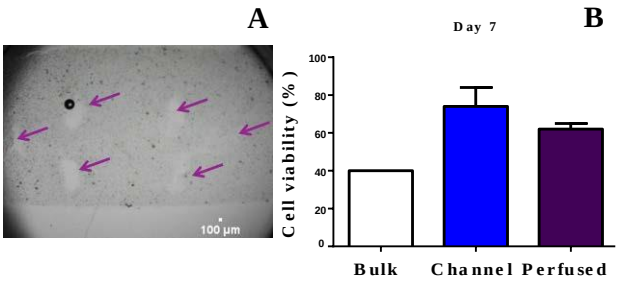

Fig. 2A: Cross section of the cell seeded hydrogel. Channels are indicated by arrows. B: Cell viability at the centre of constructs for bulk constructs and constructs with channels.

DISCUSSION & CONCLUSIONS: We have successfully produced a cellular construct with 3D interconnected channels with a sacrificial material that can easily be removed at 37°C. With this technique we aim to produce large and more complex structures that contain different materials and cells in the same construct.

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Tailoring polysaccharide hydrogels as growth factor delivery systems for intervertebral disc regeneration

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The intervertebral disc (IVD), is a fibrocartilaginous tissue composed of a peripheral network of robust collagen fibers which surround a highly hydrated gel known as the nucleus pulposus (NP). This structure undergoes an inevitable degenerative process, one of the major causes of low back pain[1].

Regeneration of the IVD has been explored with cell-based therapies [2] with limitations related to the transplantation of exogenous stem cells (autologous or allogeneic), including in vitro manipulations with high cost procedures and difficulties in regulatory approval. Repair strategies based on the intradiscal injection of therapeutic agents targeting the pathological events of NP degeneration have provided encouraging results. However, the in vivo half-life of biological factors is relatively short. Among biomaterials that could extend the residence time of these molecules after injection, and regarding the intrinsic hydration of the NP tissue, hydrogels are particularly promising. In this context, we are exploring whether biologically inspired disc repair strategies could offer an alternative to invasive spinal reconstructive surgery, while keeping away from cell therapy.

This presentation will discuss our progress in delivering bioactive hydrogels to promote IVD regenerative processes in situ. We have developed and characterized a self-setting hydrogel made of silanized cellulose[3]. This injectable and biocompatible polysaccharide based hydrogel provides a biomimetic and supportive environment to the tissue repair process. In addition, injectable microparticles based on chemically cross-linked polysaccharides are particularly interesting regarding their production in absence of organic solvents, their biocompatibility, and their ability to propose a finely tuned release of growth factors. They provide a high loading content and a high specific surface for controlled release. This presentation will highlight the ability of these hydrogel systems to clinically address low back pain early within the IVD degenerative cascade and promote IVD in situ regenerative processes.

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Biofunctional and complex hydrogel architectures fabricated via visible light photo-crosslinking with digital light processing

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INTRODUCTION: Digital light processing (DLP) is a photo-polymerisation based technology which involves projection of light into a resin in a computer driven, spatially controlled and layer-by-layer manner. The resin consists of photo-initiators combined with photo-polymerisable macromers. However, most commercially available resins are non-cytocompatible, thus inapt for fabrication of cell-laden constructs. The aim of this study is to develop a bio-resin to build cell-laden constructs with high resolution and complex architectures via visible-light DLP. Methacrylated poly(vinyl alcohol) (PVA-MA) was used as platform for DLP-3D printing and for chemical modification with gelatine-methacryloyl (gelMA), to impart bio-functionality in the resultant biosynthetic hydrogels. A secondary aim was to evaluate the bioactivity of the fabricated hydrogel constructs.

METHODS: Constructs were fabricated using the Perfactory® 3 Mini (EnvisionTec). The bio-resin used was 10wt% PVA-MA + 1wt% photo-absorber (Ponceau 4R) + photoinitiators, with or without 1wt% GelMA. Constructs were printed by exposing each layer (50µm) to 0.1mJ/cm^2 of light. Sol-gel analysis was conducted to evaluate the physico-chemical properties of the printed constructs (Ø5mm x 1mm cylinders). Mesenchymal stromal cells (MSCs) or multipotent cartilage progenitor cells (CPCs) were mixed with the resin at a final density of 5 x 10^6 cells/ml. DLP-printed cell-laden constructs were cultured for 21 days to evaluate the potential of the gel support bone and cartilage.

RESULTS: PVA-MA base resins, were fabricated into hydrogel constructs with this DLP technology. 3D constructs were produced either solid or with interconnected and self-supporting pores, with defined shape (Fig.1A), high resolution (Fig.1B&1C), and complex architectures (Fig.1D&1E). Sophisticated designs, such as the woven mat featuring intertwined struts, that cannot be obtained using other additive manufacturing techniques, were produced (Fig1D). Similar sol fraction (~25%) and mass swelling ratio (~9) were found for both PVA-MA and PVA-MA/gelMA constructs. Addition of gelMA significantly enhanced the viability of encapsulated MSCs (89±5%, Fig1F) vs. pure PVA-MA gels (78±2%). Printed MSCs and CPCs were cultured up to 21 days and expressed markers of osteogenic and chondrogenic differentiation, respectively. Histologies showed that PVA-MA gels are permissive environments for bone and cartilage matrix deposition, which were both enhanced in GelMA-functionalized constructs.

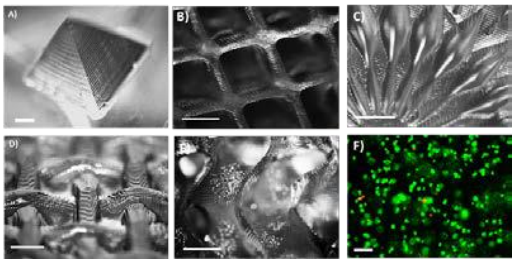

Figure 1: Hydrogel constructs printed via DLP: A) Pyramid, B) Porous lattice, C) Flower, D) Porous woven mat, E) Porous gyroid, F) Live-dead staining of MSCs in PVA-MA/GelMA gels after 14 days. Scale bar = 500 µm (A-E), 100 µm (F).

DISCUSSION & CONCLUSIONS: PVA-MA is compatible with the DLP technology as a bio-resin and addition of gelMA promotes bioactivity, cell survival and differentiation. This approach can allow to build a new generation of geometrically complex constructs with resolution higher than existing 3D-printing techniques.

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Opportunities for zonal cartilage regeneration: progenitor cell-laden hydrogels and bioprinting

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INTRODUCTION: The complex architecture of joint components and articular cartilage can be further mimicked with bioprinting, by coordinating the deposition of multiple cell types and materials [1]. Although cartilage-like tissues can be obtained with various hydrogels and tissue engineering (TE) strategies, to generate a mature construct that recapitulates the zonal tissue architecture is still a major challenge. For this purpose, cells with high potential for zonal differentiation need to be encapsulated in hydrogels that provide an instructive environment for matrix synthesis. Recently, the characterization of multipotent articular cartilage progenitor cells (CPCs), has opened new avenues for cartilage repair, however, little is known about their potential for TE [2]. This work aims to explore the potential of bioinks loaded with CPCs to promote zonal cartilage formation. Gelatin methacryloyl (gelMA) and gellan gum, which were previously characterized as printable hydrogels [3], were used as platforms for bioprinting and for 3D culture of chondrocytes, mesenchymal stromal cells (MSCs), and CPCs.

METHODS: GelMA was synthesized as previously described [3]. A library of gelMA-gellan bioinks (concentration ranges 3-25% gelMA and 0-1.5% gellan) was prepared and screened for printability and for the possibility to incorporate cells at 15-37°C. Yield stress, behaviour under shear and viscosity were assessed by rheometry. Full depth chondrocytes and CPCs were isolated from equine metacarpophalangeal joints [2], and MSCs from bone marrow from equine sternum. Biological response to several printable bioink formulations was first screened assessing cartilage matrix synthesis by encapsulated chondrocytes. One optimal ink formulation for ECM deposition was selected for encapsulation of CPCs, MSCs and chondrocytes to assess zonal differentiation. Cartilage formation was evaluated by immunohistochemistry, and the gene expression of several zonal markers was evaluated by PCR.

RESULTS: Optimal printability was found for bioinks with 10/0.5% gelMA-gellan composition, which exhibited high yield stresses, shear-thinning behaviour and allowed homogeneous cell encapsulation. All evaluated concentrations supported cartilage-like matrix production of encapsulated chondrocytes. The highest and most homogeneous cartilage matrix production was measured in the in 10/0.5% gelMA/gellan, and such system was chosen for further investigation. For all cell types, the hydrogel matrix was proven as a permissive environment for cartilage-like matrix deposition. Moreover, remarkable differences in gene expression were observed between MSCs and CPCs, the latter showing higher expression of lubricin and lower expression of collagen type X, a marker for hypertrophic chondrocytes and calcified cartilage.

DISCUSSION & CONCLUSIONS: In this work, the potential of several cell types, and in particular of CPCs to produce cartilage tissue *in vitro* was highlighted. The 10/0.5 gelMA/gellan bioink was proven as a suitable platform for bioprinting and cartilage TE. Moreover, differential expression of zonal markers was observed, according to the cell type encapsulated into the bioink. These are key findings to develop the next generation of biofabricated zonal cartilage constructs, with potential application in regenerative therapies and as more realistic 3D *in vitro* models.

ACKNOWLEDGEMENTS: The Dutch Arthritis Foundation (grant numbers: LLP12 and LLP22), the UK Regenerative Medicine Platform and the European Community (FP7/2007-2013, grant agreement n 309962-HydroZONES) are acknowledged for their financial support.

Microvesicles from amniotic cells as potential novel therapeutics in regenerative medicine: first *in vitro* result in equine stressed tendon and endometrial cells

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INTRODUCTION: The unfavorable microenvironment of injured or degenerating tissues may result in the death or apoptosis of a large proportion of implanted MSCs in the short period immediately post-transplantation. Recovery of *in vivo* spontaneous equine tendon lesions by administration of horse amniotic mesenchymal cells conditioned medium (AMC-CM) suggests a paracrine mechanisms in the regeneration process [1]. It has recently been demonstrated that microvesicles (MVs) released from cells are an integral component of the paracrine cell-to-cell communication during tissue regeneration [2]. Aims of this study were to investigate the presence and type of MVs secreted by equine amniotic mesenchymal cells (AMCs), their incorporation in equine tendon and endometrial cells and their effect on these cell lines stressed *in vitro* by lipopolysaccharide (LPS).

METHODS: MVs were obtained by ultracentrifugation at 100.000g for 1h at 4°C of the media obtained culturing AMCs isolated from three different amnion. MVs size was evaluated by Nanosight technology and transmission electron microscopy (TEM). Tendon and endometrial cells were obtained from collagenase digestion for 17h and 3h respectively and cultured in HG-DMEM with 10% fetal calf serum. To study the ability of tendon and endometrial cells to incorporate MVs, a dose-response curve was performed adding 10-20-30-40-50x10^6 MVs/ml labeled with PKH-26 for 24h, 48h and 72h. The uptake of MVs was evaluated by an Olympus BX51 microscope equipped with software for image acquisition. A dose/response curve of LPS investigated by apoptotic and MTT tests showed that 100ng/ml at 48h on tendon cells and 10ng at 24h on endometrial cells were the doses and the times most effective in inducing cellular stress. RT-qPCR expression of pro-inflammatory genes such as tumor necrosis factor-α (*TNF-α*), metallopeptidase (*MMP*) 1 and 13, and of an anti-inflammatory gene such as transforming growth factor-ß (*TGF-ß*) was evaluated in the *in vitro* LPS stressed cells by Mann-Whitney U-test.

RESULTS: Nanosight showed that AMCs secrete MVs in the range of 100-200 nm. TEM revealed budding of AMCs membrane, proving that these MVs fall within the shedding vesicles category. The MVs uptake was gradual over time (Fig. 1). The same semi-quantitative fluorescence uptake signal was obtained when 50x10^6 MVs were incorporated at 24h, or 40x10^6 MVs at 48h and 30x10^6 MVs at 72h, suggesting that an inverse correlation between concentration and time was found in MVs uptake equally by tendon and endometrial cells. MVs induced a significant ($P<0.05$) down-regulation of *TNF-α*, *MMP1* and *MMP13* expression in both cellular lines after *in vitro* LPS stress, and up-regulation of *TGF-ß* expression.

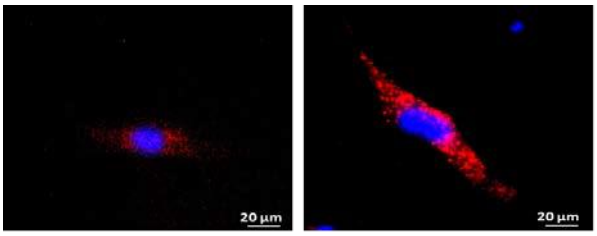

Fig. 1: Representative micrographs of internalization at 24h (A) and 72h (B) of MVs labeled with PKH-26 by tendon cells.

DISCUSSION & CONCLUSIONS: Our data suggest that MVs can be incorporated in tendon and endometrial cells and have a role in modulating inflammatory genes *in vitro*.

ACKNOWLEDGEMENTS: Grants from Università degli Studi di Milano, Italy and Università Politecnica delle Marche, Ancona, Italy are acknowledged.

Microparticle incorporation into collagen-based guiding structures for bone defect regeneration via endochondral ossification

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INTRODUCTION: To support bone regeneration, biomaterials have for a long time aimed at mimicking the intact bone tissue, with limited success. Lately, however, bone regeneration via a cartilage phase (endochondral ossification) is discussed as a promising strategy [1]. In this study we investigated the capability of a soft collagen-based biomaterial with channel-like orientated pores to induce endochondral ossification and the effect of hydroxyapatite and bioglass microparticle incorporation.

METHODS: Highly orientated collagen scaffolds S_{Co} were produced by directional solidification, freeze-drying and chemical crosslinking. Hydroxyapatite (HA) particles (Fig 1a) synthesized via a wet-chemical process and bioglass (BG) type 45S5 (Schott) were used (Fig 1b). The ability of BG particles to serve as precursor to nucleation and growth of CaP crystals was evaluated via incubation in simulated body fluid (SBF) (Fig 1c). Hybrid scaffolds S_{HA} (HA) and S_{BG} (BG) were produced via incorporation of particles in the manufacturing process. Extracellular matrix formation inside scaffold pores and expression of chondrogenic and osteogenic genes (qPCR) was investigated *in vitro* using human primary fibroblasts and mesenchymal stromal cells. Finally, *in vivo* bone formation in scaffolds implanted in a 5mm critical sized defect in rats was quantified at 2, 4, and 6 weeks via µ-CT (bone growth) and histomorphometry (tissue types).

RESULTS: HA and BG particles were uniformly distributed inside highly orientated collagen scaffolds without affecting the particle properties nor modifying the pore architecture (Fig 1d+e). As a consequence of the changed microenvironment the formation of fibrillar collagen matrix was delayed in S_{HA} compared to S_{BG} and S_{Co}. However, a strong upregulation of osteogenic genes was observed in S_{HA} compared to the other prototypes. While histology of the bone defect region revealed cartilage as a sign for endochondral ossification induced by S_{Co} and S_{BG} (Fig 1f), cartilage was absent in S_{HA}. Surprisingly, S_{BG} showed significantly higher volume of newly formed bone

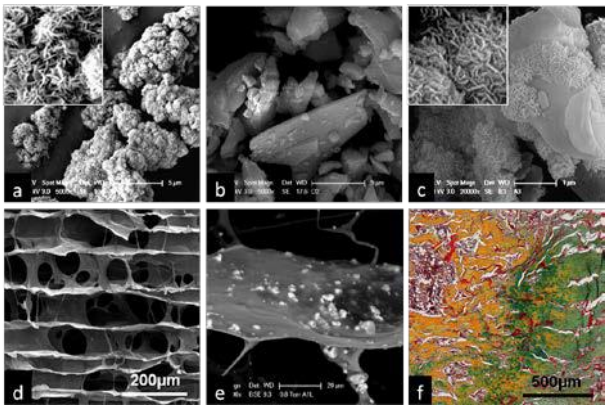

Fig. 1: Scanning electron microscopy images of HA (a) and BG particles before (b) and after incubation in SBF (c), scaffold pore architecture (d), and BG particles inside scaffold walls (e). Endochondral ossification (cartilage green) within S_{BG} in the rat bone defect model at 6 weeks (f).

at 2 weeks compared to S_{Co} and S_{HA} indicating an improved biological activity at early timepoints.

DISCUSSION & CONCLUSIONS: The absence of cartilage in S_{HA} indicated that the induction of endochondral ossification observed in S_{Co} is hindered by HA particles, which is in agreement with increased osteogenic gene expression. The positive effect of BG particles on bone growth at early timepoints is considered to be a consequence of increased biological activity supporting endochondral ossification. This might be a consequence of a temporal synchronization between tissue maturation and calcium phosphate nucleation on BG particles supporting mineralization of the fibrocartilage matrix without impairing initial cartilage formation. This study highlights the importance of an optimized architectural and biochemical environment in a purely material-based strategy for endochondral regeneration of large bone defects.

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Electrical stimulation: a tool for tissue engineers

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INTRODUCTION: The ability to influence cell activity is of paramount importance for tissue engineers. A variety of stimuli such as chemical and mechanical environments have shown to influence cell proliferation and differentiation. An additional important parameter that is often neglected is electrical fields. Electricity is an endogenous process within the body's normal function and wound repair mechanism. Understanding this process and utilising electricity in *in vitro* culture of tissues has the potential to add synergistic benefit to the end functionality of constructs created. This presentation will discuss *in vivo* approaches and subsequent *in vitro* approaches that have been used to influence cell and tissue behaviour.

METHODS: Data obtained in our research group using a laboratory-made device used for applying electrical stimulation will be presented[1,2]. The 6 well plate bioreactor has direct contact electrodes of stainless steel and is controlled using a signal generator. A variety of electrical fields have been investigated including 1us square wave pulses and 250us pulses at different voltages (70V and 210V). The cell number (picogreen, alamar blue), viability (live-dead fluorescent stain) and differentiation (RT-PCR) of primary human mesenchymal stem cells have been measured after 7 day periods. Modelling of the electrical system in the bioreactor using Finite Element Method (FEM) using COMSOL-Multiphysics software will be presented.

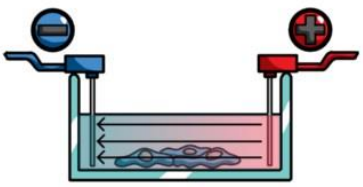

Fig. 1: Schematic demonstrating direct contact electrodes placed in well of 6 well plate.

RESULTS: We demonstrate that primary human mesenchymal stem cells significantly upregulate osteogenic genes in the presence of osteogenic supplements with little difference to cell viability.

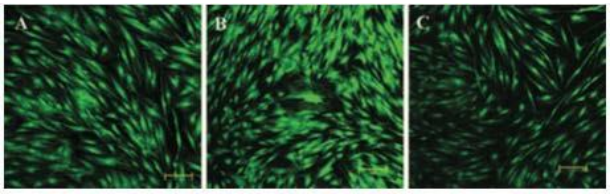

Fig. 2: Live/Dead staining of samples at Day 7. There was no sign of necrosis or significant morphological changes. A—Controls, B—Treated with 25 µs −70V regime, C—Treated with the 1µs −70 V regime. Scale bar corresponds to 200µm.

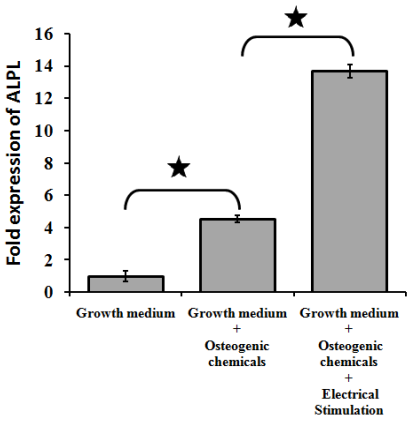

Fig. 3: Fold Alkaline Phosphatase (ALPL) gene expression in samples treated with and without chemical and direct electrical stimulation.

DISCUSSION & CONCLUSIONS: There is a growing body of evidence demonstrating the synergism of using electrical field application to improve wound healing *in vivo* and to increase the functionality of tissue-engineered constructs grown *in vitro*. This application still needs refining further to realise its potential – by optimising regimes and application methods to different tissue and cell types and to further elucidate the full mechanism by which these effects occur.

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An insight into the Glyco-environment of a murine model of hindlimb ischemia

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INTRODUCTION: Glycans play a central role in myogenesis and muscle physiology [1] and in regulating angiogenesis by binding pro-angiogenic growth factors and clustering receptors [2]. Furthermore, alterations in the *N*-glycoproteome in ischemic conditions have been reported [3]. It was hypothesised that there are overall alterations in the expression of glycosylation (glycoprofile) in ischemic conditions in mouse limb. The objective of this study is to compare the glycoprofile in healthy and ischemic muscle using a mouse model of hindlimb ischemia (HLI).

METHODS:HLI was induced in the left leg of Balb/C mice (n=12) by ligation of the femoral artery as previously described [3]. The right leg was used as control tissue. Samples were collected from both legs after 7, 14, and 21 days.
Fixed frozen sections (7µm-thick) were incubated with FITC-labelled *Sambucus Nigra* agglutinin (SNA-I, 15µg/ml) and Wheat Germ Agglutinin (WGA, 20µg/ml). Quantification of the fluorescence intensity was performed using the software ImageJ and statistical significance was determined using SPSS software. Additionally, a lectin microarray of 48 lectins spotted on a slide was carried out on healthy muscle. Alexafluor®555-labelled glycoproteins extracted from cell membrane were incubated on the lectin microarray. N-Glycans were released from both healthy and ischemic tissues via digestion with peptide N-glycosidase F (PNGase F) and quantified by mass spectrometry.

RESULTS: Lectin histochemistry of SNA-I revealed an increase in SNA-I binding sites at seven days after induction of ischemia. On the other hand, WGA binding decreased at seven days after induction of ischemia. Lectin microarray showed terminally sialylated O-linked glycans, complex type N-glycans, and high levels of high-mannose N-linked glycans. Differences in of N-glycans levels between the healthy and the ischemic samples were observed in the mass spectrometry analysis.

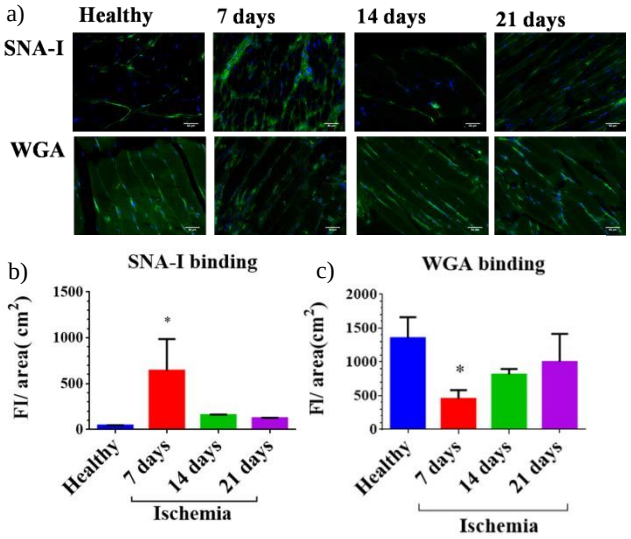

Fig. 1: a) SNA-I and WGA binding pattern on healthy and ischemic muscle at day 7, 14, 21 after the induction of ischemia; b) SNA-I and c) WGA binding fluorescence quantification. The values are expressed as mean and SD,=P value≤0.05 (n=4).*

DISCUSSION & CONCLUSIONS: The main glycan species present on cell membrane proteins of the healthy muscles are: terminal sialylated O-linked glycans, complex type N-glycans with GlcNAc, with the majority being high-mannose type. Moreover, this preliminary work suggests that early ischemic conditions can affect both α(2-6) linked sialic acid and GlcNAc presence.

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An *in vitro* rapid myelinating artificial axon system as a model of demyelinating diseases

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INTRODUCTION: The loss of the myelin sheath that insulates axons is a hallmark of demyelinating diseases. In the adult CNS myelin loss can only be partially rescued by remyelination of spared axons. The newly formed myelin is not made by oligodendrocytes surviving an episode of demyelination, but from oligodendrocyte progenitor cells (OPCs), which become activated, proliferate and give rise to remyelinating oligodendrocytes. Though the mechanisms responsible for chronic remyelination failure have not yet been identified, evidence of the presence of OPCs in chronic multiple sclerosis lesions[1] suggests the existence of a regulatory mechanism inhibiting OPC differentiation under certain pathological conditions. Using an *in vitro* rapid myelinating artificial axon system and a tissue engineered glial scar, here we hypothesize that astrocyte reactivity may play a critical role in the course of OPC differentiation into mature oligodendrocytes and that the reversion of the astrocyte phenotype to non-activated can lead to the recovery of OPC differentiation capacity.

METHODS: OPCs were cultured on electrospun poly(trimethylene-co-ε-caprolactone) (P(TMC-CL)) fibres in the presence of 3D cultured reactive astrocytes. P(TMC-CL) fibres were obtained by electrospinning[2]. A previously described alginate-based 3D tissue engineered model of astrogliosis[3], where astrocytes cultured in the presence of meningeal fibroblast conditioned medium (CM) behave similarly to glial scar astrocytes, was explored. Activated astrocytes 3D cultures (4 days *in vitro* (DIV)) were added to oligodendrocyte cells seeded on top of polymeric fibres (1 DIV), and the co-cultures were maintained for additional 5 days. All cells were obtained from brains of P2 Wistar Han rats.

RESULTS & DISCUSSION: The prepared P(TMC-CL) fibres were found to have an average diameter of 0,67±0,12 µm. OPCs were able to adhere, survive and differentiate into myelin basic protein (MBP) + oligodendrocytes when cultured on the electrospun fibres (Fig. 1), producing large membrane extensions, which in some cases contacted with multiple fibres. Differentiated oligodendrocytes were not only found to extend their processes along nanofibers but also ensheathed them (this was observed without the need to coat the polymeric fibres with any adhesive molecule). Monocultures of astrocytes within alginate hydrogels and OPCs on P(TMC-CL) fibres were initially performed in the presence of CM using freshly prepared medium as control. Astrocytes were activated by the presence of CM. However, for the OPCs cultures no significant alteration of the percentage of MBP+ cells was observed. Conversely, OPC differentiation ability was inhibited in the presence of 3D cultured reactive astrocytes, with a significant reduction of the percentage of MBP+ cells. This inhibition could be reverted by rescuing astrocytic phenotype with ibuprofen, a chemical inhibitor of RhoA[3].

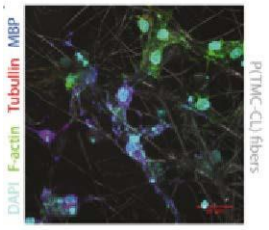

Fig 1. *OPC culture on P(TMC-CL) fibres at 7DIV.*

CONCLUSIONS: This work shows that reactive astrocytes significantly inhibit OPC differentiation, and that pharmacological inhibition of astrogliosis, enables recovery of OPC differentiation ability. Finally, the proposed *in vitro* artificial axon system may be of added value for further studies aiming to dissect the molecular mechanisms of myelination and screen new drugs, as by removing the neuronal cell contribution to the process it allows a more controlled manipulation of defined variables of the culture system, constituting a complementary approach to currently available neuron-glia cell culture methodologies.

Decoration of RGD-mimetic porous scaffold with engineered, devitalized adipose matrix

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INTRODUCTION: Numerous scaffolding materials based on either synthetic or natural polymers have been investigated in the context of adipose tissue engineering for regenerative medicine applications [1]. In parallel, devitalized adipose tissue has shown great *in vivo* potential to enhance regeneration and instruct repair of damaged soft tissues [2]. Our aim was to combine the positive features of a structural synthetic polymer and of an engineered, devitalized ECM to obtain a hybrid construct for the treatment of adipose tissue defects. Towards achieving this goal, an RGD-mimetic poly(amidoamine) oligomer foam (OPAAF) was developed, decorated with extracellular matrix (ECM) by human Adipose Stromal Cells (hASCs) *in vitro*, subsequently devitalized and implanted *in vivo*.

METHODS: RGD-mimetic poly(amidoamine) oligomers were cross-linked by free radical polymerization. Scanning Electron Microscopy (SEM) analysis was performed to assess pore size. Mechanical properties were evaluated by Dynamic Mechanical Analysis (DMA). hASCs were seeded and cultured on OPAAF, with adipogenic medium, in a perfusion bioreactor system (U-CUP, Cellec Biotek). Constructs were analyzed by BODIPY staining for lipid droplets and RT-PCR analysis for PPAR© and FABP4. Freeze and Thaw (F/T) cycles were used to devitalize the constructs and to generate a hybrid ECM-OPAAF. ECM integrity before and after F/T was assessed by SEM analysis and immunofluorescence (IF) for collagen IV. Hybrid ECM-OPAAF and nude OPAAF were implanted subcutaneously in a mouse model for one month. Histological analysis of explanted constructs was performed to investigate adipose tissue infiltration.

RESULTS: OPAAF was characterized by a highly interconnected porous network (Fig.1 A) and mechanical properties similar to those of a native adipose tissue (Young's modulus 3,2 ± 0,6 kPa). OPAAF supported *in vitro* hASCs adipogenesis, as documented by expression of PPAR© and FABP4 genes and by the formation of lipid droplets (Fig. 1B). As assessed by SEM analysis and by IF for collagen IV, F/T devitalization didn't affect matrix integrity (Fig.1 C and D). Histological analysis for OPAAF and ECM-OPAAF after *in vivo* implantation highlighted adipose tissue infiltration. Quantification of the infiltrated tissue in the two conditions is ongoing.

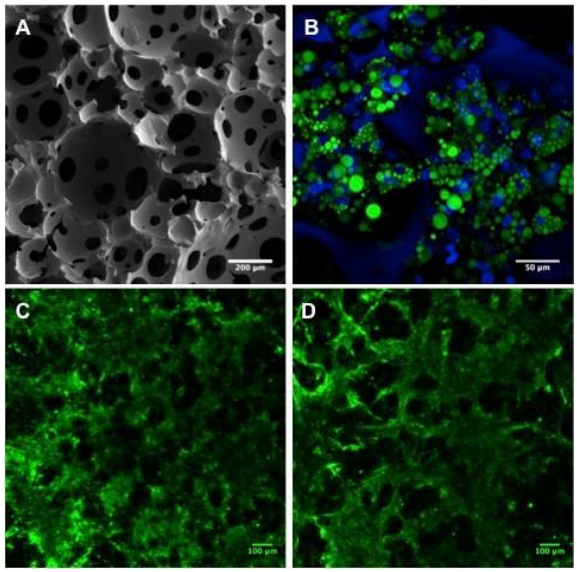

Fig. 1: *SEM image of OPAAF's porosity (A). hASC differentiated into adipocytes stained for BODIPY/DAPI, identifying fatty acid and nuclei, respectively (B). IF for collagen IV before and after F/T devitalization (C and D respectively).*

DISCUSSION & CONCLUSIONS: These results validated OPAAF as a scaffold for adipose tissue engineering and repair. Moreover, based on the presented findings, we propose that OPAAF decorated with adipose ECM should be further explored as off-the-shelf material able to regenerate defined soft tissue volumes and shapes.

DISCLOSURES: We wish to confirm that there are no known conflicts of interest associated with this publication.

Modulation of axonal growth in 3D hydrogel based environments aimed at spinal cord injury regenerative medicine

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INTRODUCTION: Spinal Cord Injury (SCI) current therapies fail to induce functional recovery. Thus, alternative strategies that can successfully promote axonal repair and regeneration are essential. The use of biodegradable hydrogels enriched with guidance cues along with cell-based therapies has been explored for establishment of novel strategies for SCI repair [1]. The aim of this work was to develop a therapeutic construct that could potentiate axonal outgrowth in a 3D environment. For this purpose Gellan-Gum (GG) hydrogels enriched with fibronectin-derived peptides (GRGDS) were used. Additionally the capability of these systems in supporting adipose tissue-derived mesenchymal stem cells (ASCs) and the role of the later in modulating axonal growth was also studied. Finally the impact of using different ECM like hydrogel matrices (NVR gel and collagen) to promote axonal outgrowth within a 3D environment was also tested, in order to determine if the GG-GRGDS could be a valuable tool for SCI regenerative medicine based strategies.

METHODS: GRGDS-modified GG hydrogels and Collagens were prepared as previously described [2,3]. NVR-gel (NVR Labs) is a liquid viscous-like hydrogel composed of HA and laminin. Dorsal root ganglion (DRG) explants were used as *in vitro* model of axonal regeneration. To study the ASCs effect in DRG neurite outgrowth, cells were encapsulated in the gels at a density of $60x10^5$ cells/mL for 7 days. DRG neurite extension was analyzed by neurofilament staining; cell morphology by phalloidin and DAPI staining.

RESULTS: GRGDS-GG hydrogel was able to support DRG neurite outgrowth by itself. This was also observed for NVR and Collagen (Fig. 1a, c, e). Moreover, the three gels were able to support ASC survival and adhesion. In fact, this is reflected by the observed potentiation in DRG neurite outgrowth cultured in the hydrogels-containing ASCs (Fig. 1b, d, f). However, from the three hydrogels used, the combination of GRGDS-GG with ASCs had a more pronounced effect on axonal growth, as observed in Fig. 1g. Finally it was also observed that ASCs growing in the GG-GRGDS hydrogels disclosed higher levels of neurotrophic factor expression, namely BDNF, VEGF, NGF and GNDF.

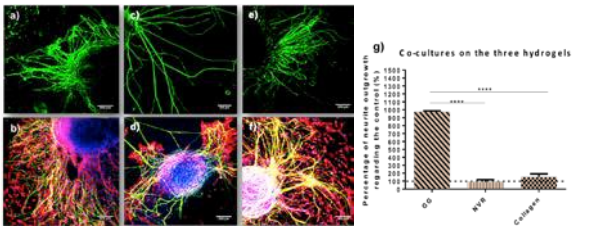

Fig. 1: DRG culture in GRGDS-GG, NVR-gel and Collagen, in the absence (a, c, e) and presence (b, d, f) of ASCs. **g)** DRG neurite outgrowth (%) in the hydrogels-containing ASCs. (Mean±SD; n=4; $p< 0.05$). Immunostaining for Neurofilament (Green), DAPI (blue) and phalloidin (Red).

DISCUSSION & CONCLUSIONS: In this study, GRGDS-GG demonstrated to be a good substrate for axonal outgrowth, which was clearly enhanced by the combination of ASCs. In line with this, the synergy observed when combining different regenerative strategies may represent a step forward on the development of successful therapies for SCI**.**

ACKNOWLEDGEMENTS: EU-FP7-Health-2011-collaborative project 278612, Biohybrid - Templates for peripheral nerve regeneration; Prémios Santa Casa Neurociências - Prize Melo e Castro for Spinal Cord Injury Research; Portuguese Foundation for Science and Technology.

DISCLOSURE: All authors declare no conflict of interest.

Bisphosphonate-modified injectable hyaluronic acid hydrogel: Optimising growth factor delivery

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INTRODUCTION: There are several disadvantages with current collagen based carriers used for the delivery of bone morphogenetic proteins (BMP). One such disadvantage is the poor retention resulting in the need to use supra physiological doses of bone morphogenetic protein 2 (BMP-2), which can result in severe side effects. Bisphosphonate chemically linked hyaluronan hydrogels have been proven to retain BMP-2 and affect osteoclast viability *in vitro*. The aim of this study was to evaluate whether conjugation of bisphosphonate to hyaluronan hydrogel-based delivery system with encapsulated BMP-2 could modulate bone formation following *in vivo* application.

METHODS: The biocompatibility of the BP-functionalised HA hydrogel (HA-BP) was screened in a chick femur defect model cultured on the chorioallantoic membrane over 8 days. In addition, the *in vivo* performance of the hydrogels was examined in a uni-cortical rat femoral defects. The defects were filled with 40 µL of HA-BP gel (left leg) and 40 µL of analogous gel without BP (right leg). The gels were loaded with 0.2 µg (5µg/ml) of BMP-2 or without BMP-2.The rats were euthanized after 4 weeks. Femurs from both *in vivo* models were harvested and examined *via* micro computed tomography and histology.

RESULTS: BP functionalisation of the hydrogels did not compromise biocompatibility in a chick femur defect model cultured on the chorioallantoic membrane over 8 days. The same treatment applied in a rat femur defect showed a remarkable increase of 25 % after 4 weeks between the HA-BP hydrogel and the control HA hydrogel both loaded with BMP-2. A substantial amount of HA-BP hydrogel was still present after 4 weeks, in contrast to HA gels without BP, which were completely resorbed.

DISCUSSION & CONCLUSIONS: BP-grafting of hyaluronan hydrogel demonstrated a positive effect on bone volume inducing 25% more bone formation compared to the analogue without bisphosphonate. HA-BP hydrogel showed a higher retention of BMP *in vitro*[1] and was more resilient to enzymatic degradation *in vivo*. Critically, the functionalization of the hydrogel can be tuned to a desired release rate of growth factor[2], which will allow optimised and controlled drug release.

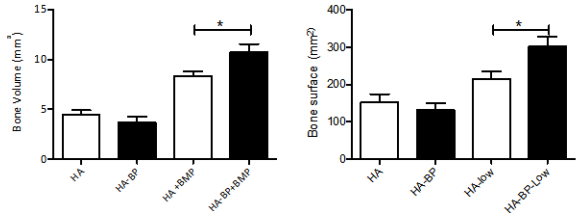

Fig. 1: Functionalisation of HA hydrogel with BP groups resulted in significantly higher bone volume and bone surface in a uni-cortical rat femoral defect model when HA-BP hydrogel was combined with BMP-2 as compared to HA+BMP hydrogel (the positive control)(Mean±SD)(=P > 0.05; Student's paired t-test).*

ntiation. Biomacromolecules, 14: 3055-3063.

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A beating heart-on-a-chip platform for the generation of functional cardiac microtissues: cyclic uniaxial strain on 3D microconstructs

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INTRODUCTION: To date, the generation of functional micro-cardiac tissues still remains an open issue. In vivo, cardiac cells undergo multiple cues deriving from the local heart tissue "niche", which instructs cells through dynamic biochemical, electrical and mechanical signaling, eventually guiding tissue development and maturation. In particular, cardiomyocytes (CMs) are subjected to periodic contraction/relaxation sequences deriving from the beating of the heart[1]. A robust and predictive in vitro model of mature cardiac tissues should thus enable the recapitulation of (i) the three-dimensional (3D) architecture of complex cell-cell and cell-extracellular matrix (ECM) interactions and (ii) electro-mechanical stimuli resembling the native myocardial environment, (iii) in the presence of controlled biochemical signals[2].

METHODS: Here, we report a new method to generate mature and highly functional cardiac microtissues within perfused microfluidic devices. To this purpose, an innovative micro-bioreactor for the culture of 3D cell constructs was designed, able to recapitulate the physiological strains experienced by cells in the native myocardium (range 10-15%)[3]. Briefly, an array of posts was implemented to confine and culture cell-laden gels, and a pneumatic actuation system was embedded to induce uniaxial cyclic strains to the 3D constructs (generated by neonatal rat or human pluripotent derived cardiomyocytes). The presence of auxiliary channels allowed to perfuse medium during culture, while providing biochemical stimulation and transporting pacing signals during the functional evaluation of tissues.

RESULTS: As shown in the figure, stimulated cardiac constructs expressed higher levels of connexin-43 (Cx43) compared to the 3D static culture condition (Figure 1a), suggesting superior electrical connection among neighboring cells and cardiac maturation. Interestingly, stimulated constructs highlighted higher cell viability with respect to control (Figure 1b). Moreover, the cyclic mechanical stimulation promoted spontaneous synchronous beating of the constructs, all over their volume (Figure 1c). Electric pacing experiments further demonstrated the superior maturation of the stimulated constructs.

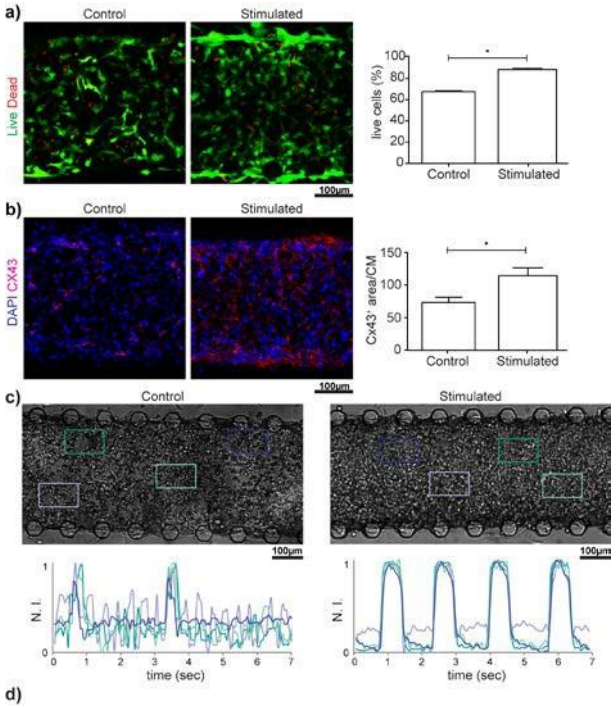

Fig. 1: Images and quantifications of LIVE/DEAD assay (a), and Cx43 expression (b). Spontaneous beating activity of mature cardiac constructs (c).

DISCUSSION & CONCLUSIONS: We introduced a novel heart-on-a-chip platform able generate functional cardiac microtissues from either neonatal rat or human pluripotent derived cardiomyocytes. This result was achieved through the application of a highly controlled cyclic uniaxial strain to 3D microtissues in culture. The compatibility with electrical pacing makes the platform suitable for both high-throughput pre¬clinical drug screenings and physio-/pathological states investigations.

Macromolecular modulation in a tissue-to-tissue model system differentially regulates the behaviour of hBMSCs

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INTRODUCTION: The simultaneous creation of multiple tissues and their functional assembly leading to complex organ systems has recently created interest in tissue engineering strategies. The structural and mechanical gradient present on tissues has its main focus at the interface since it seems that tissue-to-tissue interfaces are crucial for multitissue repair strategies. Thus, well-defined gradient model systems are of great importance in the study cell-matrix interactions at a biophysical level. It was hypothesized that by modulating the macromolecular enviroment, cells will assume a differential behaviour as a consequence of targeting specific intracellular signaling events leading to desirable cell fate patterning. Thus, cell viability and biofunctionality, as well differential paracrine secretory profiles and gene/cell marker expression, can be engineered by using human bone marrow stromal cells (hBMSCs).

METHODS: Hyaluronic acid and collagen type I formulations were produced at 1:1 ratio. Methacrylated Gellan-gum was produced and gradient hydrogels were assembled from bottom to top, layering each formulation % (w/v) sequentially. An intermediate cellular layer of hBMSCs was created. Rheology was used to assess the different mechanical properties. hBMSCs were observed under confocal microscopy for viability as well as analysis of cell morphology. ECM production as a function of cell number was evaluated. HIF-1α staining and O_2 concentration within the gradient evaluated the intrinsic hypoxic environment. Secretory cytokine measurement for angiogenesis and hypoxia factors was carried out using ELISA alongside gene and cell marker expression by RT-PCR and FACs analysis.

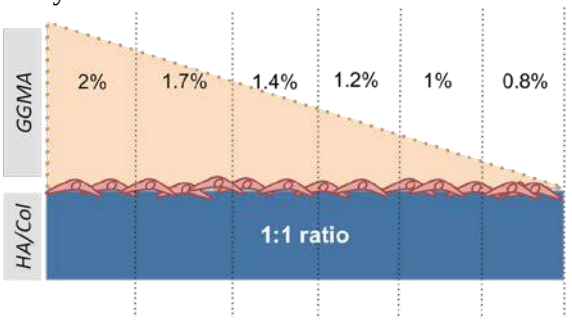

Fig. 1: Schematic representation of a tissue-to-tissue model system to differentially regulate human hBMSCs.

RESULTS: The gradient hydrogel was successfully constructed and validated by fluorescent emission of cells in the interface. Variations of oxygen concentrations were seen within a gradient over time and by HIF-1α staining. Significant differences in the levels of cytokine production in gradient hydrogels when compared to those of the control and over time were observed along with a differential genomic and cell marker expression of hBMSCs.

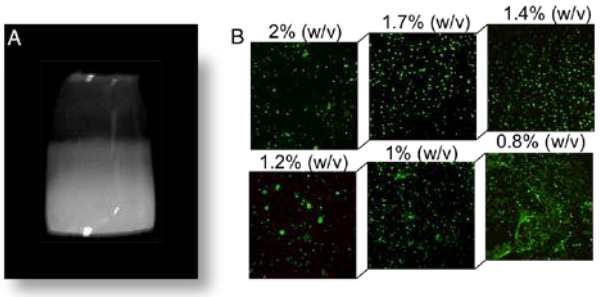

Fig. 2: (A) Image of a single hydrogel gradient; (B) hBMSCs viability was evaluated by the green emission of fluorescence for live staining (Calcein AM).

DISCUSSION & CONCLUSIONS: The macromolecular gradient hydrogel systems were successfully achieved demonstrating differentially cellular responses as a function of the macromolecules modulation.

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

Piezoelectric nanoscaffolds: mediating tendon regeneration through activation of piezoresponsive receptors.

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INTRODUCTION: The unique properties of tendon tissues are attributed to the high degree of alignment of collagen fibrils that provide topographical guidance and promote tissue organization. However, an often overlooked physical property is an inherent piezoelectric response to mechanical loading that could modulate cell function through transmembrane voltage-gated receptors. In this study, primary human tendon cells seeded on a piezoelectric fibrous scaffold developed from poly (vinylidenefluoride-co-trifluoroethylene) P(VDF-TrFE). The sensitivity of the material was further modulated through the incorporation of piezoelectric Boron Nitride nanotubes (BNNTs). The nanomaterials were evaluated as regenerative scaffolds through the analysis the expression of tendon-specific genes[1].

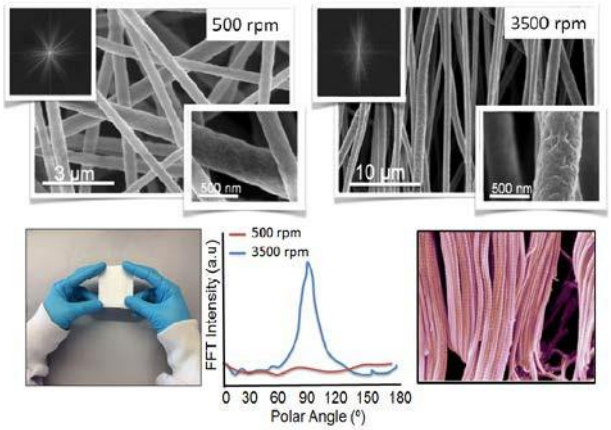

Fig.1 Scaffold structure micmicking tendon native tissue.

METHODS: P(VDF-TrFE) was dissolved in DMF/Acetone and electrospun into fine fibers. BNNTs were functionalized with a conductive polymer and incorporated into the matrix. Morphology was observed by FESEM and the electrical properties with PFM and an in-house oscilloscope/dynamic loading system. Primary Human Tenocytes were isolated and cultured for 3, 5 and 10 days onto the scaffolds under static and mechanical loading conditions. Samples were immunoassayed for focal adhesions (FA), Tenomodulin (TNMD), Collagen I (COL-I) and III (COL-III). 96-Gene expression was analyzed using a Custom RT^2 Profiler PCR array.

RESULTS: The PDVF-TrFE/BNNT scaffolds were piezoelectric, biocompatible and mechanical stimulation promoted the maintenance of a tenospecific phenotype for up to 10 days. Preliminary results shows that cell morphology and focal adhesion distribution cultured on static and dynamic conditions scaffold were modulated and piezoresponsive gene were upregulated. Despite proliferation was lower for piezoscaffolds, tenomodulin expression in piezoelectric nanoscaffolds was maintained after 7 days.

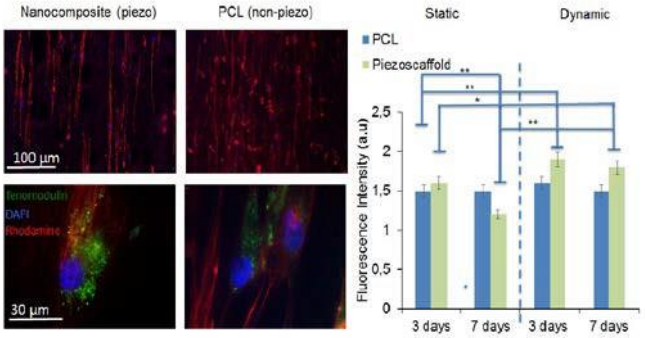

Fig.2 a) Alignment of cells after electromechanical loading and TNMD protein expression .

DISCUSSION & CONCLUSIONS: This study indicates that piezoelectrical sensitivity of PVDF-TrFE may be tuned by incorporation of BNNTs[2]. Moreover, gene and protein analyses showed different piezoelectric stimulation significantly upregulated the expression of mechanosensitive and ion channel voltage-gated genes.

ACKNOWLEDGEMENTS: SFI 11/SIRG/B2135; (FP7/2007-2013), PCIG13-GA-2013-618861.

3D bioprinting of human pluripotent stem cells and hydrogels for tissue engineering

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INTRODUCTION: In the development of new drugs, only around 16% of the drugs that begin preclinical testing are approved for human use. Some of this low success rate can be attributed to the different responses between animals and humans on the drugs being tested. A possible solution to this might be the creation of human micro-tissues derived from pluripotent stem cell sources for drug testing instead of current animal models. This would result in scalable, faster and potentially more reliable drug testing platform, and hopefully an end to animal testing. We here report the first investigation into the bioprinting of human induced pluripotent stem cells (hiPSCs), their response to a valve-based printing process as well as their post-printing differentiation into hepatocyte-like cells (HLCs) within a bioprinted 3D hydrogel structure.[1] Additionally, a new bioprinting technique was developed to produce more complex cell-laden alginate hydrogel structures.[2]

METHODS: Post-printing cellular viability and pluripotency was evaluated using fluorescence-activated cell sorting (FACS). Multiple human pluripotent stem cell lines and the pluripotency markers were tested. HLCs differentiated from both hiPSCs and human embryonic stem cells (hESCs) sources were bioprinted and examined for the presence of hepatic markers. HLCs were then printed into a 3D hydrogel matrix and the resulting cell-laden hydrogel structures were used to verify that the differentiation proceeded normally in 3D. Complex 3D structures such as branched vascular structures were printed using pre-crosslinked alginate hydrogels.

RESULTS: Examined cells were positive for nuclear factor 4 alpha and were demonstrated to secrete Albumin and have morphology that was similar to that of hepatocytes. Both hESC and hiPSC lines were tested for post-printing viability and pluripotency and were found to have negligible difference between the printed and non-printed cells. hESC-derived HLCs were tested for viability and Albumin secretion during the differentiation protocol and were found to be hepatic in nature. 3D alginate structures with 40 printed layers (Fig1a,b) containing HLCs reached peak Albumin secretion at Day 21 of the differentiation protocol. Cell-laden alginate hydrogels were formulated with tuneable mechanical properties to create more complex and continuous 3D vascular structures (Fig1c-e). Degradation time of alginate hydrogel in cell culture media was investigated and long-term stability of the alginate hydrogel can be enhanced by post-printing treatment of barium chloride.

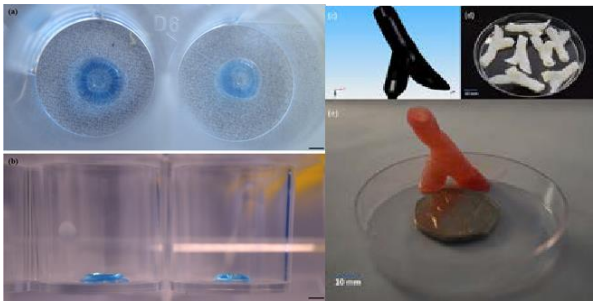

Fig. 1: 3D printed cell-laden alginate structures (a) 40 layers printed into a 24-well plate top view; (b) side view (scale bar 2 mm); (c) a CAD image of the vascular structure; (d), (e) printed vascular structures.

DISCUSSION & CONCLUSIONS: This work demonstrates that the valve-based printing process is gentle enough to print human pluripotent stem cells (both hESCs and hiPSCs) while either maintaining their pluripotency or directing their differentiation into specific lineages. The ability to bioprint human pluripotent stem cells and vascular structures will pave the way for producing organs or tissues on demand from patient specific cells which could be used for animal-free drug development and personalised medicine.

ACKNOWLEDGEMENTS: The authors acknowledge the funding support from the EPSRC (Grant No: EP/M506837/1), Innovate UK and NC3Rs.

microgrooved surface induces predominant tenogenic phenotype of human dermal fibrobalsts in vitro and aligned nanofiber scaffold enhances neotendon formation in vivo

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INTRODUCTION: Our previous study demonstrated that dermal fibroblast was a feasible cell source for tendon engineering in vitro and in vivo. Nevertheless, the underlying mechanism remained unexplored. We hypothesized that induced cell elongation and unilateral mechanical loading may contribute to this transdifferentiation. In this study, we employed a micropatterned surface and aligned nanofibers respectively as a 2D and a 3D models to investigate this hypothesis.

METHODS: In 2D model study, a silicon membrane with parallel aligned microgroove (3 μm deep and 10 μm wide) was used as an experimental group to enforce cell elongation, whereas a plain membrane was used a control. In 3D model, aligned nanofibers were used as an experimental group whereas random patterned nanofibers were used as a control. Human dermal fibroblasts(hDFs) were isolated and seeded on the topographical surface or on nanofibers for in vitro culture followed by qPCR, Elisa and TGF-β stimulation and its blocking. Patterned nanofibers were also investigated for their roles in tendon regeneration with or without cell seeding in nude mouse and rat models respectively followed by mechanical analysis, histology and scanning electron microscope examination.

RESULTS: In 2D study, parallel microgrooved topography could convert spread hDFs into an elongated shape, and induce a predominant tenogenic phenotype as the expression of biomarkers was significantly enhanced such as scleraxis, tenomodulin, collagens I, III, VI and decorin. It also enhanced the expression of TGF-β1, but not α-SMA. Elongated hDFs failed to induce other phenotypes such as adiopogenic, chondrogenic, neurogenic and myogenic lineages. By contrast, no tenogenic phenotype could be induced in elongated human chondrocytes, although chondrogenic phenotype was inhibited. Exogenous TGF-β1 could enhance the tenogenic phenotype in elongated hDFs at low dose (2 ng/ml), but promoted myofibroblast transdifferentiation of hDFs at high dose (10 ng/ml) regardless of cell shape. Elongated shape also resulted in decreased RhoA activity and increased ROCK activity. Antagonizing TGF-β or inhibiting ROCK activity with Y27632 or depolymerizing actin with cytochalasin D could all significantly inhibit tenogenic phenotype induction particularly in elongated hDFs. In 3D model, after 7 days of culture in vitro, cells seeded on aligned fibers became elongated and expressed higher levels of tenogenic markers than the cells on random fibers including SCX, MKX, TNMD, COL 1, 3 and 6, tenascin-C, BGN and FMOD. After in vivo implantation for 3 months in nude mice, cells seeded on aligned nanofibers formed much organized tissue structure with aligned collagen fibers, stronger mechanical properties and higher expression levels of tenogenic markers when compared with the tissue formed with cells on random nanofibers. When cell-free nanofiber scaffold was implanted to bridge a defect of Achilles tendon in a rat model for 3 months, the aligned nanofibers could better recruit host cells into the scaffold and regenerate a tendon-like tissue along with enhanced gene expression of tenogenic markers than the random nanofibers.

DISCUSSION & CONCLUSIONS: Elongation of cultured dermal fibroblasts can induce a predominant tenogenic phenotype likely via synergistic effect of TGF-β and cytoskeletal signalling. Aligned nanofibers could induce tenogenic phenotype in vitro and regenerate tendon in vivo.

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Platelet lysate activates endothelial cells by inducing proliferation of quiescent cells and suppresses the inflammatory response by inhibiting NF-κB activation

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INTRODUCTION: Platelet Lysate (PL) contains a cocktail of growth factors and cytokines, which actively participates in tissue repair. In previous pubblications, we have shown that PL modulates the inflammatory response occuring in wound while induces proliferation of resident cells [1,2]. The aim of this study was to assess the activity of PL on endothelial cells, the first cells that respond to the wound causing damage of blood vessels and resulting in the cascade of coagulation and release of platelet content.

METHODS: Human Umbilical Vein Endothelial Cells (HUVEC) were obtained from primary cultures derived from fresh umbilical cords (at least three patients). Cells were seeded on gelatin coated plates and cultured in M199 medium supplemented with 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 100 mg/l heparin, 10 µg/l aFGF, 10 µg/L bFGF, 10 µg/l EGF, 1 mg/L hydrocortisone. PL was produced starting from platelet rich plasma (PRP) obtained from a pool of 10, at least, healthy human blood donors in consensus with the guidelines of the institutional ethics committee. After a low speed centrifugation, platelets were washed and re-suspended in phosphate-buffered saline (PBS) at a concentration of 10 millions platelets/µl and the suspension was subjected to 3 consecutive freeze/thaw cycles followed by a high-speed centrifugation. The supernatant, containing the cocktail of factors released by the platelets (platelet lysate in PBS), was collected and stored in aliquots at -80°C until use. In the presented experiments, PL was added to complete culture medium at a final concentration as 5%.

RESULTS: PL increases proliferation of endothelial cells and induces proliferation in quiescent cells by activating the pathways of ERKs and AKT, and the synthesis of cyclin D1 (Fig.1). PL inhibits the inflammatory response induced by IL-1 by inhibition of the NF-κB activation (Fig. 2).

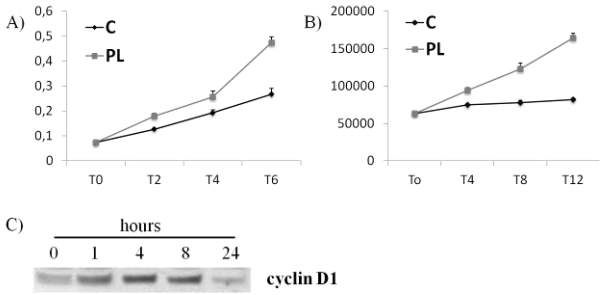

Fig. 1: HUVEC cell proliferation in the absence and in the presence of PL (average of three experiments) A) proliferating cells by crystal violet staining. B) quiescent confluent cells by cell count. C) induction of cyclin D1 by PL in confluent cells.

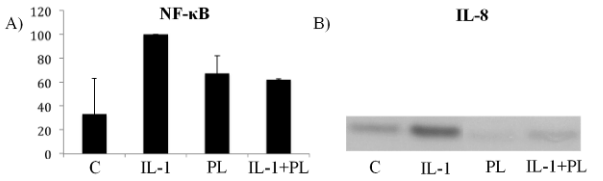

Fig. 2: HUVEC response to IL-1 in the absence and in the presence of PL. A) NF-κB induction and modulation. Values are expressed as percentage referred to IL-1 value. B) IL-8 induction and modulation

DISCUSSION & CONCLUSIONS: PL has a protective activity towards endothelial cells by inhibiting the inflammatory response to IL-1 and possibly other inflammatory cytokines released in vessels damage. Moreover, PL is able to induce in quiescent cells a proliferative response, important for vessel repair. This finding shows the possible mechanism of PL in the restoration of blood vessel functional activity in wound healing.

Unmet clinical needs for bone engineering

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The transplantation of autogenous bone is regarded as the gold standard in the surgical treatment of humans till today. Inevitable risks on the harvesting site as well as on the recipient site lead to critical consideration, especially in the treatment of multimorbid old patients. Here, the new bone tissue engineering techniques offer alternatives.
This keynote lecture assesses the clinical results of bone tissue engineering in oral surgery during the last ten years. The support we received by scientists from labs, by the pharmaceutical and medical engineering industry will be discussed critically.
Research results obtained in labs have to be verified in animal models and clinical studies before they are licensed as new products or medical devices for treatment in humans, if at all.
There is a significant difference between interests and objectives of scientists in labs and those of surgeons in clinical application. This is also forced by the influence of the pharmaceutical and medical engineering industry. Regarding the realization process of a new product, these obstacles lead to important gaps between research results in the labs and application requirements in human treatment.
The above situation will be presented in different models of bone tissue engineering (bone-TE in critical sized defects and vertical bone development).
These techniques will be explained using groups of patients that show:

1. critical sized defects, e. g. large cystic holes,
2. total bone loss of the alveolar ridges,
3. periodontal bone defects.

Bone tissue engineering techniques require:

1. matrices, should be translucent and give primary stability,
2. cells, e.g. stem cells and platelets
3. signaling molecules to induce angiogenesis and bone development.

Design and synthesis of binding growth factors

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INTRODUCTION: The immobilization of growth factors on materials is attractive because it enables cell behaviors such as growth and differentiation to be regulated through signal transduction. There has been an increase in the number of reports on immobilized growth factors since growth enhancement by immobilized growth factors was first reported.[1,2] Thus, covalently immobilized growth factors can now be used effectively. The long-lasting effect and the high local concentration on the surface induce significant biological effects on cells. Apart from these covalent immobilization on materials, design of binding growth factors has been enabled by protein engineering. [3]

Here by using conventional protein engineering, collagen-binding bone morphogenetic protein-4 (BMP4) was prepared by fusion of collagen biding domain (CBD) of fibronectin. Another is designed by conjugation of epidermal growth factor (EGF) with a peptide moiety derived from salivary statherin, a protein that adheres to hydroxyapatite.[4]

METHODS: For preparation of collagen binding BMP4, BMP4 and CDB cDNAs were connected with a spacer DNA and inserted into a DNA vector. The cDNA was expressed in *E. coli.* and the collagen-binding (CB-)BMP4 was obtained. On the other hand, the EGF derivative was prepared by a solid phase method. The peptide containing phosphorylated serine was connected to the N-terminal of truncated EGF.

RESULTS: The prepared collagen-binding BMP4 was directly injected into mouse or bound on the collagen-based matrices for implantation as shown in Figure 1. Direct injection promoted bone formation *in vivo*. On the other hand, CB-BMP4 bound to the collagen-copoly(L-lactic and glycolic acid) (PLGA) hybrid scaffold and the BMP4-immobilized hybrid scaffold supported cell adhesion and proliferation. The osteogenic induction effect of the immobilized CB-BMP4 was investigated with three-dimensional culture of human bone marrow-derived mesenchymal stem cells in the BMP4-immobilized collagen-PLGA hybrid scaffold. The *in vivo* implantation experiment demonstrated that the immobilized CBD-BMP4 maintained its osteoinductive activity, being capable of up-regulating osteogenic gene expression and biomineralization.

On the other hand, the EGF derivative had significantly higher binding affinity for both hydroxyapatite and titanium than soluble EGF, and the bound EGF significantly enhanced cell growth by long-lasting activation of intracellular signal transduction.

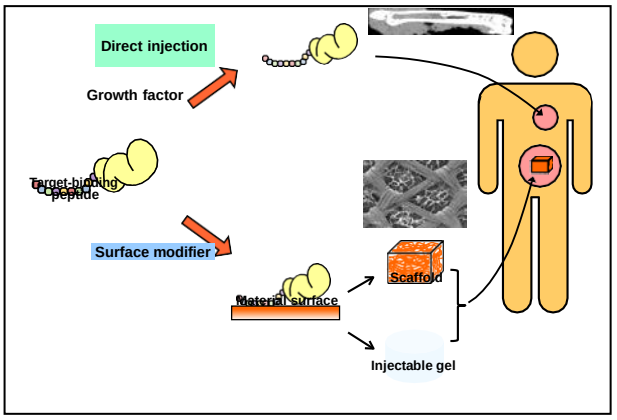

Fig. 1: Illustration of applications of binding growth factors.

DISCUSSION & CONCLUSIONS: The strong osteoinductivity of the BMP4-immobilized scaffold suggests it should be useful for bone tissue engineering, stem cell function manipulation and bone substitutes. EGF derivative significantly promoted cell growth on inorganic surfaces. The binding growth factors are useful for tissue engineering.

Generation of a 3D bone remodeling model combining human osteoblast and osteoclast precursors in a vascularized matrix enriched with CaP nanoparticles

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INTRODUCTION: Bone remodeling is a dynamic process that relies on the balance between bone resorption and formation. Osteoclasts are capable of dissolving the mineralized matrix, leaving space for osteoblasts and endothelial cells to form bone and new vessels. We have recently developed in our laboratory microscale hydrogel-based in vitro models, namely the 3D MiniTissue models, to generate vascularized bone-mimicking microenvironments [1-2]. Here, we describe a vascularized 3D MiniTissue bone remodeling model based on the tetraculture of primary human cells in a 3D collagen/fibrin (Col/Fib) matrix enriched with CaP nanoparticles (CaPn), resembling bone mineralized matrix (Fig. 1a).

METHODS: Human umbilical vein endothelial cells (HUVECs), bone marrow mesenchymal stem cells (BMSCs), osteoblast (OBs) and osteoclast (OCs) precursors were combined in different cocultures in plain and CaPn-enriched Col/Fib: 1) HUVECs-BMSCs (10:1); 2) OBs-OCs (1:2); 3) HUVECs-BMSCs-OBs-OCs (10:1:1:2). BMSCs were either used undifferentiated, to support HUVECs, or as OB precursors after 7 days in osteogenic medium. PBMCs were differentiated in OC precursors after 7 days in medium with M-CSF and RANKL. CaPn (5 mg/ml, Ø ~20 nm, length ~80 nm) were added to a collagen/fibrinogen mix (40:60). Cells were mixed with thrombin and then with plain or CaPn-enriched collagen/fibrinogen. The cell-laden mix was injected in U-shaped PMMA masks and let to polymerize. Samples were cultured for 10 days. Microvascular network formation was evaluated by confocal microscopy. OB differentiation was assessed quantifying Alkaline Phosphatase (ALP) and cell-mediated mineralization. OC differentiation was assessed quantifying Tartrate-Resistant Acid Phosphatase (TRAP) and cell-mediated phosphate release.

RESULTS: HUVECs developed a robust 3D microvascular network (Fig. 1b) and BMSCs differentiated into mural cells. OB and OC differentiation was enhanced by the presence of CaPn, as revealed by significantly higher ALP and TRAP levels, and by superior cell-mediated mineralization and phosphate release than in plain Col/Fib. ALP and TRAP levels were even higher when OBs and OCs were cocultured with HUVECs and BMSCs in the tetraculture, implying that HUVECs and BMSCs contributed to OB and OC differentiation. Remarkably, CaPn-enriched tetraculture showed significantly higher values of ALP and TRAP activity at day 3 and day 10, respectively, compared to the tetraculture in plain Col/Fib ($p<0.001$), indicating that the mineralized matrix stimulated cell differentiation (Fig. 1c,d).

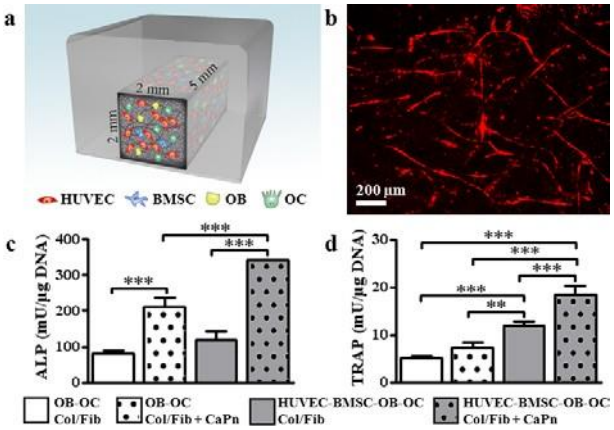

Fig. 1: 3D MiniTissue bone remodeling model (a). Microvessel formation (b). ALP (c) and TRAP (d) activity at day 3 and day 10, respectively.

DISCUSSION & CONCLUSIONS: The 3D MiniTissue bone remodeling model holds great potential to investigate bone and endothelial cell interactions. This system allows to prepare multiple samples minimizing the use of cells and reagents with a superior ease of use compared to microfluidic systems, being a suitable platform to screen drugs for bone diseases. Further, using patient-derived cells in the model will increase its relevance as personalized drug screening platform.

ACKNOWLEDGEMENTS: This project was funded by the Italian Ministry of Health. Authors have nothing to disclose.

In vitro models to investigate the contribution of cell-ECM interaction to cardiac pathologies

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INTRODUCTION: Heart failure (HF) - the condition for which cardiac muscle is not able to produce the pumping force necessary to provide adequate blood flow to the organism – can be described as the mechanical failure of the muscle. When it is not due to vascular issues, such mechanical impairment is ascribed to genetic defects of the cell contractile apparatus. Recent evidence shows that defects or modifications in the extracellular matrix (ECM) composition and mechanics and/or in the apparatus cells use to perceive such modifications (mechanobiology apparatus) can result in aberrant signalling and changes in tissue-specific cell function independently of genetic background [1]. By coupling micropatterning and microfluidics with solid biochemistry and image analysis approaches, we seek to highlight the contribution of the mechanobiology apparatus to cardiac pathologies and propose novel *in vitro* models of diseases.

METHODS: iPSC-derived cardiomyocytes were cultured onto micropatterned surfaces or hydrogels having defined stiffness. Cardiomyocyte contractility and beating frequency were evaluated by image analysis, while iPSCs were patterned to control colony size and induce cardiac differentiation. A prototype of pneumatic microfluidic chip with deformable cultivation substrate to induce and study cardiomyocyte functon is currently being developed.

RESULTS: The alignment of iPS-derived cardiomyocytes and their directional beating was induced by surface micropatterning. These experiments allowed setting the conditions to maximize the contractility and the alignment of cardiomyocyte bundles. On the other hand, we developed micropatterned cardiac microchambers from iPSC-derived cardiomyocytes. These experiments were suited to follow mechanosensor activation during the differentiation process. The information obtained by such experiments is being used to develop devices able to recapitulate the organization and the mechanical features of native cardiac tissue. In particular, a stretching machine has been adapted as to include the contribution of micropatterned cues to the arrangement of cardiac contractile units, thus improving cardiomyocyte maturation and function. Moreover, a prototype of a pneumatic microfluidic chip entailing both the stretching features and the unidirectional orientation of cardiomyocytes bundles is being manufactured.

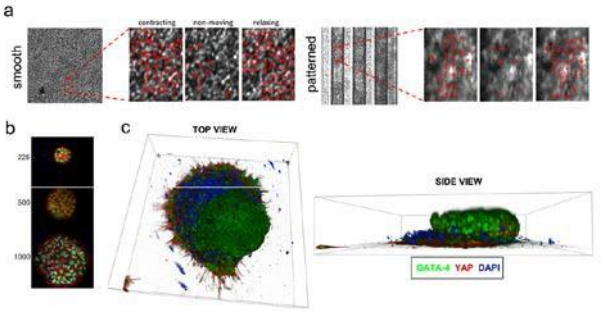

Fig. 1: (a) The alignment of iPS-derived cardiomyocytes and unidirectional beating can be controlled by micropatterned surfaces (b) Undifferentiated micropatterned iPSC colonies and (d) 3D reconstruction of a cardiac differentiated colony.

DISCUSSION & CONCLUSIONS: Investigating the role of cell mechanosensors in cardiac pathologies is crucial to unveil the contribution of ECM remodelling to the progression of such diseases. By tuning cardiomyocyte contractility and modifying the composition and the patterning of the substrate, it will be possible to: 1) set up meaningful models of cardiac pathologies; 2) investigate the contribution of cell mechanosensors to the onset of cardiac diseases; 3) highlight the role of ECM modifications in such conditions.

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).

Does artificial HIF stabilization enhance topical oxygen therapies for chronic wound healing?

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INTRODUCTION: Impaired wound healing severely impacts on the quality of life; it is estimated that between £2.3 and £3.1 billion is spent by the NHS on chronic ulcers[1] and this is the leading cause of amputation in the UK. An impaired cellular response to hypoxia (low O_2 pressure) may be the primary cause of delayed wound repair. Diabetic and elderly patients (who are most at risk of developing chronic wounds), have a reduced cellular response to hypoxia, due to an impairment of the HIF pathway. Furthermore, artificial regulation of this HIF pathway (using HIF mimetics) enhances wound repair in these patients[2]. Topical oxygen therapies (TOT) also improve chronic wound healing, possibly by increasing oxygen diffusion within the hypoxic chronic wound[3]. We hypothesise that combining TOT with artificial HIF-1α stabilization (using a HIF mimetic) will promote the normal wound healing response via the HIF pathway, whilst also allowing the benefits of increased O_2 diffusion. This approach would induce angiogenic growth factor production, stem cell recruitment, antimicrobial response and importantly may promote cell survival when exposed to the increased Reactive Oxygen Species caused by TOT.

METHODS: To mimic the diabetic wound environment, immortalized human keratinocytes (HaCaT), were pre-cultured in a high glucose (25 mmol) DMEM for a minimum of 2 weeks, prior to experimentation in the same media (with low glucose controls). DMOG (Dimethyloxaloylglycine) was selected following initial experiments with other HIF mimetics. Two normobaric hyperoxia chambers were constructed and tested. HaCaT cells were treated with DMOG (250 μM) in hypoxia (5% O_2), normoxia (21% O_2) and hyperoxia (100% O_2) for 90 min (the normal TOT treatment length), 24 h and 72 h. The chambers were sealed and flushed at a 5.5 L/min for 5 min with O_2. Media with echynomycin was used to inhibit the HIF-1α pathway as a control. Cell viability (Alamar Blue, proliferation (total DNA), cytotoxicity (LDH assay) and angiogenic factor production (VEGF ELISA) were analysed.

RESULTS: High glucose treated cells had a reduced response to hypoxia (as determined by VEGF expression, Fig. 1a). Hyperoxygen treatment in excess of 90min caused cell death and by day 7 no cells DMOG increased cell survival and metabolic activity in a hyperoxygen environment compared to those without DMOG. The addition of DMOG increased VEGF/μg DNA (P =0.05) in all oxygen conditions and by 3 fold in hyperoxygen (Fig 1b). Confirmation of the role of HIF-1α in causing these DMOG related cellular responses was investigated by the inhibition of the HIF pathway with echinomycin treatment.

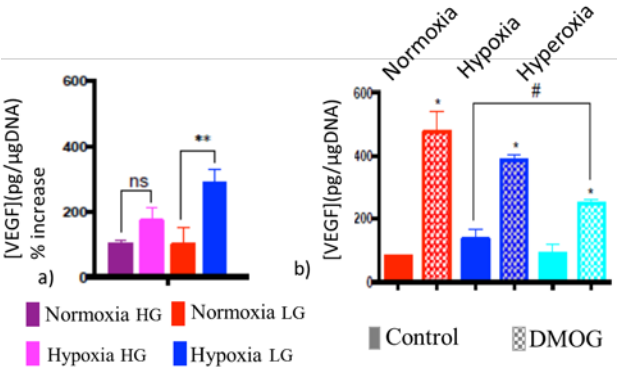

Fig. 1: a) A high glucose environment reduced the cellular response to hypoxia b) DMOG treatment increased VEGF expression in all oxygen conditions in a high glucose environment.

DISCUSSION & CONCLUSIONS: DMOG demonstrated the ability to increase wound healing responses in a diabetic environment. Indeed, artificial HIF stabilisation considerable increased VEGF expression compared to actual hypoxia controls. Furthermore, DMOG treatment promoted cell survival and caused an angiogenic response in hyperoxygen conditions, suggesting a possible combination therapy approach whereby HIF and TOT could be used together. To this end we are developing HIF mimetic releasing wound dressings that could be suitable for TOT.

ACKNOWLEDGEMENTS: This research was supported by CONACyT.

Developing confidence in the predicative ability of *in vitro* microphysiological systems for regulatory risk assessment

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INTRODUCTION: The field of toxicology is transitioning from an emphasis on animal studies and apical endpoints to *in vitro* assays that depend upon more mechanistic data. Mechanically active "organ-on-a-chip" microdevices that reconstitute tissue-tissue interfaces critical to organ function can expand the capabilities of cell culture models and provide low-cost and more informative alternatives to animal toxicology studies. With simplified designs and careful choice of biocompatible device materials, they can be useful for high-content analysis and screening of cellular responses to drugs, chemicals, particulates, toxins, pathogens, or other environment stimuli relevant to pharmaceutical, cosmetic, and environmental applications. This presentation will propose a framework to begin context-use-of-use qualification with a simple, but important, context of use, use of a "gut on a chip" for assessment of bioavailability, and hopefully pave the way for additional regulatory uses of these new tools.

METHODS: Developing confidence in the predicative ability of *in vitro* microphysiological systems begins with identifying the critical regulatory gaps to that can be addressed with these new tools, the consequences of an incorrect or incomplete answer and an assessment of the level of confidence in each of the integral parts of an *in vitro* microphysiological system. A determination needs to be made on the essential biological and functional features that are needed in the test organ on a chip to address the context of use. The test or reference library of compounds for qualification should be tied directly to the regulatory question that is being addressed in the context of use. This means that a different context of use may require a different set of test chemicals. Additionally, it should be decided up front on how to use the data to make/add to/refine a regulatory decision.

DISCUSSION & CONCLUSIONS: Often the first step towards the use of new toxicology tools is the most difficult one for regulators to take. This applies to the use *of in vitro* microphysiological systems to answer important regulatory questions. But once there is a demonstration that an *in vitro* microphysiological system can be used with confidence to answer important regulatory questions, other regulators may be more inclined to follow. This presentation defines a framework to begin context-use-of-use qualification with a simple, but important, context of use and hopefully paves the way for additional regulatory uses of these new tools. Combining together the above information for a "context of use" evaluation of the overall predictive ability of a new tool to answer critical regulatory questions can be used a framework for qualifying *in vitro* microphysiological systems in a transparent and scientifically defensible manner.

real-time monitoring of mechanical properties of 3D tissue/constructs

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INTRODUCTION: Tissue engineering is an emerging multidisciplinary field involves biology, medicine and engineering to restore or regenerate tissue or organ function [1]. As one of the main components, scaffold serves as a template for cell to delivery and support tissue remodelling, fill void and control the release of signalling molecules. The use of hydrogels in tissue engineering has become popular due to their unique properties. The mechanical strength of the hydrogel can be improved by mechanically stimulating the seeded cells, leading to remodelling within the construct in some types of cells. On the other hand, the cell proliferation and matrix production will change the property of scaffold-cell constructs continuously. Among them, the change of mechanical property in scaffolds and resulted constructs along the culture and multiple stimulations bears rich information of the cellular activities and metabolism. Thus, the monitoring of mechanical properties of hydrogel constructs non-invasively and non-destructively is of paramount importance to reveal cellular performance and optimize culture conditions for facilitating tissue engineering applications. Current mechanical assessment techniques including extensometry, compression and indentation for hydrogel are destructive, unable to measure the same sample for the entire culture period. In the recent years, we have developed a few non-destructive techniques and demonstrate that they are suitable to mornitor the mechanical property change in culture for prolonged period.

METHODS: Two types of mechanical testing techniques have been developed [2-3]. One is ball-indentation technique in which constructs were circumferentially clamped in between two transparent plastic circular rings that were held in place by tightly screwed thin stainless steel plates. The constructs were deformed by placing a sphere (indentor) with weight and diameter depending on the modulus of the scaffolds or constructs. Images of deformed constructs were acquired after placing the sphere at different culture time points up to 4 weeks using a long working distance microscope system. The Young's modulus of the samples was calculated by the extent of the deformation (δ) based on the established equation [2]. The cross-sectional thickness of each construct was measured using an optical coherence tomography (OCT) [4]. Another technique [3] is the optical coherence tomography-based micro-indentation technique in which the deformation of hydrogels under indentation of a constant weight ball is measured by OCT images and the Young's modulus and effective viscosity have been calculated by the deformed images. Various hydrogel materials including collagen, agarose and alginate have been used. Corneal stromal cells and skin cells have been used to form the constructs.

RESULTS: The ball-indentation technique has shown consistent results on the contraction behaviour of corneal stromal cells. During the culture, the collagen-stromal cell constructs continued contraction with the increase of modulus and cell elongated morphology but the decrease of thickness. These changes were cell type, cell seeding density and collagen gel quality dependent [5]. The prolonged culture and multiple measurements on the same sample do not compromised the quality of the data.

DISCUSSION AND CONCLUSIONS: The advantages of these mechanical characterization method are compelling with following advantage:
(i) the stress distribution in the deformed sample is bi-axial and axisymmetric, (ii) no need for force feedback control for creep test, (iii) applicable to permeable or semi-permeable membrane and (iv) the force and displacement resolution can be as accurate as 10 mN and 10 mm, respectively. Thus, the new tools can be extremely valuable for tissue engineering which frequently requires online, real-time and non-destructive measurement.

ACKNOELAGEMENTS: The project is supported by BBSRC, EPSRC and local charity fund.

Future prospects for human tissue engineered urethra transplantation: decellularization and recellularization-based urethra regeneration

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INTRODUCTION: The role of tissue-engineered urethra as a potential substitute for effective treatment of urethral strictures and anomalies has been previously discussed [1-2]. In the current study, we evaluated the histological characteristics of decellularized human urethra after transplantation into the rat omentum.

METHODS: Eight adult human male urethras accompanied with the surrounding corpus spongiosum were obtained in sterile condition after obtaining ethical committee approval. Then, the tissues were decellularized with detergent-based method. The efficacy of decellularization and extracellular matrix (ECM) preservation was evaluated by histopathological examinations, DNA quantification, scanning electron microscopy (SEM), and tensile test. Decellularized scaffolds were washed to remove the detergents 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 postoperatively to assess the natural recellularization. Histological examination, SEM, DNA quantification, and immunohistochemical (IHC) staining were performed.

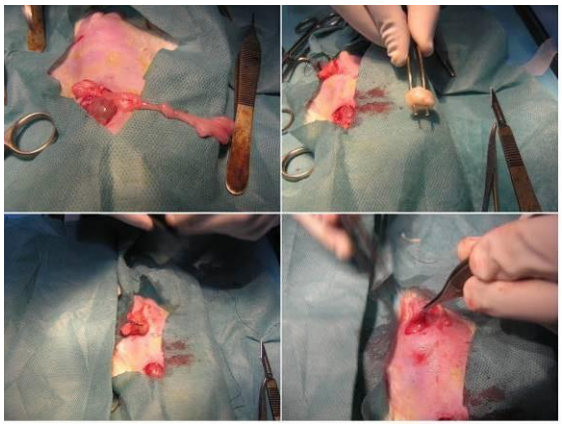

Fig: surgical technique for transplanting decellularized human urethra

RESULTS: DNA quantification and histological evaluations confirmed the removal of all cellular and nuclear components in urethral and surrounded corpus spongiosum scaffolds. Tensile test confirmed that biomechanical properties of the urethral ECM were well preserved. In-vivo recellularization revealed promising results in cell seeding of epithelium-like cells in the lining of the urethra as well as smooth muscle cells in the wall structure. Progressive angiogenesis was also detected in the transplanted scaffolds during the time-points while inflammatory markers were negative.

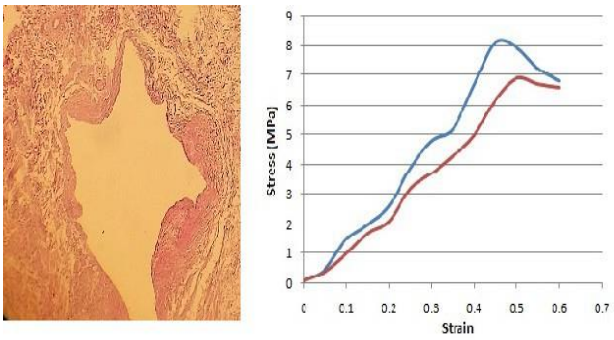

Fig 2: Histological evaluations and biomechanical properties of decellularized urethral scaffold

DISCUSSION & CONCLUSIONS: The results suggest that we can prospect to perform urethral reconstruction by using the body as a natural bioreactor that can paw the road for clinical application of acellular matrix in urological reconstructive surgery.

DISCLOSURE: Authors have nothing to disclose.

Characterization of bone marrow derived-circulating healing (ch) cells endowed with healing capacity

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INTRODUCTION: All body tissues, particularly those characterized by a high cell turnover, depend on innate regenerative events in order to function properly. The concept of adult stem cells intended as cell populations restricted to their own tissue has been challenged by reports indicating that these cells can be mobilized in response to a tissue damage [1]. A growing number of scientific reports indicate that under the influence of various pathological stimuli, tissue-specific and/or bone marrow-derived stem/progenitor cells are rapidly mobilized to the blood stream, playing a crucial role in the repair of solid organs, acting directly or enhancing the re-activation of resident stem cells [2]. However, all stem/progenitor cells isolated from the peripheral blood have been isolated only in pathological conditions or following a mobilization procedure. Recently, we reported the identification of a rare cell population of Circulating cells derived from the peripheral blood (PB) of healthy mice and actively participate in the Healing process (CH cells) [3]. Indeed, CH cells were demonstrated to be connective tissue precursors able to integrate in wounded tissues and to appropriately differentiate into a broad spectrum of tissue-specific cells. These progenitor cells were identified as small cells characterized by the lack of expression of the pan-hematopoietic CD45 antigen, of the markers expressed by differentiated blood cells, as well as of markers typically associated to well-defined progenitors circulating upon injury. In this study, we aim to implement the characterization of CH cells by means of: (i) identification of a novel candidate surface marker; (ii) definition of their compartmental origin; (iii) validation of their functional capacity to migrate toward wounded sites and to differentiate into tissue-specific cells.

METHODS: In order to find a uniquely expressed marker that could lead to the enrichment of the CH cell population, the transcriptome profile of the FACS-purified populations from the PB of healthy mice were compared to published microarray data of precursor cells characterized by varying stemness degree. To identify the compartmental origin of CH cells, immunocompetent wt mice were lethally irradiated and reconstituted with enhanced red fluorescent protein-positive (RFP^{pos}) syngeneic bone marrow cells (chimeric mice). After five weeks, we checked for the presence, in the PB, of CH cells that co-express the RFP signal. A mouse model of femur fracture was applied to test whether the circulating enriched CH cells would be recruited toward sites of injury to actively participate in the healing process.

RESULTS: The analysis of the global transcriptional profile of the purified CH cells revealed promising candidate markers that had interesting up-regulated expression patterns when compared to other cell populations. Interestingly, the FACS analysis of small-sized $Lin^{neg}CD45^{neg}$ (CH) cells confirmed the protein expression of one of these selected genes. More importantly, CH-enriched cells derived from healthy Red Fluorescent Protein (RFP)-transgenic mice and systemically injected into syngeneic fractured wild-type mice migrated and engrafted in wounded tissues, ultimately differentiating into tissue-specific cells. Lastly, using the chimeric mouse model, we demonstrated the bone marrow origin of the CH-enriched cell population.

DISCUSSION & CONCLUSIONS: In addition to conventional cell replacement therapies, harnessing endogenous repair mechanisms to promote tissue regeneration represent an emerging direction for developing powerful therapeutic strategies. The presented data provide not only a deep characterization of a unique circulating cell population, but also a starting point to design new therapies targeting CH cells in order to improve their use in the future as effectors for the repair of tissue defects.

Wnt pathway activating polymeric nanoparticles for fracture localisation

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INTRODUCTION: Worldwide, an osteoporotic fracture is diagnosed every 3 seconds. This results in a cost to the European economy of ~€100 million per day. 10% of bone fractures will not heal adequately and require surgical intervention and, as yet, there is no approved systemic drug that is effective in enhancing fracture healing. Activation of Wnt signaling offers a promising therapeutic strategy for promoting bone repair[1], but can have both positive and negative effects on bone cell function depending on the timing and site of delivery[2]. We propose that controlled activation of the Wnt signaling pathway may be achieved using drug-loaded polymersome nanoparticles (PMs)[3]. Here we examine whether BIO-loaded PMs induce Wnt signaling activity in human skeletal stem cells (hSSCs), and their biodistribution in a mouse model of injury post-fracture.

METHODS: BIO-loaded PMs were produced by nanoprecipitation of polyethylene glycol-b-polycaprolactone (5k-b-18k) block-copolymer. The ability of BIO-PMs to activate the Wnt signaling was investigated using a reporter cell-line and by qPCR in hSSCs. *In vivo* distribution of PMs in mice with a 1 mm femoral drill defect was assessed 0-10 days post intravenous injection of DiR/DiI labelled PMs using IVIS and fluorescent imaging of cryo-sectioned bone specimens.

RESULTS: BIO-PMs induced a 16.14±2.1 fold increase in Wnt pathway activation in a reporter cell line, similar to an extracellular concentration of 2 μM free BIO, but with delayed activation. BIO-PMs also induced a significant increase in the expression of the Wnt target gene AXIN2 ($p<0.05$ n=3) and in the expression of the early osteogenic marker RUNX2 ($p<0.05$ n=3) in hSSCs. DiR-loaded PMs injected intravenously localised in the fractured bone within 24 hours of administration. The maximum level of PMs accumulation was reached after 48 hours, with 73.55±17.74 fold increase in fluorescence compared to background (n=5). DiR signal declined over time, but it was still detected after 10 days from the initial injection (34.54±6.8 fold increase in fluorescence compared to background, n=3). These finding is corroborated by fluorescent images of injured femurs that show the DiI in the defect area 10 days post injection.

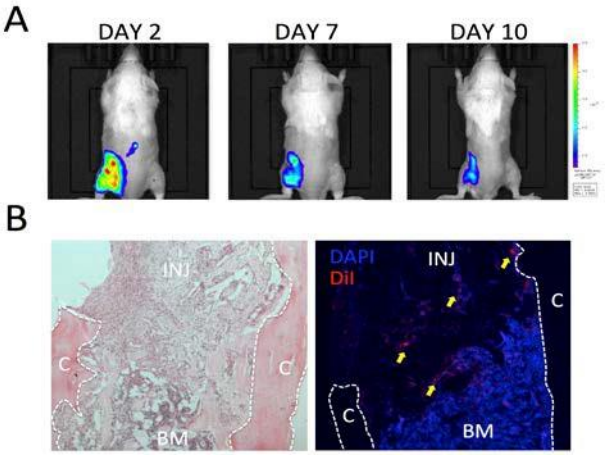

Fig. 1: A) IVIS images of mice with a bone injury showing intensity and distribution over time of fluorescent signal derived from DiR-PMs. B) H and E staining and fluorescent image showing the structure of the injury and the presence of DiI (yellow arrows) at the bone injury site following systemic injection of DiI-PMs. Inj, injury; BM, bone marrow; C, cortical bone.

DISCUSSION & CONCLUSIONS: BIO-loaded polymersomes induce the activation of the Wnt signaling pathway in hSSCs and enhance the expression of an ostegenic marker. Upon systemic injection in a bone injury model, PMs preferentially accumulate at the fracture site where they may activate the Wnt pathway. We conclude that the spatio-temporal controlled localisation of PMs loaded with the Wnt agonist BIO could represent an effective pharmacological treatment to promote bone regeneration after fracture.

ACKNOWLEDGEMENTS: We thank the University of Southampton and the Institute for Life Sciences-University of Southampton for supporting our research.

New scaffold concept for cartilage regeneration

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INTRODUCTION: Tissue engineering of articular cartilage faces the challenge that high mechanical forces impact the implanted biomaterials, the cells and the developing tissue. Therefore biomaterials should provide sufficient stiffness to withstand the varying loads during joint movement and to prevent the material from collapsing or shrinking due to cellular contraction inside the defect. The material should reduce the amount of mechanical stress transferred to the incorporated cells to promote the chondrogenic differentiation and matrix production. An optimal material should further allow even cell distribution, provide a template to guide the matrix deposition according to the requirements and architecture of articular cartilage tissue. Materials that provide high mechanical rigidity and slow degradation times are collagen and silk. The aim of this study was to prepare materials with high rigidity, providing an environment for chondrocytes or progenitor cells and compare it to native and decellularized cartilage.

METHODS: Silk hydrogels and silk sponges, created by salt leaching, were seeded with bovine chondrocytes or human adipose derived stem/stromal cells and cultivated under static condition or in a hydrostatic pressure bioreactor. Silk scaffolds were compared to cartilage tissue in its native state and after removal of glycosaminoglycans (GAGs) as well as commercial scaffold materials, which served as control. Histology, real time PCR and glycosaminoglycan assays were performed in order to investigate distribution, matrix deposition and differentiation of the cells. Samples were further mechanically tested for stiffness by compression tests.

RESULTS: Cells in silk sponges and gels showed even distribution and an increased GAG deposition or collagen type II expression compared to the time point of seeding and standard monolayer cultures. These conditions were achieved even without the addition of growth factors. Mechanical stimulation further increased the chondrogenic marker expression. The material kept its shape over the observation period of four weeks and was not contracted as it is reported for commercial scaffolds including hydrogels. Mechanical stiffness was above that of commercial materials but lower than cartilage tissue, even under GAG removal. The materials showed minor signs of degradation in the course of five weeks.

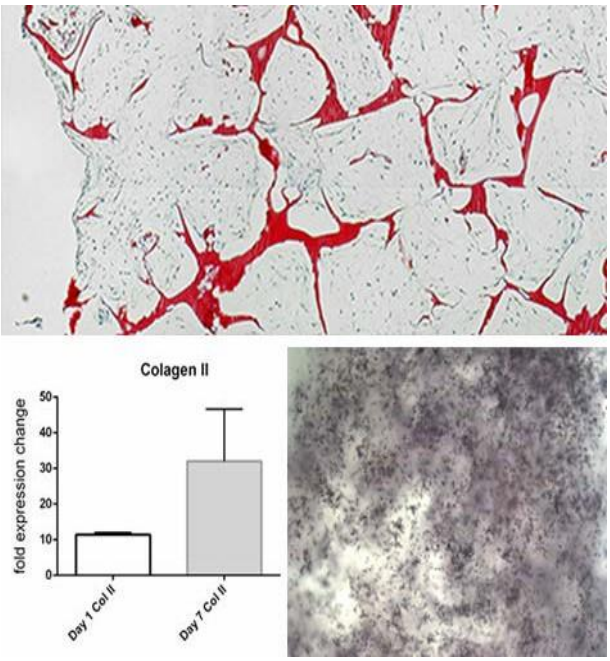

Fig. 1: MSB staining of histological sections of silk sponges (upper row) reveals an even distribution of cells in between the spongous lumina of the scaffold. In the silk hydrogel (lower row) cells revealed an increasing chondrogenic differentiation and formazan stained cells a high physiological activity.

DISCUSSION & CONCLUSIONS: Silk hydrogels and sponges developed in this study are promising candidates as temporal cell environment for cartilage regeneration since they retain their shape and mechanical resistance over several weeks and likewise provide a stable, long term degradable, suitable environment supporting chondrogensis.

ACKNOWLEDGEMENTS: The study was funded by the FFG Bridge early stage „CartiScaff“ 842455 project.

Correlation between *in vitro* and in *vivo* assessments of biomaterials for bone-regeneration – a multi centre study

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INTRODUCTION: Research on biomaterials for bone regeneration generates a surfeit of new biomaterials requiring evaluation with reliable and comparable methods of biocompatibility and functionality for clinical translation. To reduce the burden of in vivo assessment, there is a need for refined in vitro assays that are predictive of in vivo outcomes. This retrospective study correlated in vitro results with in vivo outcomes observed for a range of biomaterials for bone regeneration.

METHODS: Assessors from 8 universities in the European consortium BioDesign scored both *in vitro* and *in vivo* outcomes of individual biomaterial experimental variables (1=poor to 5=very good) on the basis of assessor-defined criteria particular to each assay. The data included 36 *in vivo* studies 47 *in vitro* assays and 93 materials. Data were sorted into sub-groups of different *in vitro* assays.

RESULTS: There was no significant overall correlation between in vitro and in vivo outcome. The mean in vitro scores shared 58% of variance to the in vivo scores. The mean in vivo scores shared 51% of variance to the in vitro scores. All combinations of in vitro assays showed less than 10% covariance, except for one combination of alkaline phosphatase and biocompatibility assays, which shared 95% covariance though low statistical power limits the conclusions that can be drawn.

DISCUSSION & CONCLUSIONS: The conventional biomaterial-testing pipeline tends to selects variables with positive *in vitro* outcomes for testing in subsequent *in vivo* studies. This makes the poor correlation surprising. The inadequacies of the current *in vitro* assessments highlighted here, further stress the need for the development of novel approaches to *in vitro* biomaterial testing and validated pre-clinical pipelines.

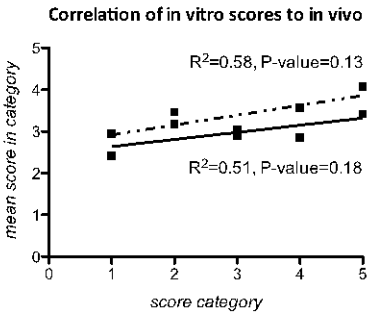

Fig. 1: Overall correlation between in vitro and in vivo study outcomes in a European multicentre study. The mean in vitro scores had a covariance of 58 % to the in vivo score (dotted line). The mean in vivo scores shared 51% of variance with the in vitro scores (solid line).

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Overcoming Endosomal Escape: Addressing the Key Challenge of Nucleic Acid Delivery

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INTRODUCTION Gene based therapies using plasmid DNA (pDNA) or interfering RNA holds great promise in treating diseases that are not treatable with conventional treatment modalities. These methods rely on finding suitable ways to deliver functional biomolecules to the site of action i.e., nuclear delivery of pDNA and cytosolic delivery of short interfering RNA (siRNA). Cellular delivery of these functional molecules requires suitable delivery carriers that are generally cationic polymers that condense such large negatively charged molecules by ionic interactions [1]. Though such a strategy improves intracellular delivery, it does not guarantee efficient endosomal release of the cargo molecules. In fact, with well-known lipid based cationic polymers only 1–2% of the biomolecules escapes the endosome compartment [2]. We have developed a unique method to improve cellular delivery of pDNA and siRNA using two different strategies that improves endosomal release of nucleic acids drugs.

METHODS: We have developed two different strategies to deliver pDNA and siRNA that are described as follows:

Designing pDNA delivery strategy: To improve cellular delivery of pDNA, we developed chondroitin sulphate (CS) coated DNA-nanoplexes using a modular approach where CS was coated post pDNA/PEI nanoplex formation [3]. To ensure good stability of the nanoplexes, we exploited imine/enamine chemistry by reacting aldehyde-modified chondroitin sulphate (CS-CHO) with free amines of pDNA/PEI complex. To test the effect of coating on endosomal release, we performed transfection experiments in different human cell lines in the presence and absence of chloroquine, an endosomolytic reagent.

Designing siRNA delivery strategy: In our efforts to develop a siRNA delivery strategy, we developed a novel siRNA design having a specific DNA overhang at the 3'-end of the sense strand (cell penetrating RNA or cpRNA). We also tested the gene knockdown efficiency of siRNA design where the DNA strand was incorporated at the at the 3'-end of antisense strand (anti-cpRNA). Gene knockdown experiments using modified RNA molecules were performed in MG63 cells with and without MATra, a magnetic nanoparticle based transfection reagent.

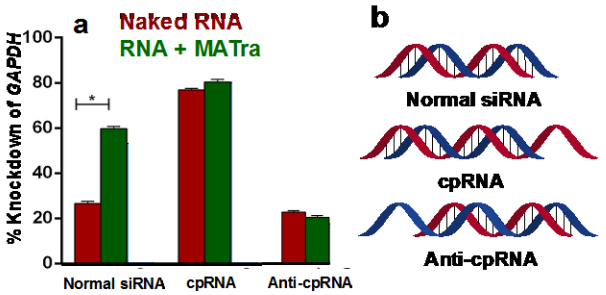

Fig. 1: (a) GAPDH gene knockdown efficiency of different modified RNA sequences with and without transfection reagent (MATra), as determined by qPCR experiments; (b) Structural design of different siRNA that were tested in this study.

RESULTS:

pDNA delivery: With our rational design of the supramolecular gene carrier system we observed efficient cellular uptake, and controlled endosomal pDNA release without any cytotoxicity. On the contrary, burst release of pDNA from endosome (using chloroquine) resulted in significant reduction in gene expression.

siRNA delivery: Our siRNA design gave an unprecedented cellular delivery efficiency without any cationic delivery carrier. The cpRNA design prompted selective recruitment of functional strand, which resulted in higher gene knockdown efficiency than standard siRNA. The anti-cpRNA design gave significantly lower gene knockdown efficiency, validating the selective strand recruitment due to the unique design of RNA.

DISCUSSION & CONCLUSIONS: Our pDNA and siRNA delivery strategy clearly suggests that anionic polymers having a unique structural design can promote cellular delivery and control endosomal release of the cargo molecules. Such an approach will therefore have major implications in designing next-generation functional nucleic acid drugs.

efficacy of conventional versus innovative therapies for treating skin wounds in veterinary medicine.

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INTRODUCTION: The skin is the largest organ of mammals. The loss of skin integrity may induce important dysfunctions or even death. For superficial wounds, the endogenous healing mechanisms in combination with traditional wound care are sufficient to achieve functional repair. In contrast, in larger wounds, like third and fourth degree burns, chronic wound or deep ulcers it is difficult to obtain the *restitutio ad integrum* and fibrosis and/or scar tissue develops[1,2]. The aim of this study was to verify the efficacy of conventional and innovative topic treatments on skin regeneration, induced experimentally in sheep. To achieve this goal different types of investigations (clinical, molecular, histological, immunohistochemical) were performed.

METHODS: Six skin lesions (4x4cm) were surgically created on the back of six healthy adult sheep; every single wound was destined, in a randomized way, to one of the following treatments: Acemannan gel, Manuka Honey, hyaluronic acid, Plasma[3] (ionized gas), allogeneic mesenchymal stem cells isolated from peripheral blood (PB-MSCs). The sixth wound was the placebo. Biopsies were collected with a surgical punch (0,6x0,6 cm) at time T0, T15 and T40 days. Lesions were clinically evaluated considering the presence and color of wound fluid, the state of hydration, the wound surface/surroundings and other parameters. Histological examinations considered crust formation, re-epithelization and epidermal thickness, dermis edema, extension of granulation tissue, acute and chronic inflammation. Immunohistochemistry for evaluation of inflammation, vascularization and cell proliferation was performed using CD3, CD20, MHCII, von Willebrand factor (vWF) and KI67 antibodies. Furthermore, Real time-PCR investigated genes as Vascular endothelial growth factors (VEGF), Transforming growth factor beta 1(TGFβ1), Vimentin (VIM), Collagen 1α1 (Col1α1) and hair Keratin (hKER).

RESULTS: Clinically, the lesions treated with plasma healed more rapidly respect to other treatments and a reduced bacterial load was observed. At T7 wounds treated with stem cells and plasma were less macerated than lesions treated with other therapies**.** At T15 the wounds treated with hyaluronic acid showed a normal state of hydration while lesions treated with Manuka Honey exhibited a normal hydration from the third week only (Acemannan gel at fourth week). From the second week onwards all wounds did not show presence of fluid and exhibited a dry and clean secondary layer. All lesions, excluded wounds treated with acemannan gel, presented a red (hyaluronic acid and plasma) and dark red (Manuka Honey, PB-MSCs) granulation tissue starting from the first week. Molecular analysis showed a correspondence between clinical and molecular/histologic results. For instance, VEGF mRNA expression confirms angiogenetic events observed at histological level while TGF-β, CD3 and CD20 mRNA/protein expression indicated the presence/absence of inflammation in the used treatments.

DISCUSSION & CONCLUSIONS: Innovative therapies led to surprising results regarding regeneration of mammalian skin. Indeed, on the basis of clinical analysis, wounds treated with plasma and MSC healed more rapidly. Further examinations are ongoing in order to elucidate possible mechanisms explaining these differences.

biophotonics tools for tissue engineering and regenerative medicine

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INTRODUCTION: Tissue engineering and regenerative medicine are emerging fields at the interface of materials science and medicine. As with any other branch of materials science, process monitoring tools are required to ensure that tissue engineered constructs are formed with the correct properties and become successfully integrated into the body. Biophotonics is exceptionally well able to meet the requirements for monitoring in tissue engineering, because of its unique combination of high speed, low cost, high spatial resolution and excellent molecular contrast. This talk highlights some key biophotonics tools and shows how they can provide unique measurements in tissue engineering.

METHODS: Optical coherence tomography (OCT) systems operating at 890 and 1300 nm have been used to obtain structural images of tissue engineered models of invasive and dyplastic epithelial tumours. These systems offer label-free virtual biopsy images by providing depth-resolved imaging via coherence gating.

Combining OCT with polarized light yields PS-OCT, which uniquely can obtain depth-resolved images of tissue collagen content and organization, without the need for invasive staining eg. using picrosirius red.

OCT can be made sensitive to minimally invasive labelling agents such as FDA-approved magnetic iron nanoparticles using magnto-motive OCT. Transplanted stem cells potentially can be tracked in this way.

By transilluminating tissue with a 100 fs pulse of laser light the transient power density becomes high enough to drive two-photon fluorescence and second harmonic generation processes (SHG). SHG comes predominantly from ECM collagen and muscle myosin. SHG thus permits label-free, longitudinal and bleach-resistant assay of collagen production in tissue engineered constructs.

RESULTS: OCT structural images show key processes (e.g. barrier keratinization) in tissue engineered skin and help quantify wound healing rates in tissue-engineered wound models. 890 and 1300 nm OCT display complimentary performance when imaging tissue-engineered oral mucosa cancer models.

PS-OCT is used successfully to image the 3-D collagen structure of type-II collagen fibrils in articular cartilage. Such studies can offer insights into the correct tissue engineering scaffold morphology that should be designed.

Magneto-motive OCT is able to detect iron-oxide-labelled corneal epithelial cells seeded onto intact rabbit cornea.

SHG microscopy successfully images the production of collagen by dermal fibroblasts and bone osteoblasts, without sample damage. Changes in collagen abundance and alignment can be detected in response to mechanical stimuli such as fluid flow.

DISCUSSION & CONCLUSIONS: Biophotonics tools such as OCT and SHG microscopy have great potential to assist the development of next-generation tissue engineered products and therapies. Key to success will be successful size- and cost-reductions in the hardware.

Pluripotent stem cells for cartilage repair and disease modelling

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INTRODUCTION: Pluripotent stem cells (PSPs) are a promising source of cells for regenerative cell therapies and disease modelling because they can self renew indefinitely and form cell types of all three germ layers. However their plasticity can be a problem and one barrier to their use is the difficulty in targeting differentiation to a single desired cell type. New methods to generate high quality hyaline like repair cartilage would be a major advantage in orthopedic medicine especially if tissue typed stocks of committed progenitors can be banked for off the shelf therapy. Here we show that PSCs can differentiate to chondrogenic cells([1]) which give cartilage repair tissue in animal cartilage defect models([2]) and hard tissue from patient iPCs in vitro.

METHODS: Human ES cells (HUES1: Harvard, or MAN lines, North West Embryonic Stem Cell Centre, Manchester: derived under UK HFEA licence and informed consent. IPSCS were derived from human ligament, cartilage, dermal fibroblasts and blood under NRESC ethical approval. They were differentiated according to 10 and 2) by a three step 14 day defined serum-free protocol using a series of growth factors (Wnt, BMP, FGF2 GDF5) to give chondroprogenitors, or via an alternative 35-40 day protocol to deliver hESC-MSC-like cells. After 14 days some cells were embedded in fibrin gels for 3D culture to day 25 or subjected to classic pellet culture. Surgical osteochondral 2mm defects were made in the patella groove of the Stifle joint in the RNU rat (under UK home office licence). After 4-12 weeks joints were fixed decalcified and embedded in paraffin wax for histology and immunostaining.

RESULTS: hESCs/iPSCs were differentiated through two routes to generate chondrocytes. 1- Recapitulating early development, through mesendoderm, mesoderm and aggregates resembling condensing limb bud chondroprogenitors ([1]). 2- Induced to form MSC-like cells which formed cartilage nodules in pellet culture. After 14 days the defined protocol gave 94-97% SOX 9+ cells, expressing COL2A1 and 11a1, COMP, Matrilin3 in Safranin O+/chondroitinase sensitive aggregates indicating abundant Chondroitin sulphate proteoglycan. Osteochondral defects in the hind limb joint of immunocompromised RNU rats were implanted with hESC-chondrogenic cells in fibrin gels, and monitored histo/immunologically. HESC-chondrocytes repaired osteochondral defects in these rats, giving good quality hyaline-like cartilage matrix in which human cells were detected at 12 weeks ([2]). Through interrogation of Illumina-RNAseq data on 2 different hESC-lines we identified matrix synthesis/assembly pathways as targets for modulation including by micro RNA regulators. This protocol is now being converted to cGMP. HESCs and iPSCS from diverse sources were also induced to form MSC-like cells which formed cartilage nodules in pellet culture from both wild type iPSCS and those with genetic mutations. affecting cartilage (e.g.in COMP).

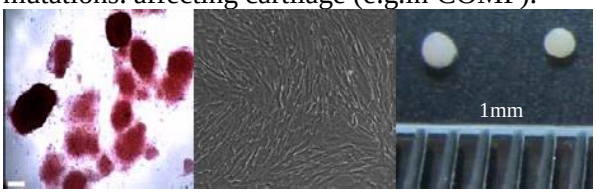

Fig.1: L-R Safranin O +MAN7 chondrogenic aggregates at 14d in defined protocol; hPSC-MSC like cells and cartilage pellets from differentiated hESCs/iPSCs-MSCs. Bar 100um

DISCUSSION & CONCLUSIONS: PSPs can be driven to chondrogenic cells through different routes. By recapitulating aspects of early limb development ([1], [3]) they can generate repair tissue in vivo ([2]). Through a PSC-MSC-like cell they can produce hard tissue in vitro. Our protocols allow us to gain complimentary evidence to that from transgenic animal models for understanding human chondrogenesis and genetic diseases affecting cartilage. With new safer methods to generate virus-free iPSCs this may open the way to iPSC-cartilage therapy.

ACKNOWLEDGEMENTS: supported by grants from MRC UK, EuFP7, Arthritis UK.

Niche factors for 2D and 3Dculture of hESC-chondroprogenitors

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INTRODUCTION: Cartilage is an aneural and avascular tissue consisting of around 3% chondrocytes, the rest being extracellular matrix, made by the cells and water**.** Several studies have reported using pluripotent stem cells (PSCs) to generate chondroprogenitors in vitro e.g.([1]). Some of these protocols have produced cells which can also contribute to the repair of osteochondral defects ([2]). Authentic matrix assembly to form hyaline-like cartilage in vitro in a reasonable time span has proved challenging. Many of the protocols are extremely lengthy or fail to generate fully assembled extracellular matrix networks. Thus, improved protocols to generate stable chondrocytes which assemble high quality 3D hyaline-like cartilage would be a major advance in terms of generating disease models from induced PSCs, and research on factors affecting cartilage matrix assembly, of benefit to orthopedic medicine. We describe evaluation of different natural and synthetic substrates and scaffolds for PSCchondrogenesis starting from our 2D protocol.

METHODS: Human ESCs (HUES1, Harvard) or MAN lines (Manchester) derived in North West Embryonic Stem Cell Centre under an HFEA licence were initially differentiated by a 3 step 14 day defined serum-free protocol using a series of growth factors (Wnt, BMP, FGF2 GDF5) to give chondroprogenitors ([1],[2]). These were transferred to a 3D environment at day 8 or 14 and were embedded at 2-5 X10e5 cells per 50µl gel in fibrin, alternative hydrogels or synthetic scaffolds for 3D culture. Constructs were assessed for viability/apoptosis, sectioned and stained with antibodies to SOX9 and collagen II or stained and viewed by confocal microscopy. Transcript expression was analysed by Illumina RNA-seq or RTPCR. Flow cytometry was used to evaluate integrin expression as in [3].

RESULTS: hPSCs generated chondrogenic cells in the 14 day protocol giving >94% SOX 9+ positive cells, expressing COL2A1 with little difference between cells grown on a fibronectin or a vitronectin substrate, although but with some variation between lines. Vitronectin N-terminal peptide (VnN) or fibronectin cell binding peptide (Fnbp) were as effective as plasma fibronectin in promoting 2D differentiation. Fibrin proved a useable 3D scaffold after 14d, supporting cell viability, low apoptosis, nuclear SOX9 expression and modest collagen II protein production. Immunofluorescence/confocal microscopy showed a relatively uniform distribution of SOX9 positive cells. Other hydrogels supported lower survival and we are now assessing composite scaffolds. The chondroprogenitors express an array of integrin cell-matrix receptors albeit at relatively low levels including α1, 3,5,6,9 and 10 integrins.

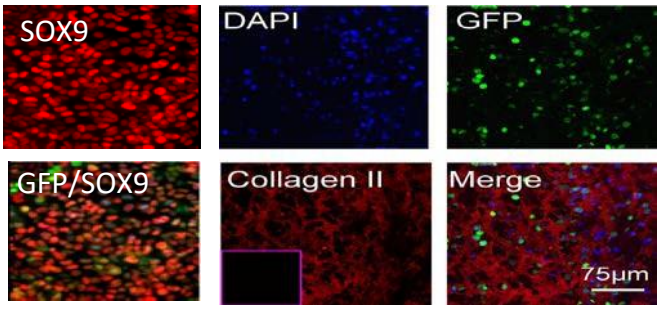

Fig. a: hESC-chondroprogenitors in fibrin gels at d21; sections stained with primary antibodies as indicated and AlexaFluor secondary antibodies. Constitutive GFP label

DISCUSSION & CONCLUSIONS: Use of defined peptides such as Fnbp and VnN provides a more cost effective defined substrate for chondrogenesis than plasma Fn. Although Fibrin is a suitable scaffold for maintaining hESC-chondroprogenitors for generation of cartilage repair in vivo ([2]), additional factors are needed to maintain the chondroprogenitors in longer term 3D culture to allow generation of authentic cartilage matrix with expected mechanical properties in a reasonable time scale. Composite scaffolds may be part of the solution to this challenge.

ACKNOWLEDGEMENTS: supported by funding from UKRMP platform hub (Niche), MRC UK, and Arthritis Research UK.

A differential role for paralog Hippo effectors YAP and TAZ in controlling cell mechanics

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INTRODUCTION: The downstream effectors of the Hippo pathway, YAP and TAZ, act as cellular mechanotransducers by integrating the mechanical cues from the extracellular matrix (ECM) and cell-cell interactions into intracellular signals in order to control cell proliferation, growth and differentiation [1]. Given their structural homology, YAP/TAZ have been so far deemed to have similar functions as mechanosensors [2]. By using micropatterned surfaces, gene silencing and CRISPR/Cas9 technologies, we demonstrate that YAP controls adipose-derived mesenchymal stem cells (AD-MSCs) mechanics and differentiation, and highlight crucial differences in the biological functions of TAZ paralog protein. In turn, we show for the first time that the effect of YAP is exerted by tuning actin cytoskeleton integrity and propose a possible mechanism by which YAP controls cell stiffness in response to ECM mechanics.

METHODS: Single human AD-MSCs were cultured on micropatterned substrates coated with ECM components of the ECM (fibronectin, laminin) or proteins involved in cell-cell interaction (N-cadherin, E-cadherin). YAP and TAZ knockdown was obtained in AD-MSCs by a retroviral vector expressing short hairpin RNA (shRNA), while YAP- and TAZ-deficient Cal51 lines were produced by CRISPR/Cas9 technology. Single cell mechanics was evaluated by Atomic Force Microscopy (AFM).

RESULTS: The function and localization of YAP/TAZ can be controlled by cell spreading over ECM-coated surfaces, thus tuning cell shape and differentiation. Silencing experiments and gene expression analysis show that YAP and TAZ elicit distinct roles in AD-MSCs, with the first controlling cell differentiation, the latter being more important for cell spreading and shaping. In fact, cells deprived of TAZ lose the ability to acquire a defined morphology, as dictated by the micropatterned surface, while those missing YAP expression show a derangement in actin cytoskeleton pattern. Nonetheless, when the elasticity of YAP/TAZ silenced cells was tested, both YAP- and TAZ-deficient cells showed a similar reduction in their Young's modulus.

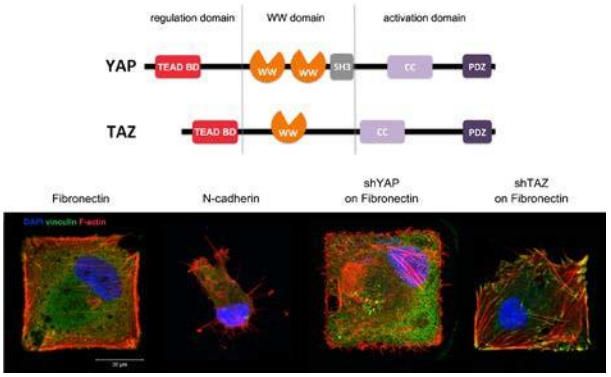

Fig. 1: YAP and TAZ are paralog proteins sharing a common architecture. Growing cells on cadherin-coated surfaces impairs both YAP and TAZ function and leads to a dramatic change in cell phenotype. The disruption of either YAP or TAZ leads to the acquisition of two different phenotypes in AD-MSCs: YAP-silenced cells display a disorganized actin cytoskeleton while TAZ-silenced cells completely fail to spread.

DISCUSSION & CONCLUSIONS: Cell response to ECM mechanics is mediated by intracellular molecular systems, like the one involving YAP and TAZ proteins. Our results indicate that, although being paralog proteins with mechanosensor activity, YAP and TAZ exert differential roles in gene regulation, cytoskeleton integrity and AD-MSC differentiation.

ACKNOWLEDGEMENT: 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).

mesenchymal stem cells modulate tissue repair responses after local injection within the scarred vocal folds

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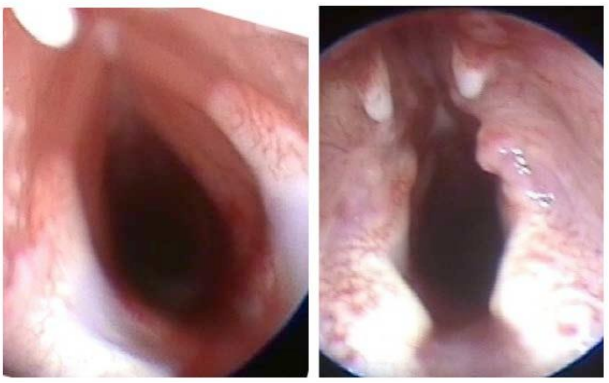
INTRODUCTION: Vocal fold scarring is often caused by trauma, surgical procedure and post radiotherapy. It damages to the vibrating layers present in the vocal folds, which leads to decrease viscoelastic properties, turns into severe voice problems (1,2). Currently there is no valid treatment available. Human mesenchymal stem cells (hMSCs) are known to be immnosuppressive or immunomodulatory effects, which minimize the inflammation in the scarred vocal fold tissue and promote the wound healing. The aim of this study to identify whether local injection of hMSCs can modulate the early inflammatory response within the scarred rabbit vocal fold and promote the wound healing process. This project was designed based on how MSCs work towards possible regulatory effects on immune cells.

METHODS: Scarring was induced within the VFs of New Zealand white rabbits (*n*=20) to make the inflammation in the vocal fold tissue and immediately injected hMSCs in the scarred area to verify the immune response with MSCs in the scarred vocal fold tissue. The total 40 vocal folds from 20 animals were operated with bilateral resections in the lamina propria, 20 vocal folds were injected with hMSCs (80,000 to 100,000 in 100ul of human AB plasma) immediately after scarred, and 20 vocal folds were only scarred used as controls. The animals were sacrificed at day2 and day4. The vocal folds were removed and separated from the larynges, and one vocal fold was taken for histological stainings for Hemotoxylin and Eosin (H&E), fluorescent in situ hybridization (FISH) was used to identify the cell survival after injection and Immunohistochemistry (IHC) for anti-inflammatory macrophages (M2, CD163), and other vocal folds used for qPCR studies.

RESULTS: Injected hMSCs were found to have engrafted into the vocal folds at both time points using FISH experiment. The total engraftments of MSCs were analyzed from the vocal fold tissue at both time points (3.3% day2 and 0.6% on day 4). Increased number of CD163 positive cells were observed in the vocal folds where hMSCs were injected, close to vasculature, switching to an anti-inflammatory M2 phenotype adjacent to the injection site at both time points compared to control animals. H&E staining were used to verify the tissue architecture, and pathologist qualitatively graded the inflammation rate in both treated and untreated samples. The qPCR studies has given indication that MSCs shows an anti-inflammatory effect which reduce the expression of pro-inflammatory cytokines such as interleukin (IL)1a/b, IL6 and tumour necrosis factor (TNF)a.



1.Normal vocal folds 2. Scarred vocal folds
Fig 1. Rabbit VFs were monitored before (1) and after (2) the surgical scarring procedure.

DISCUSSION & CONCLUSIONS: This study provides evidence that local injection of MSCs can aid in the reduction of scar formation within the VF. Increased levels of M2 macrophages and an accompanying decrease in inflammation with MSC treatment suggests enhanced resolution of the wound. The findings from this study indicate that local injection of MSCs may offer a novel cell-based therapeutic for the treatment of scarred VFs.

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The fabrication of pure fibrin fibers with cellular alignment following induced topography.

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INTRODUCTION: Many disease and disorders may be treated using tissue-engineered (TE) constructs. Tissues such as cardiac and skeletal muscle, nerve and bone rely on cellular alignment for optimal functioning. TE constructs designed to repair or replace such tissues will need to feature similar cellular organisation. In this study we present a simple method for the production of fibrin fibers with aligned embedded cells.

METHODS: Fibrin (5mg/ml after mixing of components) fibers were produced by filling silicon tubing with a hydrogel-cell mixture. Following polymerization of the gel the fiber was ejected from the tube by injection of liquid. An initial longitudinal stretch was sufficient to induce a longitudinal aligned topography of the fibrin fibers. We hypothesized that cells embedded in these stretched fibers would organize themselves along the longitudinal axis of the fiber. Different cell types were embedded in the fibers, including endothelial cells, fibroblast and Schwann cells. Cell-laden fibers were either cultivated free-floating in medium or incorporated in a secondary fibrin matrix. The latter served as a model to investigate the potential of the fibers for bottom-up TE of multicomponent constructs.

RESULTS: Fibrin fiber dimensions could be adapted as needed, with diameters ranging between 0.5-3 mm and with lengths of up to 1 meter. All the tested cell types displayed longitudinal alignment when embedded in stretched fibers, that were either incorporated in a secondary matrix or free-floating. Cells in fibers that were not stretched did not align. Fibroblast cell-laden fibers showed >65% of cells to be aligned within 10 degrees of the longitudinal axis of the fiber. Fiber cultivation conditions strongly affected endothelial cell behaviour: when floating in medium they migrated to the surface of the fiber, forming a confluent layer on both stretched and unstretched fibers. When incorporated in a secondary matrix the endothelial cells formed tubes of type IV collagen which were longitudinally aligned in the stretched fibers.

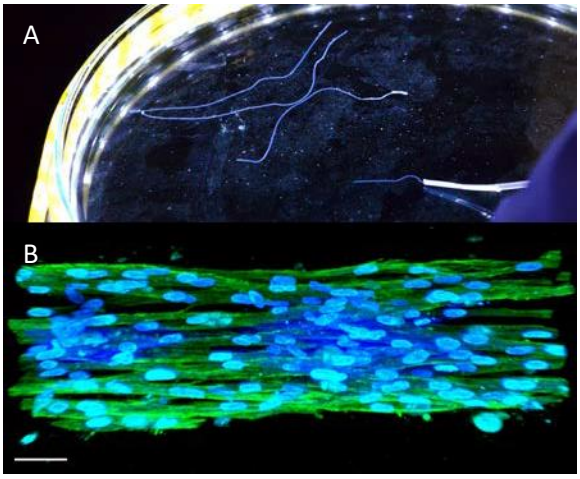

Fig. 1: Production of fibrin fibers (A) and 3D rendering of image stack (B) of longitudinally organized fibroblasts stained for smooth muscle actin (green). DAPI nuclear counterstain. Scale bar: 50µm.

DISCUSSION & CONCLUSIONS: Cells embedded in a fibrin hydrogel align when a short initial strain is applied to the gel. The present technique provides a scalable, economical, simple and versatile approach for producing fibrin fibers with an aligned topography. The low concentration of fibrinogen allowed for proliferation and migration of embedded cells. By avoiding expensive machinery or additional components such as alginate, our method marks an improvement in fibrin fiber production[1,2]. The cell responses to the fibers shows their potential, as well as that of the general technique, for bottom-up tissue engineering and modeling strategies to investigate cell behavior in 3D environments.

ACKNOWLEDGEMENTS: This research was funded by the Stiftung Kinderhertz.

Improving safety and efficacy of cancer drugs: Biopolymer based drug delivery

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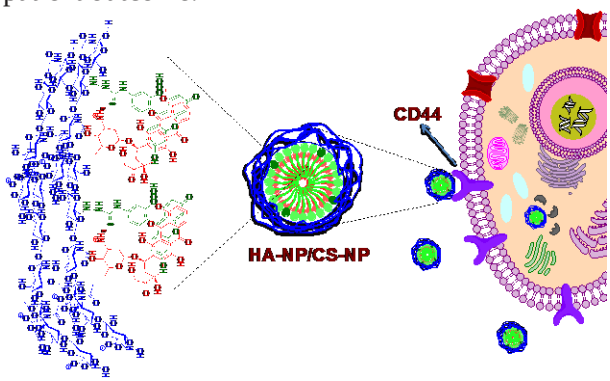
INTRODUCTION: Delivering chemotherapeutic agents to malignant cells without triggering innate immunity, is greatly needed in anticancer therapy. This is a daunting task, as these agents are extremely toxic not just to cancer cells but also to healthy cells[1] and trigger innate immunity.[2] One of the most promising strategies to achieve this is nanoparticles based delivery. However, recent studies have revealed the chemotherapeutic agents[3] and nanoparticles[4] trigger undesired complement and coagulation cascade, which results in immune activation and thromboinflammation. Thus methods of suppressing innate immune activation while improving the therapeutic efficacy of the drug is greatly needed, which will significantly benefit patient outcome.



Schematic representation of engineered HA and CS nanoparticles undergoing CD44 mediated cellular uptake.

METHODS: Hyaluronic acid (HA) and chondroitin sulphate (CS) are two biopolymers of human origin. HA is non-sulphated and possess N-acetyglucosamine and glucuronic acid repeat units, while CS is sulphated and possess (N-acetylgalactosamine and glucuronic acid repeat units. This minor difference has a profound impact on its bioactivity and physical properties. We have engineered nanocarriers by developing amphiphilic polymers and loaded a chemotherapeutic drug, Doxorubicin. To design these nanocarriers we used fluorescein as the hydrophobic probe, which induced self-assembly and inherent imaging modality. Subsequently, we evaluated the complement and coagulation activation and studied their drug loading and cytotoxicity in human cancer cell lines.

RESULTS & DISCUSSION: The self-assembled HA and CS nanoparticles (NPs) possessing the fluorescein molecule as a hydrophobic component, efficiently stabilized anticancer drug doxorubicin (DOX) and promoted near zero-order drug release. Interestingly, cytotoxicity studies of these NPs showed that CS-DOX-NP displayed higher dose dependent cytotoxicity than HA-DOX-NP in different mammalian cells tested. Immunological evaluation of these nanocarriers revealed that unlike unmodified polymers, HA-NP and CS-NP showed platelet aggregation and elicited significant levels of thrombin-antithrombin complex formation at high concentrations (0.8 mg/mL). Investigation of early- and late-stage complement activation (C3a and sC5b-9) suggested that both CS and CS-NP triggered significant complement activation at high concentrations (0.08-0.8 mg/mL), unlike HA.

CONCLUSIONS: Our study reveals that HA is superior over CS in terms of biocompatibility while CS based NP possess higher cellular uptake and cytotoxicity. To the best of our knowledge, our study is the first report of direct comparison of hemocompatibility and anticancer properties of HA- and CS-based nanomaterials.

ACKNOWLEDGEMENTS: The authors acknowledge Dr. Oommen Varghese for advice and suggestions, the authors also acknowledge Swedish research council grant '*StemTherapy*' and EU-FP7 project '*DIREKT*' for financial support.

Extracellular matrices simultaneously mimicking stepwise osteogenesis-co-adipogenesis of mesenchymal stem cells

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INTRODUCTION: The extracellular matrices (ECMs) are not only tissue-specific but are also dynamically remodeled and balanced during the development and aging process to maintain the activity of each tissue and organ. In this study, four novel stepwise development-mimicking matrices were developed to mimic the dynamically changing ECM derived from human bone marrow-derived mesenchymal stem cells (MSCs) undergoing simultaneous osteogenesis and adipogenesis.

METHODS: To induce MSCs undergoing simultaneous osteogenesis and adipogenesis, MSCs were cultured in mixture media composed of osteogenic medium (OM) and adipogenic medium (AM) at different ratios of 95/5, 85/15, 70/30 and 50/50. The cells were cultured in the mixture media for 7, 14 and 21 days and media were replaced every 3-4 days. The different stepwise stages of simultaneous osteogenesis and adipogenesis of MSCs were obtained by controlling the OM/AM ratio and culture time. Four types of cells at different stepwise osteogenesis-co-adipogenesis stages were obtained. They are early osteogenesis and early adipogenesis stage (EOEA) cells, early osteogenesis and late adipogenesis stage (EOLA) cells, late osteogenesis and early adipogenesis stage (LOEA) cells, and late osteogenesis and late adipogenesis stage (LOLA) cells. The different differentiation stages were confirmed by histological staining (alkaline phosphatase and Alizarin red S) and analysis of differentiation process related genes encoding *ALP*, *IBSP* and *LPL*. After differentiation culture, the cells were decellularized to prepare their respective matrices. Removal of cell components was confirmed by actin cytoskeleton and cell nuclei staining before and after decellularization. The obtained matrices were used for culture of MSCs to investigate their effect on adhesion, proliferation, osteogenic and adipogenic differentiation of MSCs.

RESULTS: Four ECMs from the cells at the four stages of EOEA, EOLA, LOEA and LOLA were prepared. The stepwise osteogenesis-co-adipogenesis-mimicking matrices had different compositions depending on the stage of osteogenesis-co-adipogenesis. They supported adhesion and proliferation of MSCs. They had different effects on differentiation of MSCs. LOEA and LOLA matrices promoted osteogenic differentiation but not adipogenic differentiation of MSCs. EOEA matrices promoted adipogenic differentiation but not osteogenic differentiation of MSCs. EOLA did not promote either osteogenic or adipogenic differentiation of MSCs.

DISCUSSION & CONCLUSIONS: The stepwise osteogenesis-co-adipogenesis-mimicking matrices had different compositions and different effects on the osteogenic and adipogenic differentiation of MSCs. The matrices could provide very useful tools to investigate the interaction between ECM and stem cells and the role of ECM on stem cell differentiation.

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Design criteria for 3D printable hydrogels and recombinant spider silk as model

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INTRODUCTION: Biofabrication is a rather young and promising research field that relies on the use of automated layer-by-layer deposition of biomaterials, bioactives and cells, encompassing both bioprinting- and bioassembly-based techniques, to generate hierarchical cell / bio-material composite structures [1]. This approach has the potential to overcome a number of challenges relating to organization, personalized shape and mechanical integrity of generated constructs.

As biofabrication originated from the (bio-) engineering community and did not evolve from materials science, the field has for long worked with the most established hydrogel systems available in quantities that are necessary for the processes. Hence, the vast majority of literature studies use (modified or supplemented) alginate and gelatine as base material. Although this has allowed achieving some remarkable successes, it has recently become evident that the lack of variety in printable hydrogel systems is one major drawback for the complete field [2,3].

The main objective of this contribution will thus be to introduce the most important rheological aspects of printing and give an overview of possibilities to create tailored molecular building blocks for printable hydrogels [4]. At the example of recombinant spider silk proteins, the advantages of defined building blocks for biofabrication will be discussed [5].

METHODS: Hydrogel were prepared from recombinant spider silk proteins (eADF4(C16)) based on the repetitive core sequence of dragline silk fibroin 4 (ADF4) of the European garden cross spider (Araneus Diadematus) by storing a 3 wt% solution. Robotic dispensing was performed with a 3D Discovery Bioprinter (RegenHU). Gels were characterized using standard methods, with special attention to conformational changes of the peptides during gelation using FT-IR. Human dermal fibroblasts were cultured for cell printing and cell seeding. Cell viability was evaluated after printing using live/dead staining and monitored by confocal fluorescence microscopy.

RESULTS: Hydrogels were obtained from eADF4(C16) through storage of a 3 wt% solution in an incubator at 37°C overnight. FT-IR analysis showed a doubling of beta-sheet content in the peptides during gelation, indicating intermolecular beta-sheet interaction as main gelation force. These gels could directly be used for 3D printing due to the rapid reversible nature of these supramolecular interactions that are characterized by a fast regain of the initial structure after relaxation of shear. Dispense plotting of more than 10 layers was possible purely relying on supramolecular beta-sheet mediated interaction between the peptides. For printing of cell loaded constructs, cells were mixed into the gels before the overnight gelation. A quantification of cell viability 48 h after printing and comparison to cell culture in cells immobilized in cast gels showed that the printing process did not negatively influence cell viability.

DISCUSSION & CONCLUSIONS: Using recombinant spider silk proteins we demonstrate that physical crosslinking by β-sheet structures is a promising strategy for bioink development, especially due to the rapid regain of structure after release of shear stress. This allows for 3D printing of stable constructs without the need of thickeners or crosslinking additives.

ACKNOWLEDGEMENTS: This work has received funding from the European Union (FP7, grant agreement 309962; project HydroZONES).

Cell Lineage Perspectives of Cutaneous Scarring

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Fibroblasts remain poorly characterized but are central to the fibrotic response to injury. We reveal the existences of multiple embryonic lineages in mammalian cutaneous tissues that underlie the diversity of scar formations across developmental stages and between anatomic sites. We further identify a highly fibrogenic lineage defined by embryonic expression of Engrailed-1 that plays a central role in dermal development, wound healing, radiation-induced scarring, and cancer stroma formation. These results hold promise for the development of therapeutic tactics aimed at ameliorating wound healing and fibrotic diseases.

Precise bioengineering of functional materials and biomimetic environments

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INTRODUCTION: There is widespread agreement of the central role the extracellular matrix (ECM) plays in biological processes. This "complex hydrated matrix material" is capable of optimizing molecular presentation and providing structural hierarchy, chemical anisotropy, biological selectivity, adaptability, and appropriate mechanical properties. A major goal in the biomaterials community is the generation of functional hydrogel materials that exhibit these kinds of properties.[1]

METHODS, RESULTS, DISCUSSION: The talk will focus on novel technologies that enable the fabrication of 2D and 3D bioactive and/or biomimetic hydrogel-like materials with potential application in tissue engineering, drug screening, and regenerative medicine. The first project utilizes a protein-based hydrogel to control and guide hydroxyapatite biomineralization for applications in bone[1] and enamel regeneration. The second project exploits the dynamic self-assembling properties of a peptide-protein system capable of growing functional tubes with potential application in vascular tissue engineering. The system permits access to non-equilibrium to create tubular structures that are robust, exhibit dynamic behaviour, display controlled dis-assembly and re-assembly capabilities, can seal to surfaces, and have the capacity to be morphed into desired shapes (Fig. 1a).[2] The third project introduces a simple 3D molecular printing method named 3D Electrophoresis Assisted Lithopgraphy (3DEAL) to create complex 3D patterns within readily available hydrogels. We demonstrated the potential of 3DEAL by creating anisotropic environments with parallel and perpendicular columns, curved lines, gradients of molecular composition, and patterns of various proteins ranging from tens of microns to centimeters in size and depth using readily available hydrogels and proteins like elastin, fibronectin, and IgG (Fig. 1b).

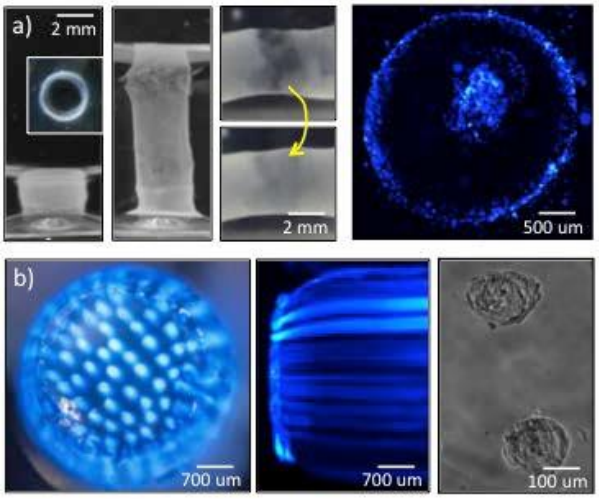

Fig. 1: a) Top and side view of a 3D hydrogel patterned with proteins that selectively guide cell growth. b) Peptide-protein self-assembled membranes undergoing morphogenesis into tubes with dynamic properties and capable of supporting cell growth.

ACKNOWLEDGEMENTS: We would like to thank our collaborators Mr Juan Pablo Aguilar, Dr Helena Azevedo, Dr Ronit Bitton, Prof. Molly Stevens, Prof. Carlos Rodriguez-Cabello, Dr Lorenzo Botto, and Dr Andy Bushby.

Bioengineering Dermo-Epidermal Skin Grafts with Blood and Lymphatic Capillaries

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As the importance of the blood, and particularly the lymphatic vascular system, is increasingly recognized, it is attractive to engineer both human blood and lymphatic vessels in one tissue or organ graft. We show here that functional lymphatic capillaries can be generated within the dermal compartment of dermo-epidermal skin grafts. Like normal lymphatics, these capillaries branch, form lumen, and take up fluid in vitro and in vivo after transplantation onto immunocompromised rodents. Formation of lymphatic capillaries could be modulated by both lymphangiogenic and anti-lymphangiogenic stimuli. Blood and lymphatic endothelial cells never intermixed during vessel development, nor did blood and lymphatic capillaries anastomose under the described circumstances. After transplantation of the engineered grafts, the human lymphatic capillaries anastomosed to the nude rat's lymphatic plexus and supported fluid drainage. Successful preclinical results suggest that these skin grafts could be applied on patients suffering from severe skin defects.

Novel bio-engineered dermo-epidermal skin grafts: Combining first clinical and new experimental data

E Reichman

TissueBiologyResearch Unit, Zurich, Switzerland.

Every year more than 50 million people worldwide suffer from deep skin defects. These defects can originate from a variety of conditions such as burns, congenital disease, tumors, scar corrections or chronic wounds. The present standard of care too often leaves these patients with disfiguring, debilitating scars that may require repeated corrective surgery and/or intense homecare. In this symposium we will report for the first time on a finalized phase I clinical study in which we appliednovel dermo-epidermal skin grafts on paediatric patients. Furthermore we will elute on the future of these skin grafts by introducing a new generation of bio-engineered pre-vascularized and pigmented skin substitutes. In addition we will describe the identification of distinct dermal cell lineagesdiffering significantly in their potential to cause cutaneous scarring after excisional wounding. In this symposium we will present both novel clinical data and new basic findings that in combination hold promise for a significant progress in the treatment of severe skin defects in human patients.

Anti-Inflammatory sulfated hydrogels enable chondrocyte proliferation without de-differentiation

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INTRODUCTION: Articular cartilage is a tissue which profits little from the body's natural healing mechanisms. Its low capacity to regenerate means that cartilage injuries due to trauma lead to progressive joint deterioration, a condition known as post-traumatic osteoarthritis. Current cell-based therapies require a cartilage biopsy from which chondrocytes can be isolated. However, the low cell yield requires expansion in plastic flasks to increase their number. These expanded cells become fibroblast-like in phenotype and therefore have an impaired capacity to regenerate cartilage. We hypothesize that growing cells in a glycosaminoglycan-rich microenvironment would benefit the preservation of the chondrocyte phenotype.

METHODS: Sulfation of alginate (Pronova UP LVG, *FG* = 0.67, *NG* < 1 = 14, Novamatrix) was carried out using increasing concentration of chlorosulphonic acid to achieve 3 degrees of substitution. Gels were crosslinked with CaCl2 and characterized by swelling, rheology and SEM. Freshly isolated chondrocytes were encapsulated in the materials and evaluated for gene expression of cartilage matrix proteins and immunostaining. In certain studies, gels were exposed to IL1-β stimulation to determine the effect of the hydrogel on markers of inflammation.

RESULTS: Sulfation of alginate potently promoted proliferation of chondrocytes in 3D compared to cells in unmodified alginate (Figure 1). This mitogenic effect was found to increase with increasing sulfation. Highly sulfated hydrogels also showed a significantly higher retention of FGF2 compared to control hydrogels, indicating that the increase in proliferation was linked to activated fibroblast growth factor signaling.

Interestingly, despite the strong proliferation, there was a strong staining for collagen 2 in sulfated alginate hydrogels. Expression of collagen 1 was also inhibited in pure sulfated alginate hydrogels, whereas as mixes of unmodified and sulfated gels, still induced the de-differentiated phenotype. Finally, sulfated gels showed extensive anti-inflammatory and anti-catabolic effects upon IL-1β stimulation. Cytokine-stimulated gene expression of pro-inflammatory markers such as IL-6, IL-8, COX-2 and ADAMTS-5 were lowered in the sulfated gels.

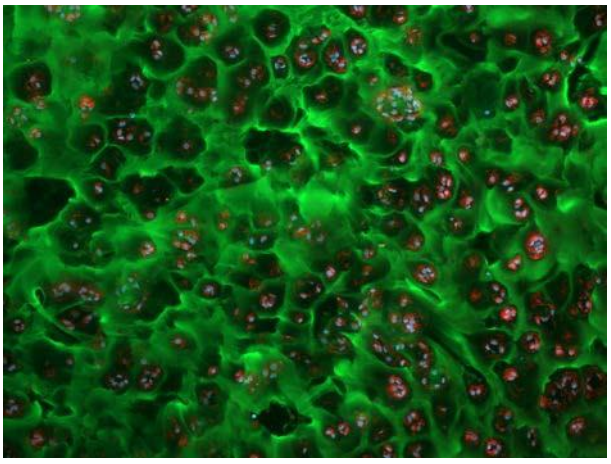

Fig. 1: Freshly isolated bovine chondrocytes were cultured in sulfated alginate hydrogels, where they proliferated and secreted significant amounts of collagen 2 (green).

DISCUSSION & CONCLUSIONS: Alginate sulfate hydrogels promote extensive proliferation of chondrocytes without loss of phenotype. The biomimetic hydrogels act as an active signaling scaffold that not only entraps growth factors through high affinity binding but also mediates an interaction between the growth factors and their receptors to activate chondrogenic signaling pathways. Furthermore, we found evidence that the sulfated alginate protected the resident cells from inflammatory insult. This study shows the importance of biomimetic cues in engineered hydrogels used in regenerative medicine.

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Microfluidic system with integrated 3D cell culture matrix - towards more *in vivo*-like organ-on-chip models

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INTRODUCTION: Organ-on-chip is an emerging research field with the ambition to mimic organs by culturing cells in microfluidic devices [1]. In barrier models, cells are usually cultured on synthetic porous membranes. The biocompatibility of such membranes is however far from those provided by the *in vivo* extracellular matrix (ECM). Here, we show the use of a 3D collagen matrix integrated in a microfluidic platform to provide the cultured cells with an ECM-like environment.

METHODS: The 3D collagen cell culture matrix was prepared by mixing 80 v/v% of 2.16 mg/ml rat tail collagen type I with 10 v/v% DMEM media 10x and jellified at 37°C for 30 min. For culturing of cells inside the collagen matrix, the DMEM media was supplemented with a cell suspension of $1x10^6$ cells/ml.

The collagen matrix was mechanically supported by a 10-µm polystyrene membrane and bonded in-between two microfluidic channels, 20 mm *x* 1 mm *x* 0.2 mm. The channels were prepared in PDMS using Teflon moulds and soft lithography. For leakage-free bonding, the PDMS pieces were treated with corona plasma before the collagen matrix was bonded in-between the channels.

Mouse brain endothelial cells (bEnd.3 cell line) were cultured in the chip ($1x10^6$ cells/ml) on top of the collagen matrix and mouse brain astrocytes (C8-D1A cell line) were cultured inside the collagen. Cells were stained with green cell tracker before seeding for visualization purposes. Both channels of the microfluidic device were connected to syringes and continuously flowed with DMEM media supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin at a flow rate of 40 µl/h. The devices were maintained in an incubator with a humidified atmosphere of 5% CO_2 in air at 37 °C. Cells were monitored by means of fluorescent microscope and their viability was evaluated with AlamarBlue assay.

RESULTS: Microfluidic devices with integrated collagen matrix were successfully made. The good bonding and no leakages of a device were tested using dyed water (Figure 1).

Fig. 1: Microfluidic device with coloured liquid in the channels.

Both endothelial cells and astrocytes proliferated inside the microfluidic channel, on top of collagen or inside, respectively. The endothelial cells spread well already after at 24h (Fig. 2a-b), whereas the astrocytes showed a rather round morphology even 72h after seeding the cells (Fig. 2c-d). Both cell types showed to be alive by AlamarBlue assay.

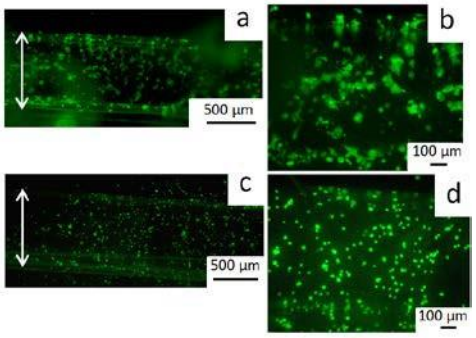

Fig. 2: a,b) Endothelial cells on top of collagen; c,d) astrocytes inside of collagen. Arrow in a) and b) shows the width of the channel.

DISCUSSION & CONCLUSIONS: We have successfully integrated a 3D collagen cell culture matrix into a microfluidic platform and showed cell viability over a period of (at least) 3 days.

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Spontaneous and induced tendon disease models in the horse and sheep

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INTRODUCTION: Spontaneous, or naturally-occurring, tendinopathy is common in a limited number of animal species – the human, the horse, and the greyhound. Given that these three species are athletic while the absence of the disease in other species suggests that athleticism is the common factor in the pathogenesis of the disease. Many animal models have been developed to mimic the disease encountered in humans [1] but all induced models have limitations in reflecting all the features of human tendon disease. This presentation will review these similarities and differences in disease between the two species and models that we have developed to investigate regenerative therapies.

The aetiopathogenesis of tendinopathy is complex and multi-factorial with exercise, age and mechanical loading as key disease drivers. The tendons affected by both species involve both extra-thecal and intra-thecal (within a synovial sheath) portions of tendons. The most common injuries in horses are 'subcutaneous' overstrain injuries of the weight-bearing flexor tendons of the distal limb while the most common injuries in humans affect both weight-bearing tendons (e.g. Achilles tendon) and non-weight bearing tendons which are intra-thecal (e.g. rotator cuff tendons). When comparing the anatomy of these lesions between the two species (biped vs quadruped), it is more relevant to relate tendons with similar mechanical environments and functionality rather than anatomical parallels. Thus equine superficial digital flexor tendinopathy is functionally more closely related to the human (Achilles) as is equine deep digital flexor tendinopathy to the human rotator cuff where the tendon is intra-thecal and under compression.

Injuries often are mid-substance (core) tears which commonly occur in the superficial digital flexor tendon (SDFT) of the horse, making it a good model for this type of injury. Although the horse deep digital flexor tendon (DDFT) can be used to model human rotator cuff intra-thecal injuries the DDFT in the sheep similarly lies within a synovial sheath and may represent a better and more tractable large animal model.

METHODS: Recent treatments have involved biological options such as stem cells and platelet-rich plasma in both medical and veterinary fields. We have investigated the efficacy of autologous bone marrow-derived MSCs (BM-MSCs) in naturally-occurring over-strain injury of the equine SDFT [2] and in an ovine model of intra-thecal injury that we have developed [3] where a partial thickness longitudinal lesion was surgically created in the forelimb lateral DDFT.

RESULTS: In an experimental study of equine over-strain injuries (n=12) [2], MSC treatment 'normalised' a number of key tissue parameters towards untreated tendons compared to saline-injected controls. In a clinical study [4] of MSC-treated racehorses (n=113), the re-injury percentage was 27.4% and a significantly reduced re-injury rate (about 50%) compared with two other studies in racehorses with identical inclusion and follow-up criteria. In sports horses treated with MSCs (n=68) the re-injury rate was only 19% and also about 50% that previously documented for sports horses. In the sheep, the lesions did not heal by 6 months and inflammation was not a major feature. MSCs injected into the digital sheath of the injured tendon demonstrated selective engraftment to the synovium and the most distal and proximal regions of the tendon but did not adhere to the lesion.

DISCUSSION & CONCLUSIONS: These findings support the horse as a large animal model for assessing regenerative treatments for spontaneous SDFT injuries. The induced model of intra-thecal injuries in the sheep shows similarities to Achilles injuries with poor spontaneous healing and minimal signs of inflammation. Our results demonstrate that the synovial fluid environment around the lesion represents a major challenge and the validation of this novel model offers opportunities to develop therapeutic strategies that overcome this environment.

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Plasma-coated polycaprolactone scaffold in ovine model of osteochondral defect

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INTRODUCTION: Cold plasma processes at both low and atmospheric pressure are very useful for functionalizing material surfaces without changing the bulk material. These processes can tailor the surface compositions of scaffolds and improve their cytocompatibility, and can be used to synthesize functional surfaces for direct cell growth and biomolecule immobilization. They can also be employed for the deposition of non-fouling coatings, nano-composite bacterial resistant coatings, or micro/nano-structured surfaces [1-3]. The aim of this study was to conduct in vivo testing of a plasma-coated polycaprolactone (PCL) scaffold in an ovine model of osteochondral defect.

METHODS: Plasma modifications of materials were performed by solvent casting/particulate leaching to obtain three-dimensional (3D) PCL scaffolds (7 mm diameter, 10 mm thick). The samples were plasma coated with radio frequency glow discharges, in a low pressure plasma reactor. C_2H_4/N_2 deposition was performed (at 47 Pa, 50 W, 30 min), followed by H_2 plasma post-treatment (53 Pa, 20 sccm, 20 W, 3 min). Twenty sheep were randomly assigned to the experimental groups detailed below. Groups sacrificed at 3 months: (a) group implanted with untreated PCL scaffolds; (b) group implanted with PCL scaffolds treated by PDE with N/H_2; and (c) group implanted with PCL scaffolds treated by PDE with N/H_2 and incubated in culture for 15 days with bone marrow mesenchymal cells (BMSCs). Groups sacrificed at 6 months: (a) group implanted with untreated PCL scaffolds; (b) group implanted with PCL scaffolds treated by PDE with N/H_2; and (c) group implanted with PCL scaffolds treated by PDE with N/H_2 and kept in culture for 15 days with bone marrow mesenchymal cells. An osteochondral defect measuring 7 mm in diameter was made in the medial condyle of the right femur, and then filled with the scaffold as per the specifications outlined for each group. After surgery, each sheep was subjected to X-ray examination of the knee joint in the left orthogonal projection. The micro-morphology of 3D PCL scaffolds and new tissue formation were examined using micro-computed tomography (Skyscan 1172, Bruker MicroCT) and immunohistochemical analysis.

RESULTS: At 3 months after implantation, the scaffolds treated with plasma (PCL + PDE) exhibited better osteointegration, particularly new bone formation on the scaffold and on the boundary, where the bone tissue was organized with an architecture similar to that of the healthy bone. On the cartilaginous side, we also detected the presence of dense material, which moved from the periphery to the center of the scaffold, where it was supported by subchondral bone.

DISCUSSION & CONCLUSIONS: The present results demonstrated that after 6 months, the group treated with PCL + PDE showed more clustering of biomaterials and thickening of the trabeculae, particularly on the scaffold-bone interface. This phenomenon may have been related to bone remodeling over time because of greater maturity of the newly formed bone than in the group treated with PCL + PDE at 3 months. The groups treated with PCL + PDE + BMSC exhibited better osseointegration than the other groups, thereby demonstrating the superior ability of the biomaterial coated with plasma to support BMSCs and ameliorate the healing process.

Monitoring osteogenic differentiation of mesenchymal stem cells in a microfluidic device

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INTRODUCTION: The convergence of microfabrication-based technologies with cell biology has laid the foundation for the development of advanced *in vitro* diagnostic systems that are capable of analyzing cell cultures under physiologically-relevant conditions. In this talk we present state-of-the-art lab-on-a-chip platforms for *in vitro* assessment of stem cell cultures and discuss their potential future applications [1]. We highlight in particular the tangible advantages of microfluidics to overcome many of the current challenges faced with stem cell identification, expansion and differentiation.

METHODS: Lab-on-a-Chip Fabrication: A detailed description of our microdevices fabrication methods can be found elsewhere [2,3]. The biochip was manufactured either from polydimethylsiloxane (PDMS) or OSTEmer containing four cell cultivation chambers each consisting of individual addressable interdigitated gold electrode structures (IDEs) as impedance sensors. IDEs were used either directly or after coating with a passivation layer (ZrO_2) [4]. Perfusion was realized by means of i) a syringe pump, ii) a peristaltic pump or iii) pneumatically operated microvalves.

RESULTS: Of all three perfusion modi, micropumps were best suited for minimizing working volumes and for the application of well-defined small doses of soluble stimuli (IL1β). While stem cells are highly sensitive towards shear forces, mechanical stimulation also promotes osteogenic differentiation. We identified an optimized perfusion strategy using micropumps for 100 pump strokes every 60 min to ensure nutrient supply while maintaining stable cell layers. A new biosensor for improved impedimetric cell analysis has been developed based on a 15 nm thin biocompatible ZrO_2 coating. The ultrathin passivation thickness in combination with high-k dielectric material yielded minimal sensitivity loss while providing a homogeneous surface chemistry and improved distribution of the electric field. Practical application of the ZrO_2 nanopassived IDEs for non-invasive and label-free cell analysis included nanotoxicological model studies as well as identification of osteogenic and adipogenic stem cell differentiation.

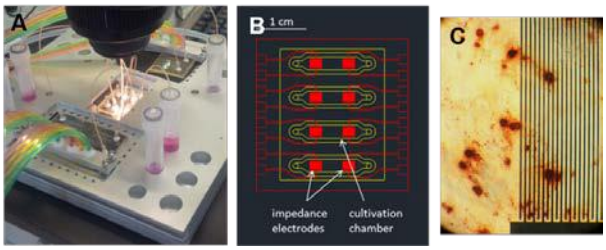

Fig. 1: A) StemCellChip station, B) schematic top view of cell chip, C) stem cells on IDE after 11 days osteogenic differentiation (Alizarin Red).

DISCUSSION & CONCLUSIONS: In the light of an increasing demand of stem cells for cytotoxicity screening, disease modelling, pharmaceutical compound testing and cell-based therapies, the manufacturing industry will need to supply a large number of highly characterized and documented stems cells on an international basis. The necessity for advanced *in vitro* cell analysis systems to characterize stem cell cultures has therefore provided the opportunity to develop automated cell culture systems capable of monitoring stem cell proliferation rates and differentiation capacity. Lab-on-a-chip technology is considered an enabling technology that is amiable for miniaturization, automation and large volume testing−all crucial parameters in stem cell experiments.

ACKNOWLEDGEMENTS: This work was funded by DECHEMA Max Buchner Research Foundation (MBFSt 3423) and Vienna Anniversary Foundation for Higher Education (H-301919/2014).

Electroconductive pericardial derived hydrogels doped with multiwalled carbon nanotubes for cardiac applications

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INTRODUCTION: Electrical stimulation and electrically conductive scaffolds showed promising results in cardiac tissue engineering. We have developed an injectable pericardial matrix derived scaffold enhanced with multi-walled carbon nanotubes and investigated the cardiomyocyte and human mesenchymal stem cells (hMSCs) response to these gels. 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]. COOH-MWNTs were modified with hydrazide groups, dispersed with pericardial matrix (PM) at 0.5 wt% (PMNT) and incubated at 37°C to induce gelation. HL-1 cardiomyocyte cells and passage four hMSCs were cultured on these gels (PM and PMNT gels) and evaluated for viability, expression of connexin43 and α-actinin and beating properties of the cells investigated [2] using AB assay, immunostaining, and Fluo-4 monitoring, respectively. Stemness markers of hMSCs evaluated by PCR for Oct-4 and HIF-2 genes.

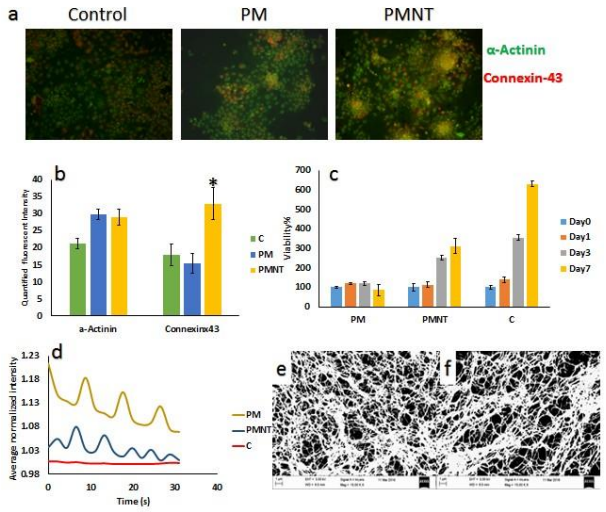

Fig.1:(a,b)Marker expression, (c)Viability and (d)beating of HL-1 cells on (e)PM and (f)PMNT.

RESULTS: Cells showed higher viability and connexion43 expression on PMNT displaying superior electrical coupling between cells. PMNT gels caused a two-fold increase in the beating frequency of cardiomyocytes than those on the PM alone. Decreased level of Oct-4 and HIF-2 genes along with elevated expression of cardiac markers in hMSCs shows they are differentiating into cardiac lineage.

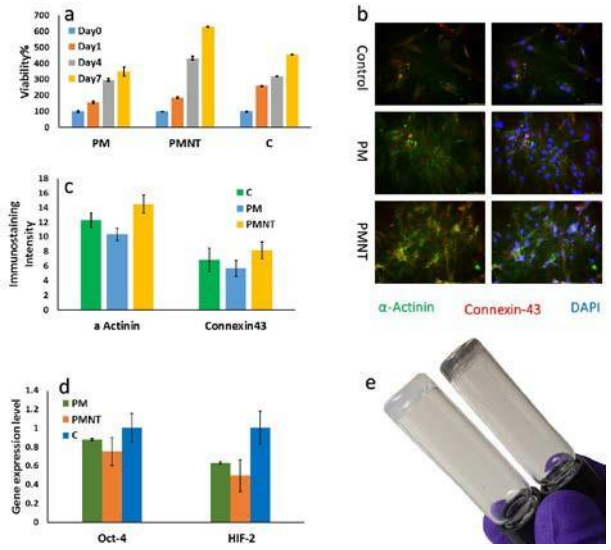

Fig.2: (a) Viability and (b,c) marker expression and (d) PCR gene expression of hMSCs on (e) PM and PMNT.

DISCUSSION & CONCLUSIONS: The PMNT gels promoted good viability and cell beating properties (Fig.1), suggesting electrical conduction between cells. Our study suggests that PMNTs are excellent scaffolds cardiac applications.

Spatial signalling at the membrane: using nanotools to control ligand position and lateral mobility

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INTRODUCTION: Cells communicate with other cells in multicellular organisms to coordinate their activities into functional units. Membrane proteins are key sensor components in cell-to-cell communication and form the largest class of proteins in the druggable genome. Cells that are nearest neighbours can communicate through direct interactions between membrane proteins called ligands and receptors. Dyregulation of these interactions in cancer is involved in allowing cells to acquire abnormal functions such as aberrant proliferation and invasion of neighbouring tissues.

METHODS: DNA origami is a nanofabrication technology that uses DNA self-assembly to drive the precise formation of nanostructures. We have recently shown that DNA origami can be used to tailor the spatial distribution of protein assemblies (*1*). This new tool allows for display of well-defined protein nanoclusters in solution and is therefore amenable to the study of 3D in vitro tissue models and presents an opportunity for future translational applications. Therefore, DNA origami/ligand nanoclusters form a nanotool that is well suited to investigate the roles of the biophysical properties of ligand/receptor interactions on downstream signaling and cellular outcomes.

RESULTS: We are analysing receptor activation with single cell resolution using *in situ* proximity ligation assay (*in situ* PLA). We have shown that applying this assay to single cells grown on substrates with micropatterned fibronectin islands of defined size allows for quantification of the levels of receptor activation with high reproducibility. We have validated this method for the analysis of EphA2 receptor activation (1) and showed that the levels of EphA2 receptor activation depend on the spatial distribution of ephrinA5 ligands at the nanoscale. To investigate the morphological and dynamical aspects of the assembly of the receptor on the cell membrane, we are using Stochastic Optical Reconstruction Microscopy (STORM).

In order to identify the downstream signaling pathways modulated by EphA2 receptor clustering, we are using RNA-sequencing and functional assays.

DISCUSSION & CONCLUSIONS: We developed tailor-made ligand nanoclusters that have the potential to contribute to understanding of the fundamental mechanisms of action of physical variables in ligand/receptor signalling. We are applying these tools to understand signaling mediated by cell-cell contact in neural stem cells, cancer cells and immune cells.

osteogenic differentiation of human mesenchymal stem cells within a β-sheet peptide hydrogel induces mineralisation *in vitro*

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INTRODUCTION: The recovery of the integrity and functionality of damaged tissues is a challenging goal for medicine. Peptide based fibrillar hydrogels are promising platforms for the development of materials that mimic the extracellular matrix[.1] In this study, the capability of β-sheet forming peptide hydrogels to encapsulate human mesenchymal stem cells (hMSCs) and induce their osteogenic differentiation is demonstrated. Cell viability and proliferation were sustained within peptide hydrogels over two weeks. In addition, assays showed that hMSCs committed into an osteogenic profile where they produced key bone proteins and mineralised within the gels over two weeks of culture.

METHODS: FEFEFKFK hydrogels were prepared at 2.5 % w/v. Oscillatory rheology was used to determine hydrogels' mechanical properties. hMSCs were grown according to manufacturer's instructions. Hydrogels' biocompatibility was assessed using Live/Dead assay. Cell proliferation within hydrogels was quantified by Picogreen assay. Spectrophotometric assays and confocal microscopy were used to study the production of bone proteins and mineralisation.

RESULTS: hMSCs showed a sustained viability and proliferation inside gels over two weeks of culture (Figure 1). Hydrogels' stiffness decreased over time mainly in the presence of cells (Figure 2). hMSCs deposited type I collagen within peptide gels and produced other bone proteins such as alkaline phosphatase (ALP) and osteocalcin mainly under osteogenic stimulation (Figure 3). Moreover differentiated hMSCs were capable of mineralising inside gels mainly under osteogenic stimuli as evidenced by the bony-like nodule deposition in gels over two weeks of culture (Figure 4).

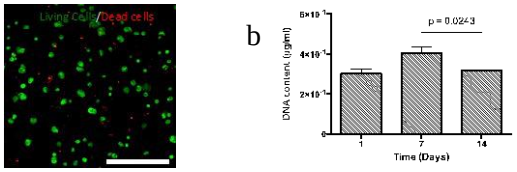

Fig. 1: hMSCs viability. Scale bars=250µm (a). DNA content representing cell proliferation (b).

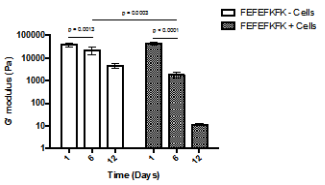

Fig. 2: FEFEFKFK hydrogel's mechanical properties under cell culture conditions.

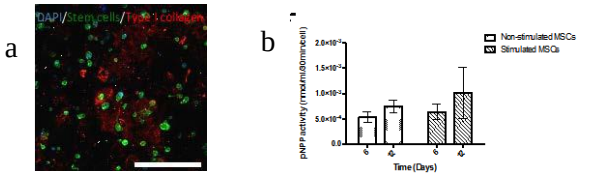

Fig. 3: Collagen I deposits in gel over two weeks of culture. Scale bars=250µm (a). ALP production over two weeks of culture (b).

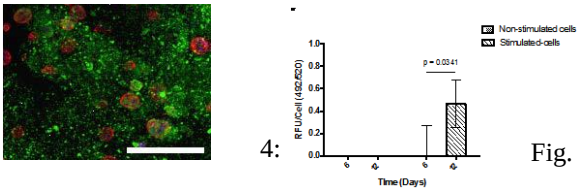

Fig. 4: Mineralisation in hydrogels after 7 days of culture. Scale bars=250µm (a). Quantification of mineralisation inside gels over two weeks (b).

DISCUSSION & CONCLUSIONS: Our findings demonstrated that the FEFEFKFK peptide hydrogel supports the 3-D culture of hMSCs. Gel mechanical property decrease suggests scaffold remodeling by cells and gel degradation. More significantly, the production of bone proteins such as alkaline phosphatase, and mineralisation by stimulated hMSCs confirm the osteogenic differentiation over two weeks of culture. The β-sheet forming peptide gel is a promising scaffold to potentially induce the regeneration of bone tissue.

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Towards a Human-on-a-Chip – Where are we?

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INTRODUCTION: Microfluidic systems have proven to be a powerful tool for recreating tissue- and organ-like functions, providing the basis for preclinical assays with great predictive power. The development of new drugs is strikingly expensive, time-consuming and only a few substances can stand up to the long testing phase. Animal tests often fail to forecast the effects of new drugs, because the phylogenetic distance between laboratory animals and humans is too large. Whereas, current monolayer or suspension cell culture assays are failing to emulate the human cellular microenvironment and, therefore, lead to a rapid dedifferentiation and loss of function in primary human cell cultures. Thus, it is essential to find new technologies to evaluate potential drug candidates at an early stage of the process. Microfluidic culture devices combining human microtissues in an organ-like arrangement could well become a translational solution for that testing dilemma. Currently, the number of microfluidic platforms available which mimic the human situation in vivo is steadily increasing.

METHODS: The new opportunities for the application of human- and organ-on-a-chip systems, as well as important challenges in realizing the full potential of this technology will be addressed.

RESULTS: Although it is an ambitious project, the development of human-on-a-chip systems has been recognized as a promising route towards optimizing the drug development process. Therefore, two essential requirements have to be met: i) the improvement of the organotypical nature of single organ equivalents, and ii) the linking of different organs into a systemic arrangement reflecting that of the human organism at a miniaturized scale. Recently, standard in vitro cell culture techniques have evolved from two-dimensional monolayer cultures toward three-dimensional multi-cellular models, aiming to mimic the in vivo tissue microenvironment. These systems have already shown major improvements towards more accurate prediction of the mode of action of compounds. Furthermore, adapting the in vitro culture conditions to the highly specialized needs of cells, such as controlled nutrient and oxygen supply, removal of accumulating products, and mechanical force acting on the cells was shown to further enhance physiological relevance. Using these well-characterized medium flow rates around the tissues to combine several single organ-on-a-chip devices to a single system emulating multi-tissue behavior leads the way towards a human-on-a-chip.

DISCUSSION & CONCLUSIONS: Many promising single and multi-organ-chips have been published during the last years. Combining the generated knowledge and expertise's of the various groups, the generation of a fully functional human-on-a-chip appears to be an achievable goal within the next 5 years.

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Transplantation of gene supplemented autologous hepatic stem cell organoids in a COMMD1 deficient dog

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INTRODUCTION: Liver cell transplantation can be an alternative for liver transplantation. We asked whether liver organoids, a liver stem cell culture system, would be a suitable cell source. Dogs were used as a large animal preclinical model for autologous liver organoid transplantation to predict safety and efficacy for human application.

Organoids are 3D mini-organs expressing various cell types of a specific organ. Canine liver organoids could be grown and functional correction of a deletion in the *commd1* gene, leading to hepatic copper accumulation (similar to Wilsons disease) by means of gene supplementation in organoids from COMMD1 deficient dogs was successful[1]. It remained to be seen if these corrected organoids could be used in autologous cell transplantation in dogs with copper storage disease.

METHODS: Canine liver organoids were cultured from digested liver biopsies of COMMD1 deficient dogs (n=3), a genetic model of copper toxicosis. Cells were transduced with a construct containing DsRed, a puromycin resistance gene and canine COMMD1. Corrected cells were expanded as organoids and differentiated towards hepatocytes using a defined medium. Dogs were anesthetized and a liver lobectomy was performed to stimulate regeneration. In the same procedure a port-a-cath was placed in the portal vein, providing permanent intraportal access through a subcutaneous port. On the day of surgery and the two consecutive days a total of 4.5-7.3x10^8 autologous cells were infused intraportally under CT or ultrasound guidance and portal pressure monitoring (n=2 dogs). One dog received vehicle. Liver biopsies were taken after 1 week, 1 month, 3 months and 6 months up to 2 years post-transplantation (ongoing study). On the same time points biliary copper excretion was measured upon intravenous administration of Cu^{64}. Cells are traced back in biopsies by immunohistochemical staining for DsRed, COMMD1 and keratin 19.

RESULTS: Organoid-derived liver cell infusion did not result in alterations in portal pressure, signs of discomfort or any adverse events. Preliminary results indicate engraftment of organoid-derived cells in the liver. Immunohistochemistry for COMMD1 positive cells, rubeanic staining for copper accumulation and functional copper excretion studies are underway.

DISCUSSION & CONCLUSIONS: No adverse effects of the transplanted cells was observed. The transplanted cells could be tracked in the liver with biopsies. This animal model allows for long term longitudinal studies to verify functional recovery on the one hand and to observe possible side effects of autologous cell transplantation. The number of dogs in the transplantation and control group will be increased in the near future.

ACKNOWLEDGEMENTS: Research grants form the Dutch Research Counsel NWO ZON/MW (92003538 and 16004121).

Microvascular TE by co-cultures of blood vascular and lymphatic endothelial with mesenchymal stem cells

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INTRODUCTION: Vascularization still remains as one of the challenges in engineering tissues. Besides technical approaches like 2-photon polymerization or 3D-printing to solve this problem, recently co-culture systems of endothelial cells with supporting cells have been developed. In our lab we aim to combine both, blood vascular and lymphatic endothelial cells (ECs) with mesenchymal stem cells (MSCs) in a fibrin matrix to construct blood microcapillaries for oxygen- and nutrient supply but also for waste removal through lymphatic vessels[1,2].

METHODS: In order to facilitate visualization of vascular tube formation we retrovirally infect primary cells (ECs, MSCs) with cDNAs for different fluorescent proteins (YFP, GFP, mCherry). ECs are derived from umbilical veins (HUVEC), peripheral blood (ECFC), or human foreskin (LEC). MSCs are isolated from human fat tissue after liposuction. We routinely characterize our cells by flow cytometry and immunofluorescence. Subcutanous implantation of prevascularized fibrin clots into nude mide was used to evaluate in vivo functionality of the generated microcapillaries.

RESULTS: Co-cultures of blood vascular ECs derived from umbilical veins and peripheral blood with MSCs resulted in vascular network formation in fibrin. The characteristics of these structures depended on growth factors, serum and the addition of the serin protease inhibitor aprotinin. Moreover, we show the kinetics of fibrin degradation as well as the influence of thrombin on vascular tube formation. By angiogenic protein profiling we show paracrine signalling from MSCs towards ECs. Furthermore, integration of LEC resulted in the formation of a lymphatic vascular network. Finally, MSC/EC co-cultures in fibrin resulted in perfusable microcapillaries, as evidenced in an in vivo mouse model.

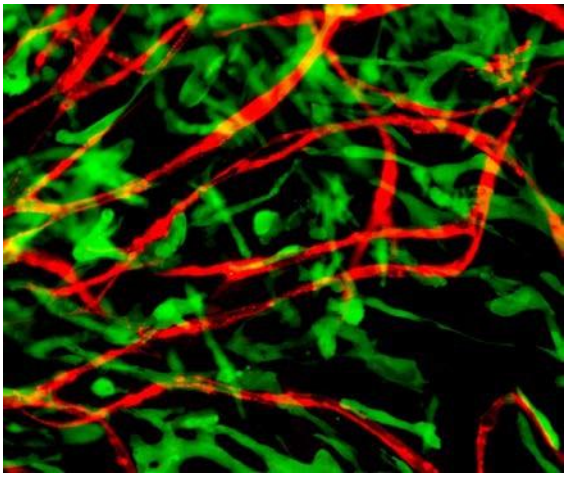

Fig. 1: Co-cultures of lymphatic ECs (green) and blood vascular ECs (red) with ASCs in fibrin result in separate capillary-like tube formation. Scale bar=

DISCUSSION & CONCLUSIONS: Co-cultures of EC and MSC in a fibrin matrix represent a suitable approach for microvascular tissue engineering. Blood vascular, as well as lymphatic ECs can be used to construct both microcapillary beds. However, for clinical translation further molecular insights in these vascular beds are necessary.

ACKNOWLEDGEMENTS: Research in our lab is partly funded by EU-projects Biodesign and VASC-MOC.

Non-mulberry silk fibroin based smart nanofibrous wound dressing for chronic cutaneous ulcers

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INTRODUCTION: The current statistics showing rising cases of traumatic injuries and pathological chronic wounds has led to a huge demand for smart dressing materials to combat impaired healing. Silk fibroin (SF) as biopolymer has been extensively explored in regenerative medicine since centuries [1]. However, limited study is available on the potential of SF from *Antheraea assama* (AA), an endemic Indian non-mulberry silk variety. In this study, SF from both *Bombyx mori* (BM) and AA were electrospun by blending with poly vinyl alcohol (PVA) and were further functionalized with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) to aid healing of chronic cutaneous ulcers.

METHODS: Three types of mats were fabricated namely PVA+AA, PVA+BM and pure PVA. Mats were characterised by SEM, AFM, and FTIR. Further, tensile strength, swelling capacity, degradation profile and water vapour transmission rate (WVTR) were probed into. Biocompatibility of mats was assessed both *in vitro* and *in vivo*. Investigation of wound healing ability of mats was done *in vivo* in a rabbit model through histology.

RESULTS: The e-spun nanofibers ranged between 50-300 nm in diameter. Amide peaks at 1625 cm^{-1} and 1532 cm^{-1} suggested homogenous blending of SF with PVA. Desirable Young's modulus (3±0.5 MPa) along with high swelling ratio and water vapor transmission rate (WVTR, 2330 $gm^{-2}day^{-1}$) were documented. The mats showed very low immunogenicity as confirmed by both *in vitro* TNF-α release and histological studies by implanting subcutaneously in mice for 6 weeks. PVA+AA showed higher proliferation of human dermal fibroblasts (HDF) and keratinocytes (HaCaT) as compared to PVA+BM and pure PVA by MTT and Alamar blue assay (p<0.01). Further, nanofibers incorporated with EGF and FGF showed sustained release leading to enhanced proliferation of HDF/HaCaT cells as compared to control (p<0.01). Post 07 day of operation, wound contraction rate by PVA+AA was higher than PVA+BM (75% vs. 45% respectively; p<0.01).

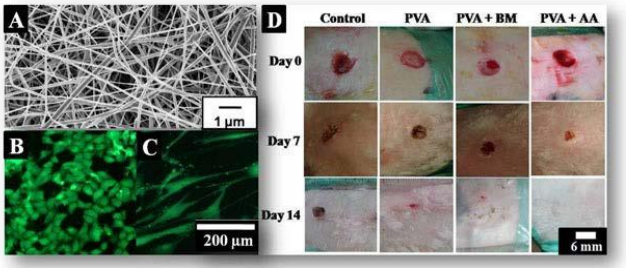

Fig. 1: A) SEM image of electrospun PVA+AA mat B) Live/dead assay of HaCaT and C) HDF cells on mats and D) In vivo wound closure on rabbit.

DISCUSSION & CONCLUSIONS: Nanofibrous matrix of PVA+AA showed remarkable morphology, tensile strength, biodegradability and biocompatibility favoring its use in wound dressing applications. High swelling capacity of mats along with WVTR would help keeping the wound-bed moist and absorb wound exudates. Presence of RGD motifs in AA-SF showed greater cell recruitment and cell migration within wound area, thereby accelerating wound healing process [2]. High wound healing rate achieved by synergistic release of EGF and bFGF suggests possibility of sustained growth factor delivery from nanofibrous mats [3]. In this study, we conclude that functionalized nanofibrous blends of PVA and non-mulberry AASF have promising wound healing attributes and can be used as affordable wound dressing patches to treat chronic wounds.

ACKNOWLEDGEMENTS: We are thankful to Department of Biotechnology, Government of India for their generous funding. Central Instrumentation Facility of IIT Guwahati is acknowledged for use of high-end instrumentation.

Regenerating bone by multifunctional coatings: the blending of cell adhesive and antibacterial properties on the surface of implant biomaterials.

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INTRODUCTION: An implant is considered as osseointegrated when there is no progressive relative movement between the implant and the surrounding bone. It is well established that the use of cell adhesive sequences derived from extracellular matrix proteins, such as RGD, promotes higher adhesion of osteoblasts [1]. However, the clinical success of a dental implant requires not only an optimum osseointegration, but also a powerful antibacterial surface to prevent biofilm formation. To this end, antimicrobial peptide coatings are a promising strategy to prevent bacterial adhesion [2]. Hence, the aim of this work is to develop a multifunctional coating by means of a peptide-based dual platform [3] with the ability to simultaneously enhance osteoblast adhesion and antibacterial activity on the dental implant (Figure 1).

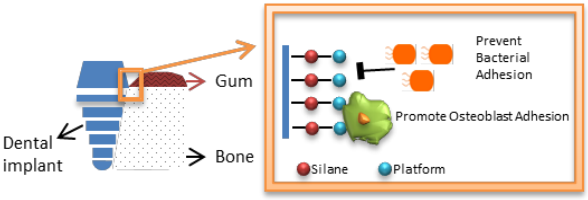

Fig. 1: Multifunctional coating for dental implants

METHODS: The dimeric platform was manually synthesized on solid phase according to reported protocols [3]. RGDS was chosen as cell adhesive peptide, while hLf1-11, a peptide derived from lactoferrin [4], was selected as antibacterial motif. Biofunctionalization method was based on silanization with 3-(aminopropyl)-triethoxysilane. Finally, the peptide platform was covalently attached to the surface by means of a crosslinker agent. Physicochemical characterization was performed with contact angle analysis, interferometry, X-ray photoelectron spectroscopy and quartz crystal microbalance. The response of sarcoma osteogenic cells (Saos-2) to the biofunctionalized surfaces was evaluated in vitro by means of cell adhesion, proliferation and differentiation assays. Bacterial assays were performed using *Streptococcus sanguinis* as model of oral bacteria. To describe the antibacterial effect of the platform, live/dead and bacterial adhesion assays were performed and then verified by SEM and confocal microscopy.

RESULTS: The results of early adhesion events (number of cells attached and their spreading) are shown in Figure 2 (left). After 4 h of incubation, the presence of bioactive coating molecules increased the number of cells attached and their spreading onto the surfaces compared to control samples, as well as cellular proliferation and differentiation (data not shown). Moreover, the percentage of viable bacteria was strongly reduced in treated samples (Figure 2-right). This result was confirmed by live/dead assay and confocal laser scanning microscopy (CLSM).

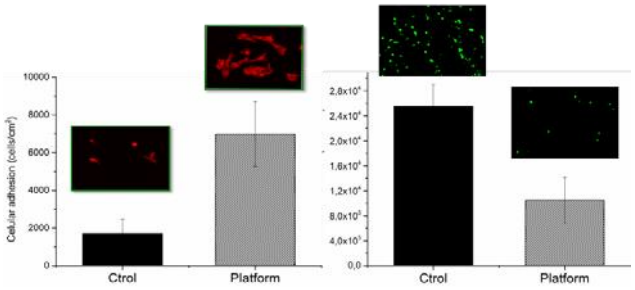

Fig. 2: Osteoblasts adhesion and spreading (left). Results collected from bacterial adhesion assay (right). The images acquired by CLSM show viable S. sanguinis (green dots).

DISCUSSION & CONCLUSIONS: Our innovative strategy significantly reduces the adhesion of the oral bacterial strain *S.sanguinis*. At the same time, the presence of this peptide-based platform, promotes osteoblast cells adhesion, proliferation, mineralization and alkaline phosphatase activity. Thus, our findings confirm that this multifunctional coating has potential applications to promote bone regeneration and overcome limitations of implant materials, such as the lack of an optimal osseointegration or the development of bacterial infection in dental implants.

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A therapeutic window of VEGF doses ensures both effective vascularization and efficient bone formation in osteogenic grafts

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

METHODS: Recombinant VEGF was engineered with a transglutaminase substrate sequence (TG-VEGF) to allow cross-linking into fibrin hydrogels [2]. Osteogenic constructs were prepared with primary human BMSC seeded on hydroxyapatite granules in a fibrin hydrogel containing 4 different TG-VEGF concentrations (0.1, 1, 10 and 100 µg/ml) and optimized to ensure the controlled release of the factor over 4 weeks [3]. Control grafts were generated with BMSC only and no TG-VEGF, or retrovirally transduced BMSC that constitutively express VEGF linked to the cell-surface marker truncated CD8 (VICD8). Histological analysis 1, 4 and 8 weeks after ectopic subcutaneous implantation in nude mice was used to determine vascularization (CD31 immunostaining), bone formation (H&E and Masson Trichrome) and osteoclast recruitment (TRAP staining and Cathepsin K immunostaining).

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

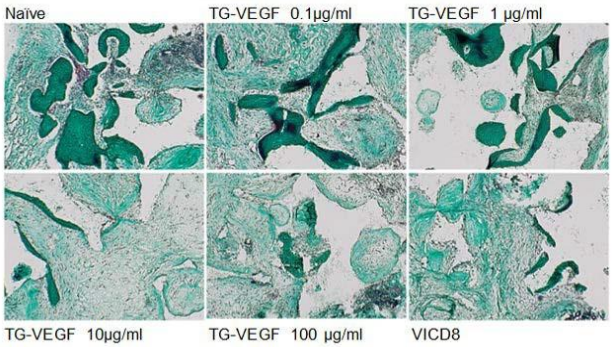

Fig. 1: Masson's Trichrome staining shows that increasing VEGF doses gradually impair bone formation 8 weeks after in vivo implantation of osteogenic grafts.

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

ACKNOWLEDGEMENTS: This work was supported by a Marie Heim Vögtlin grant (158312) by Swiss National Science Foundation to N.D.M. and an intramural grant by the Department of Plastic and Reconstructive Surgery (Basel University Hospital).

A multifactorial approach towards cell phenotype maintenance and enhanced matrix deposition for *in vitro* organogenesis

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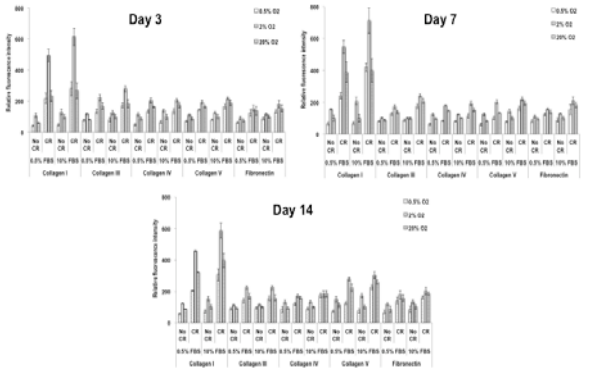
INTRODUCTION: Tissue engineering by self-assembly strategies have yet to be extensively adopted in clinical and commercial setting due to the prolonged time required to develop an implantable device. Recent data have demonstrated that macromolecular crowding (MMC) [1], a biophysical phenomenon that governs the intra- and extra- cellular milieu of multicellular organisms [2] accelerates extracellular matrix (ECM) deposition by several orders of magnitude. Herein, it is hypothesised that MMC coupled with low oxygen tension (2 %) [3] will further enhance ECM deposition in permanent differentiated and stem cell culture, whilst maintaining cell phenotype.

METHODS: Human bone marrow stem cells, dermal fibroblast and corneal fibroblasts were cultured for 3, 7 and 14 days with 100 µg/ml of carrageenan (MMC) at 20 %, 2 % and 0.5 % oxygen tension. Collagen I deposition was assessed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and complementary densitometric analysis. Phenotypic assessment was performed by flow cytometry for the expression of cell surface markers CD90, CD105, CD73, CD44 and lack of expression of CD34, CD11b, CD19, CD45 and HLA-DR. Staining for Oil Red O, Alizarin Red S and Safranin O / Fast green was used to assess adipogenic, osteogenic and chondrogenic differentiation. Numerous ECM molecules were assessed immunocytochemically.

RESULTS: ECM deposition was increased in the presence of MMC in all cell populations. Oxygen tension preferentially affected ECM deposition. Neither MMC nor oxygen tension affected cell phenotype an dfnction.

Fig. 1: *Relative fluorescence intensity analysis revealed that human dermal fibroblasts deposited significantly more collagen type I, collagen type III, collagen type IV, collagen type V and fibronectin ($p < 0.005$) at 2 % oxygen tension than at 0.5 % and 20 % oxygen tensions, at all time points (3, 7 and 14 days), FBS concentrations (0.5 % and 10 %) and presence (75 µg/ml) or absence of CR. Further, MMC (75 µg/ml CR) significantly increased ($p < 0.005$) the deposition of all ECM molecules assessed, at all time points (3, 7 and 14 days), oxygen tensions (0.5 %, 2 % and 20 %) and FBS concentrations (0.5 % and 10 %).*



DISCUSSION & CONCLUSIONS: MMC and low oxygen tension can be used effectively to enhance extracellular matrix deposition in human stem cell an permanently differentiated cell culture, without detrimental effect on cell phenotype and function. This multifactorial approach paves the way for the development of scaffold-free cell therapies for various clinical targets.

ACKNOWLEDGEMENTS: The authors would like to acknowledge the Irish Research Council, the Health Research Board, the Science Foundation Ireland and the EC for financial support. This template was modified with kind permission from eCM Journal.

Online monitoring of mechanical properties of three-dimensional tissue engineered constructs for quality assessment

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INTRODUCTION: To regenerate damaged tissues, various approaches have been established including the delivery of cell-seeded three-dimensional scaffolds. Their development and maturation is promoted through the utilisation of bioreactors mimicking the physical *in vivo* growth environment of cells and tissues. Monitoring the maturation of tissue constructs during culture and their performance prior to implantation into the patient is important for defining their quality and manufacturing criteria. Key properties include scaffold integrity and mechanical properties. Therefore, novel non-invasive three-dimensional (3D) imaging modalities are required providing rapid results and translational solutions. Elastography allows the mapping of mechanical properties by measuring the deformation in correlation to external mechanical loading. However, it is limited in terms of resolution and sensitivity. To overcome these drawbacks elastography has been coupled with optical coherence tomography known as optical coherence elastography (OCE) and a hydrostatic pressure bioreactor into a new image modality, HP-OCE.

METHODS: Scaffolds were fabricated from several techniques[1]**.** Scaffolds were prepared by Fused Deposition Modelling (FDM) using poly (lactic acid) (PLA). Hydrogels were prepared by dissolving agarose in distilled water to final concentrations of 0.5%, 1.0% and 1.5%. hMSC ($1x10^5$) were incorporated before gelation to prepare cellular gels. Salt-leached scaffolds were prepared by dissolving PLA in chloroform and casting the polymer solution over sodium chloride particles with diameters between 250-350 µm. Scaffolds were air dried and infiltrated with 0.5% agarose. All scaffolds were cultured in cell culture medium and imaged in PBS. Scaffolds were imaged utilizing OCT during mechanical stimulation at 1, 5, 10, 15 and 25 kPa at 1Hz frequency in the hydrostatic force bioreactor[2]. Displacements maps were generated using elastography algorithms[1].

RESULTS: Due to its improved sensitivity phase-resolved OCE is capable of detecting small displacements in heterogeneous tissue phantoms. Clear differences in displacement were detected for hybrid hydrogels prepared from 0.5% and 1.5% agarose compared to 1.0% and 1.0% hydrogels (Figure 1). Interfaces between stiffer and softer gels can be identified.

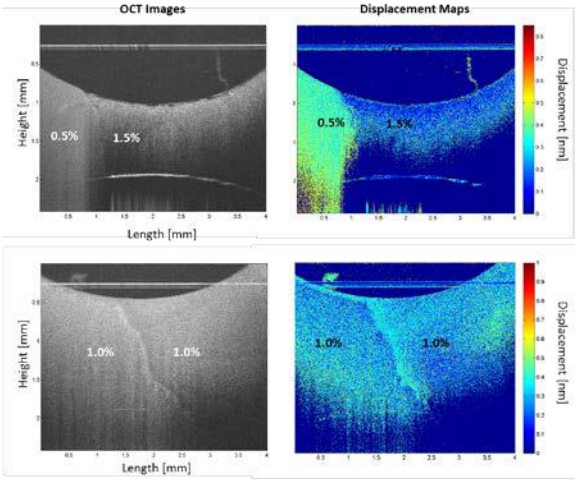

Fig. 1: Monitoring hydrogels during mechanical stimulation in the hydrostatic force bioreactor. Relative displacement maps were generated by elastography algorithms. Colour represents displacement.

DISCUSSION & CONCLUSIONS:

A novel dynamic OCE technique, HP-OCE, with cyclic compression as the external excitation generated by hydrostatic pressure was established. Phase-resolved OCE algorithms were applied to determine the scaffolds displacement in OCT phase-based images of various tissue phantoms. Our results indicate that HP-OCE allows real-time non-invasive monitoring of the displacement and strains of tissue phantoms. It enables the investigation of scaffold degradation, material interfaces and heterogeneity as well as changes in scaffold porosity. It further allows the investigation of the effect of mechanical forces on cellular activities during dynamic culture. Future experiments will investigate the maturation of tissue engineered constructs during dynamic culture.

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Electroactive biomaterials for drug delivery and tissue engineering.

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INTRODUCTION: Electrical fields affect a variety of tissues and play important roles in a multitude of biological processes, which inspired the development of electroactive biomaterials (e.g. bionic ears/eyes, cardiac pacemakers, neural electrodes), some of which have been clinically translated [1]. The tuneable properties of conducting/electroactive polymers (CPs or EAPs, respectively) such as derivatives of polyaniline, polypyrrole or polythiophene make them attractive components of electroactive biomaterials for drug delivery devices, electrodes or tissue scaffolds [1].

METHODS: Drug delivery: solution processable electroactive block copolymers were prepared on a multigram scale; a clinically relevant drug was loaded and its delivery in the absence/presence of electrical stimulation was assessed by UV absorption [2].

Tissue scaffolds: Polymers (e.g. polycaprolactone or silk) were processed into various materials morphologies (e.g. films, fibers, foams), rendered electrically conductive with derivatives of polypyrrole or polythiophene, and cells (human stem cells, human fibroblasts, or rat Schwann cells) were cultured thereon in the absence/presence of electrical stimulation, and studied using biochemical assays and histochemistry [3].

RESULTS: Electrical stimulation (ES) of drug-loaded EAPs triggered drug delivery from the materials (Figure 1). ES of scaffolds for stem cells enhanced their differentiation towards osteogenic outcomes (Figure 2); ES of scaffolds for fibroblasts enhanced their alignment; ES of scaffolds for Schwann cells increased nerve growth factor production [2,3]

DISCUSSION & CONCLUSIONS: I present the first examples of degradable EAP-based drug delivery devices [2], the largest CP-based tissue scaffolds thus far used for tissue engineering [3] the first examples of biomineralized CP-based bone tissue scaffolds, and other examples of the results of 1 granted patent and 8 patents pending. Such materials have prospects as chronotherapeutic devices for drug delivery and tissue engineering.

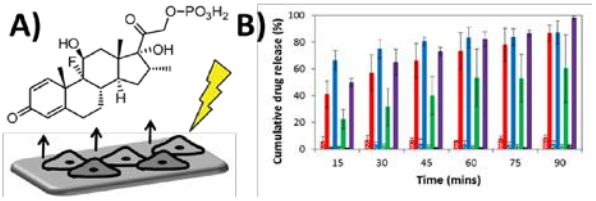

Fig. 1: CP-based materials for drug delivery. A and B) Electrical stimulation enhances drug delivery from biodegradable electroactive polymers. Polyethylene glyocol-based polymers red & blue; polycaprolactone (PCL)-based polymers green & purple; checked bars – no stimulation; solid bars with stimulation.

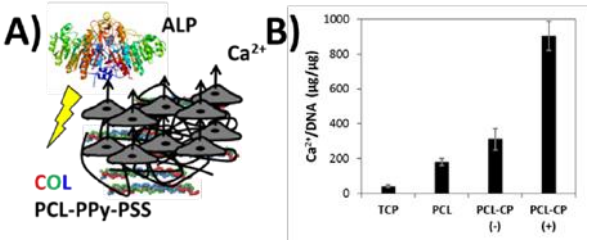

Fig. 2: CP-based materials for tissue engineering. A and B) Electrical stimulation of stem cells on CP-based biomaterials results in increased Ca^{2+} deposition. TCP tissue culture plate control, PCL polycaprolactone control, PCL-CP (-) conductive PCL without electrical stimulation, PCL-CP (+) conductive PCL with electrical stimulation.

ACKNOWLEDGEMENTS: I warmly acknowledge my mentors Prof. Christine E. Schmidt (University of Florida) and Prof. David L Kaplan (Tufts University), and Lancaster University for financial support in the form of a 50th Anniversary Lectureship and a Faculty of Science and Technology Travel Grant.

Multi-parametric imaging of oxygen in scaffold-free and scaffold-based three-dimensional tissue models

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INTRODUCTION: Molecular oxygen (O_2) is one of the key metabolites of aerobic cells, playing a multitude of roles in tissue physiology. Lower than normal O_2 environment, termed "hypoxia", affects cell death and proliferation in stem cell niche. Higher than normal O_2, "superoxia", often leads to oxidative damage and production of reactive oxygen species. The gradients of O_2 in live tissue are dynamic, being dependent on cell respiration and diffusion. The monitoring and manipulation of O_2 supply within 3D tissue models are therefore important for better mimicking *in vivo* conditions occurring in cultured tissue and further progress in stem cell physiology [1].

METHODS: To achieve *in situ* high-resolution quantitative analysis of O_2 in tissue models, we employed method of Phosphorescence Lifetime Imaging Microscopy (PLIM) [1]. This photophysical process relies on the specific and reversible quenching of phosphorescence of organic dyes complexed with heavy metal ions. In order to achieve high brightness, photostability and reliable calibration, phosphorescent indicator dye must be protected from self-quenching and environmental interferences by: (i) converting it to cell-penetrating small molecule or nanoparticle probe, or (ii) embedding in solid-state 3D porous scaffold material. Using click-modification of Pt-porphyrins we developed a panel of small molecule and versatile polymeric nanoparticles compatible with one- and two-photon excitation modes and harboring different charged groups. To test the application potential of prepared O_2-sensitive materials, we chose multicellular spheroid aggregates made from rat primary neurons (neurospheres), tumor cells (PC12, HCT116), *ex vivo* cultures of rat brain slices and mouse intestinal organoids. These 3D cultures were grown using conventional techniques, stained with different fluorescent dyes (live cell imaging and immunofluorescence) and analyzed by multiplexed PLIM imaging [2-4].

RESULTS, DISCUSSION & CONCLUSIONS: With small molecule probe Pt-Glc we achieved efficient in-depth staining of neurospheres, live brain slices and intestinal organoids [2]. Nanoparticle probes showed better performance with continuous staining protocol [1, 3]. O_2-sensitive scaffolds displayed ease of use with medium-size 3D models, such as multicellular aggregates of PC12 cells [4]. We found that multicellular aggregate models often experience size-dependent and dynamic pattern of deoxygenation, which can be affected by their formation method. We successfully combined O_2 imaging with analysis of such parameters as cell death (necrosis and apoptosis), viability (mitochondrial membrane potential), cell proliferation (staining with BrdU), pH and Ca^{2+} (fluorescent probes), and distribution of specific cell types. Compared with semi-quantitative methods of assessing hypoxia, such as immunostaining of hypoxia-specific proteins or labeling with pimonidazole, O_2 PLIM imaging demonstrated superior performance. We also observed high heterogeneity of O_2 gradients within tissue models, which dictates the use of live cell imaging methods, rather than "averaging" high- and medium throughput approaches (e.g. PCR or western blotting). Collectively, our method shows necessity of O_2 imaging within 3D tissue models, being a powerful tool for quality and viability control and advanced tissue engineering.

ACKNOWLEDGEMENTS: Supported by Science Foundation Ireland grants 13/SIRG/2144 and 12/RC/2276.

BIOART/kidney and liver

Protein-bound anionic uremic toxins efficiently cleared by a bioengineered renal tubule

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INTRODUCTION: Chronic renal failure is a severe health problem with a high morbidity and mortality rate as adequate therapy is currently not available. The development of a biotechnological platform for the removal of protein-bound waste products (e.g. uremic toxins) is a prerequisite to improve current treatment modalities for patients suffering from end stage renal disease.

METHODS: Conditional immortalized proximal tubule epithelial cells overexpressing the organic anion transporter 1 (OAT-1) transporter (ciPTEC-OAT1[1]) were seeded on the outside of double coated hollow fiber membrane (HFM). The fibers were biofunctionalized using a coating combination of 3,4-dihydroxy-L-phenylalanine (L-DOPA) and collagen IV[2]. To investigate the barrier function, the fibers were mounted on a tailor made flow system. Unseeded or seeded double coated HFM were perfused with FITC-inulin and diffusion was measured in real-time. To determine cells polarization on HFM, the expression of the tight junction protein zonula occludens-1 (ZO-1) was investigated. To study the activity of the main transporters involved in anionic uremic toxins handling (OAT1), breast cancer resistance protein (BCRP) and multidrug resistance-associated proteins 4 (MRP4), the tubules were perfused with the substrate fluorescein (1 µM) in the presence or absence of specific drug transporter inhibitors (probenecid blocks OAT1, and efflux pump (EP) inhibitors KO143 (BCRP blocker) and MK571 (MRP4 blocker)) and two anionic uremic toxins: indoxyl sulfate (IS) and kynurenic acid (KA). Finally, transepithelial transport of IS and KA free and bound fraction (using human serum albumin) was studied using a similar perfusion set-up as was used for the barrier function assay.

RESULTS: Within 1 min of perfusion, unseeded-HFM showed a sustained leakage compared to the seeded HFM (no cells: 89 ± 4 % vs. cells: 10 ± 3 %; $p<0.001$). In addition, the presence of ZO-1 along the boundaries of the cells further endorsed the epithelial character of a homogenous and polarized cell monolayer on HFM. Fluorescein uptake, in the presence of EP inhibitors, was inhibited by 100 µM IS (45 ± 13%; $p<0.001$) and 30 µM KA (83 ± 3%; $p<0.001$). In the presence of 100 µM probenecid, fluorescein uptake was clearly attenuated as well (96 ± 3%; $p<0.001$). The transport tests revealed a clearance of 44 ± 6 µl.min-1.cm-2 and 72 ± 20 µl.min-1.cm-2 for IS and KA, respectively. In presence of 66 µg/ml HSA the clearance of IS and KA were 74 ± 10 µl.min-1.cm-2, $p<0.01$ and 101 ± 23 µl.min-1.cm-2, ns, respectively..

DISCUSSION & CONCLUSIONS: In summary, a successful bioartificial renal tubule was established which presented a clear barrier function and facilitated transepithelial transport of protein-bound IS and KA. This provides an innovative basis for regenerative nephrology through advanced function replacement.

ACKNOWLEDGEMENTS: This research was performed as part of the Netherlands Institute for Regenerative Medicine (NIRM, grant no. FES0908), funded by the Dutch Ministry of Economic Affairs and the Marie Curie ITN project: BIOART (grant no.316690, EU-FP7-PEOPLE-ITN-2012). The authors declare that they have no conflict of interest.

Innovative cell deposition techniques for tissue model development

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INTRODUCTION: The availability of validated and reproducible in vitro micro-tissue models could provide a step-change in the ability to investigate biological processes, including investigation of underlying disease mechanisms and new treatment strategies, and 3D culture models have been a growing area of recent research. Whilst many models have shown promise, and some models are now commercially available, the methods used to create them generally remain labour intensive and the models themselves can be inconsistent [1]. From a scientific perspective, the principal need is to develop the processes which will allow micro-tissue models to be reliably and consistently produced on a large scale for a low cost. From a clinical perspective, the principal need is to understand the value and limitations of the models when compared to established models, where these exist, and to establish the potential for the models to be used for both disease modelling in a general sense (e.g. for drug screening), and as a diagnostic or theranostic tool to support personalised therapy. The need for models to be appropriately validated means that the need to start scale up is not at the point of commercialisation: batches of hundreds or thousands of models will typically be required to demonstrate proof of concept.

The first step in the development of a tissue model is the deposition of a controlled number of cells, normally either in media or in a gel. Most functional models require more than one cell type to be present in order to recapitulate relevant aspects of tissue behaviour. Bioprinting [2] and biofabrication techniques have been explored as automated methods through which these micro-tissue pre-cursors can be formulated. However the techniques for automated cell deposition in general remain slow and unreliable.

This presentation will outline two approaches to the development of high precision, high productivity methods of creating micro-tissue precursors.

METHODS: The first approach uses a novel bio-ink formulation technique in order to avoid issues of agglomeration. Single cell encapsulation using poly-L-lysine (PLL) has been evaluated for both inkjet and micro-valve printing techniques.

The second approach utilises micro-valves to create two impinging jets of material, which can then be used to achieve mid-air mixing of gel precursors which then react to create the gel which can be deposited on the substrate, as illustrated in Fig. 1. This approach has been evaluated for alginate and fibrin gels, both acellular and cell filled.

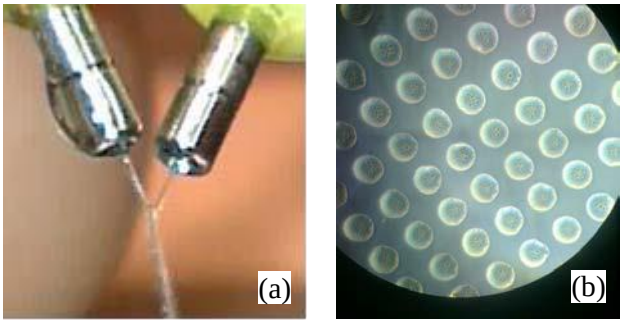

Fig 1: (a) Twin Micro-Valve Impingement Mixing; (b) Inkjet Printed Cell Aggregates

RESULTS: We have demonstrated for multiple cells types that temporary encapsulation of cells in PLL allows them to be printed through inkjet heads at rates approaching 1000 cells per second. The ink can be maintained in the printer for periods of at least an hour, with cell viabilities no different from an unprinted population, and with the ability to deposit single cells and cell aggregates (see Fig 2). Micro-valve deposition allows for higher rates of deposition (hundreds of cells in a single deposition), but with less control over the precise number of cells being deposited. As agglomeration can be avoided the numbers of jets or valves can be increased to increase productivity. A single twin micro-valve impingement head can deposit alginate gels at a rate of 30 mm^3/min, with potential for higher deposition rates.

DISCUSSION & CONCLUSIONS: Innovative cell processing techniques can deliver the high precision, high productivity processing that the development of scalable micro-tissue models requires.

ACKNOWLEDGEMENTS: The research has been supported by the Arthritis Research UK Tissue Engineering Centre, the UK EPSRC Centres for Innovative Manufacture in Medical Devices and Additive Manufacture, and Newcastle University.

Combining glyoxal cross-linked type II collagen based scaffolds with infrapatellar fat pad stromal cells for articular cartilage repair

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INTRODUCTION: Articular cartilage lacks an intrinsic repair mechanism and subsequently external factors must be applied to induce cartilage repair post injury. Current treatments options for articular cartilage repair such as micro-fracture remain sub-optimal, with the repair tissue generally consisting of bio-mechanically inferior fibro-cartilage. Extra cellular matrix (ECM) derived biological scaffolds have been used for various tissue engineering applications in clinical settings due to their ability to both provide structural support and biological cues to aid tissue repair. In order to not elicit an immune response from the recipient, effective decellularization is of vital importance. <50ng DNA per mg of ECM dry-weight has been proposed as one criteria which must be reached to confirm sufficient decellularisation [1]. The infrapatellar fat pad (IFP) is a tissue which contains a large number of chondro-progenitor cells [2]; considering the anatomical location of the tissue, this makes the IFP an ideal source of stromal cells for cartilage repair applications. This study details the coupling of type II collagen based scaffolds derived from decellularized porcine articular cartilage with IFP-derived stromal cells as a possible treatment for the repair of articular cartilage defects.

METHODS: To generate type II collagen scaffolds, articular cartilage was harvested from 4 month old pigs post sacrifice. The cartilage was finely diced and subsequently solubilised using pepsin. Salt precipitation was then performed to purify the type II collagen from the sample. The type II collagen was then cross-linked with glyoxal and transferred to 5x3mm moulds prior to freeze-drying to produce porous scaffolds. The collagen concentration and degree of glyoxol cross-linking were varied to identify the optimal parameters for promoting stem cell chondrogenesis. To test the ability of the scaffolds to promote chondrogenesis *in vitro*, 500,000 IFP-derived stromal cells (FPSCs) were seeded onto the scaffolds and stimulated with transforming growth factor (TGF)-β3 for 28 days.

RESULTS: Biochemical analysis of the fabricated type II collagen scaffolds revealed sufficient decellularization with a mean DNA content of 39ng per mg of dry-weight. Glyoxal cross-linking produced scaffolds with remarkable shape-memory properties, returning to their original shape even when compressed to over 90% of their original thickness. As expected, the scaffolds stained strongly for type II collagen (Fig. 1; Top row). When seeded with FPSCs, the scaffolds promoted robust chondrogenic differentiation as observed by high levels of GAG and collagen deposition (Fig.1).

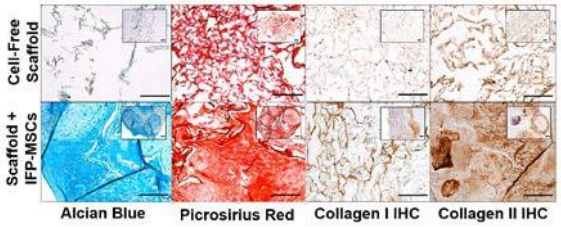

Fig. 1: Histological and immunohistochemical analysis demonstrates robust chondrogenesis of FPSCs seeded on collagen II scaffolds after 28 days in vitro. Scale bar = 200µm.

DISCUSSION & CONCLUSIONS: Development of xenogeneic-derived biological scaffolds that are sufficiently decellularized while maintaining mechanical integrity remains a challenge in tissue engineering. This study demonstrates that solubilisation and purification of porcine articular cartilage results in the creation of type II collagen scaffolds with a DNA content lower than the currently accepted decellularization threshold. The resulting scaffolds were observed to be highly chondroinductive in vitro.

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Microengineered hydrogels for stem cell bioengineering

Ali Khademhosseini

Harvard-MIT's Division of Health Sciences and Technology, Wyss Institute of Biologically Inspired Engineering, Brigham and Women's Hospital and Harvard Medical School.

Engineered materials that integrate advances in polymer chemistry, nanotechnology, and biological sciences have the potential to create powerful medical therapies. Our group aims to engineer tissue regenerative therapies using water-containing polymer networks called hydrogels that can regulate cell behavior. Specifically, we have developed photocrosslinkable hybrid hydrogels that combine natural biomolecules with nanoparticles to regulate the chemical, biological, mechanical and electrical properties of gels. These functional scaffolds induce the differentiation of stem cells to desired cell types and direct the formation of vascularized heart or bone tissues. Since tissue function is highly dependent on architecture, we have also used microfabrication methods, such as microfluidics, photolithography, bioprinting, and molding, to regulate the architecture of these materials. We have employed these strategies to generate miniaturized tissues. To create tissue complexity, we have also developed directed assembly techniques to compile small tissue modules into larger constructs. It is anticipated that such approaches will lead to the development of next-generation regenerative therapeutics and biomedical devices.

Re-engineering developmental processes for regenerative medicine

I Martin

Department of Biomedicine, University Hospital Basel, University of Basel, Switzerland.

Biological processes leading to tissue formation during embryonic development are characterized by the stability and reproducibility of events, typically referred to as 'robustness'. Would regenerative medicine approaches be more repeatable and effective if they targeted the recapitulation of pathways and mechanisms typical of tissue development? Following the exemplifying context of cartilage and bone regeneration, this lecture will describe and discuss the concept of engineering regenerative strategies by mimicking developmental processes, exploiting the own body as the in vivo bioreactor. Rather than engineering a tissue, the strategy targets the use of cells (e.g., mesenchymal stromal cells) to engineer the different stages of processes, recapitulating events of development (e.g., endochondral ossification or joint cavitation). The product would be a modular construct containing all necessary and sufficient cues to autonomously remodel into the target repair tissue upon grafting.

In this perspective, however, shouldn't tissue regeneration strategies be inspired by development but adapted to be effective in a context, which is different from the embryo? In fact, cells in adults may strongly differ from multipotent embryonic cells, and typically reside in an environment, which is tightly regulated by post-natal mechanical conditioning or immune/inflammatory processes. Thus, the developmental machinery may need to be re-designed for regenerative purposes by establishing artificial events or conditions. Will 'developmental re-engineering' offer a chance for regeneration to those tissues with limited capacity to recover from injuries?

A secret weapon of ECM bioscaffolds

SF Badylak

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The extracellular matrix (ECM) represents the composite accumulation of secreted structural and functional molecules of the cells of every tissue and organ. Logically therefore, the ECM constitutes the ideal "microenvironmental niche" for cell maintenance, proliferation and/or differentiation. Bioscaffolds composed of ECM are prepared by decellularization of a source tissue, and with proper care the decellularization process can largely preserve the ultrastructure and composition of the native matrix.

The use of ECM bioscaffolds for repair and reconstruction of damaged soft tissues such as the body wall, gastrointestinal tract, lower urinary tract, cardiovascular structures, and musculoskeletal tissues has yielded promising results. The process of ECM scaffold remodeling upon implantation involves a robust infiltration of host cells and degradation of the scaffold itself, with subsequent recruitment of endogenous

stem/progenitor cells and modulation of the local innate immune response. Collectively these events can stimulate the partial restoration of vascularized, innervated, site-appropriate functional tissue, a process referred to as "constructive remodelling". The mediators of these events are the subject of intense interest and investigation. A recently discovered "secret" mechanism utilized by the extracellular matrix appears to be a critical determinant of remodeling outcomes.

Macrophage-induced rapid scaffold remodeling of a cell-free resorbable supramolecular electrospun scaffold into a neo-artery

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INTRODUCTION: One of the challenges of *in situ* vascular tissue engineering using synthetic scaffolds is to exploit the natural wound-healing cascade to obtain functional blood vessels by providing an instructive resorbable scaffold that is able to guide and control neo-tissue formation [1]. Recent studies suggest that this cascade is an inflammation-driven process in which the involvement of macrophages is of great importance to coordinate the process of scaffold degradation as well as neo-tissue formation and to avoid detrimental outcomes such as stenosis [2-3]. However, a full mechanistic insight in the process of neo-tissue formation, and specifically the role of circulating cells, remains elusive. The objective of the current study was to gain advanced insight in the remodeling of a cell-free degrading supramolecular scaffold used for *in situ* vascular tissue engineering. We therefore implanted a rapid resorbable scaffold, as an interposition graft in a rat model and studied neo-tissue evolution up to 2 months.

METHODS: Polymeric vascular grafts were made of fast-resorbing, highly porous electrospun bisurea-modified polycaprolactone (PCL-bisurea) tubular scaffolds and were shielded by non-degradable expanded polytetrafluoroethylene (ePTFE) to avoid transmural or transanastomotic ingrowth [4]. The polymeric grafts were implanted in 60 healthy male Lewis rats as an interposition graft into the abdominal aorta (Fig. 1A). The grafts were explanted after 1 day (n=9), 3 days (n=9), 1 week (n=8), 2 weeks (n=8), 1 month (n=13), and 2 months (n=13). Samples were cryosectioned and immunohistochemically analyzed for matrix formation (Elastica van Gieson) and cell specific markers, including CD68, iNOS, αSMA, and vWF.

RESULTS: Already after one week, scaffold resorption was observed, starting at small spots in the center of the vascular graft wall. These spots expanded into larger areas (at day 14), in which scaffold had been replaced by extracellular matrix (ECM). After two months, all scaffold was resorbed and replaced by ECM (Fig. 1C). Cells infiltrated the scaffold from day one, with a dominant presence of pro-inflammatory macrophages at sites of resorption in the first month (Fig. 1D). When most of the scaffold was resorbed, a major reduction in macrophages was observed. Noteworthy, an endothelial lining at the luminal site was already present after one week, while the first tissue producing cells (alpha smooth muscle actin positive (α-SMA$^+$)) cells were detected at day 14. However, only two-month explants displayed a medial layer of α-SMA$^+$ cells consistent with native tissue (Fig. 1E).

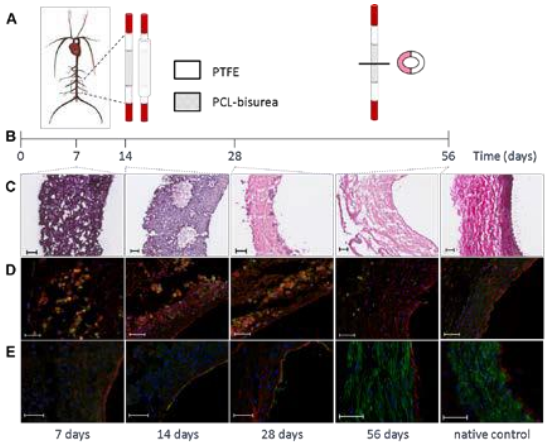

Fig. 1: (A) Graft composition, (B) Time line, (C) Scaffold degradation and ECM formation in time (scaffold: purple; ECM: pink), (D) Immuno-fluorescent co-staining of pan-macrophage marker CD68 (green) and pro-inflammatory macrophage marker iNOS (red), (E) Immuno-fluorescent co-staining of α-SMA (green) and von Willebrand factor for endothelium (red). Scale bars: 50µm.

DISCUSSION & CONCLUSIONS: In only two months time, we observed the fast transformation of a polymeric vascular graft into a native-like neo-artery in which no scaffold was present. Furthermore, we demonstrated that macrophage-induced rapid scaffold resorption led to neo-tissue development *in situ*, followed by resolution of inflammation.

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Pancreatic islet cell transplantation in the clinic

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INTRODUCTION: Type 1 Diabetes **(**T1D) is in Sweden the most common chronic disease in children for which there is no cure. The average diabetes incidence in children has been increasing by approximately 2 % per year over the last 70 years, and now at least 800 children are annually diagnosed with T1D in Sweden. Exogenous insulin treatment is by no means able to fully mimic the fine-tuned regulation of insulin secretion in normal individuals and only a minority of T1D patients registered in the Swedish National Diabetes Registry (NDR) reaches target HbA1c levels of 52 mmol/mol.

DISCUSSION & CONCLUSIONS: Clinical islet transplantation is currently being explored as a treatment for persons with T1D and hypoglycemia unawareness. Although “proof-of-principle” has been established in recent clinical studies the procedure suffers from low efficacy. At the time of transplantation, the isolated islets are allowed to embolize the liver after injection in the portal vein, a procedure that is unique in the area of transplantation. A novel view on the engraftment of intraportally transplanted islets is presented that could explain the low efficacy of the procedure. Finally, novel approaches to optimize the outcome in clinical islet transplantation are presented.

where's the cultured skin?

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INTRODUCTION: The written evidence of the first use of skin substitutes dates back to some thousands year BC, as mentioned in the Papyrus of Ebers. Since then numerous biological and synthetic skin substitutes have seen the light of day but very few have managed to become an integral part of the clinical treatment options in wound healing.

RESULTS:

Fig 1. The Papyrus of Ebers. First described use of skin substitutes

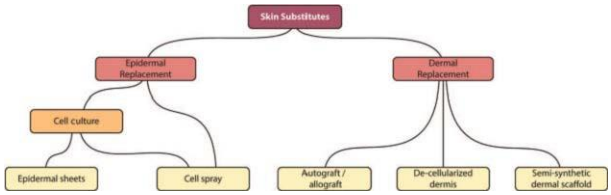

Fig 2. Categories of skin substitutes (from [1])

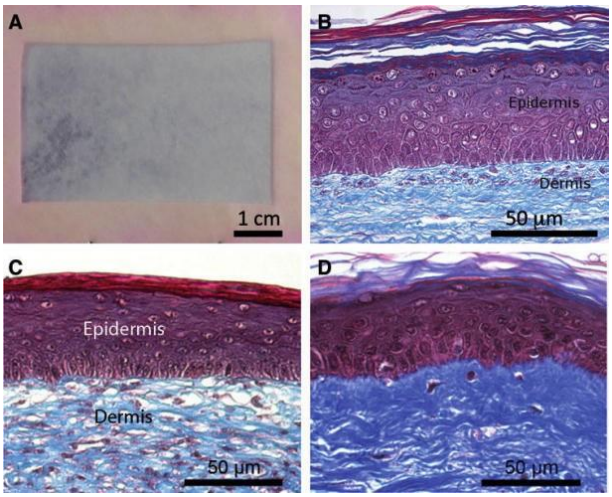

Fig 3. Skin substitute from a self-assembled dermal matrix A, B, C; normal skin D. (from [3])

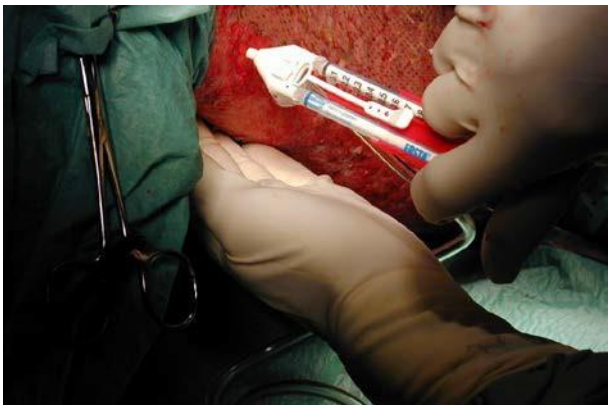

Fig 4. Cultured autologous keratinocytes being spray painted onto excised burn wound.

DISCUSSION & CONCLUSIONS: The advent and progress in tissue engineering and bio-technology has promised plenty but delivered less. Why have we not come further? We make a temporal journey whilst reviewing attempted approaches, (bio)materials, and cell therapies.

Fig 3. Skin substitute preparation steps (from [2]

Autologous cultured keratinocytes in the clinic

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INTRODUCTION: Gold standard of burn care is early excision of eschar and autologous split-thickness skin grafting to the surgical wound areas. [1] However, already at 20-30% of the body surface area burned the access to healthy skin for transplantation starts to be limited. Techniques are available to maximise the amount of skin grafts. However, cultured autologous keratinocytes (CAK) is often the last resort for the most severely burned, and a useful tool also in significant burns. Since the introduction of autologous cultured keratinocytes to burn care in the 80's expectations have been high. [2,3] CAK-sheets have been widely available and used. The culture/application technique has been further developed into spraying CAK with tissue glue. Numerous other applications of CAK with membranes, matrices, etc ± dermal substitutes have been used. During these, soon four, decades of availability the experience and support for the use of CAK has varied, from 'very useful' to having 'no effect'.

The introduction of the European Tissues and Cells Directive (2004/23/EC) has also changed the stage for the use of (autologous) cultured cells.

This presentation will describe the evolution and current uses of cultured autologous keratinocytes in burn care.

METHODS: Clinical experience and critical review of the literature was used to paint a picture of the current 'consent' regarding the use CAK in burn treatment.

RESULTS: In brief:

The level of evidence supporting the use of CAK in burn care is limited and frequently restricted to case series without level 1 evidence.

The use of CAK can be summarized as being time consuming, expensive, complicated, to involve plenty of manual labor, and with a crucial lack of a dermal component.

Some case series show good results, whereas literature reviews show more negative results.

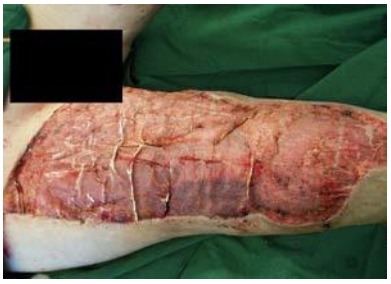
Fig 1.

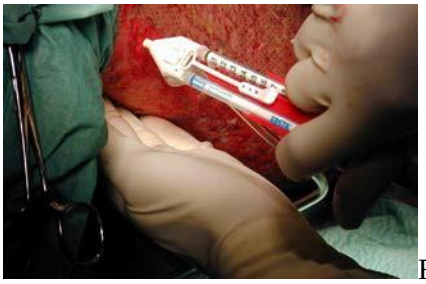
Fig 2.

Fig. 1: CAK-sheets 9 days after application to wound bed. Fig. 2: CAK in single-cell suspension being spray-painted on wound bed in tissue glue.

DISCUSSION & CONCLUSIONS: The main characteristics of CAK-use are:
Inconsistency
Unpredictability of engraftment
Extremely variable take, from poor to excellent.

Good graft take has been observed as the exception rather than the norm.

The obvious main question still stays virtually unanswered: 'Does CAK have a role in burn treatment?'

There is an indisputable need for future higher level research.

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Acceleration of wound healing using biological and physical means

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INTRODUCTION: During the life cycle tissue are several times damaged and need to be repaired or better regenerated. The intrinsic properties of the tissues allow a good regeneration or may lead to repair tissue with restoration function, but not of form. Co-morbidities such as diabetes mellitus, vascular diseases, obesity, osteoporosis, ageing etc. may impair proper wound healing. Therefore, wound healing often needs to be supported to have at least a reparation with restoration of function. Depending on the underlying pathology, different approaches can be chosen. E.g. in case of vascular pathologies, neo-angiogenesis could be stimulated in order to overcome the impaired flow of oxygen and nutrients and thereby allow physiologic wound healing to occur. Activation of tissue specific cells is also possible. These stimulation of properties can be achieved by different approaches using biological stimulation, physical stimulation or adding biomaterials as start points of regeneration.

STIMULATION METHODS: Biological stimulation comprises different levels of possibilities. Proteins such as growth factors can be used to e.g. stimulate vascularization. Delivery of these proteins can be performed using different drug delivery systems. For example, vascular endothelial growth factor (VEGF) can be delivered using fibrin. The release kinetics results in expression of the inducible VEGF-R2 with subsequent better perfusion of the wound area [1]. Instead of proteins, genetic information can be used. Delivery methods transfer the information to target cells in the tissue to be healed. Cell intrinsic pathways lead then to local production of proteins with subsequent stimulation of healing processes. This genetic information can be in form of plasmids or messenger RNA transferred via viral or non-viral means [2].

Furthermore, physical stimulation of the wound area can accelerate regeneration. Possibilities are shock wave, light, mechanical stimulation, magnetic waves etc. LED light of different wave lengths shows distinct effects in fibroblasts or keratinocytes. Blue light induces proliferation and migration of those cells. This can be quantified and studied in vitro using a scratch wound assay [3].

Another stimulation means is magnetic waves. These are different frequencies on harmonic Schumann waves. Exposing anterior cruciate ligaments to a certain waves, proliferation was increased and expression of ligament specific genes was induced [4]. Similar results could be obtained with another wave programme in adipose-derived mesenschymal stem cells. These cells could be stimulated to osteogenesis. The magnetic wave stimulation results in activation of the MAPK and JNK pathways.

CONCLUSION: Impaired wound healing is a burden for the patient. Tissue regeneration can be stimulated using different means. The optimal protocols still need to be developed. Combination therapies or subsequent performed stimulations may also be of benefit. Moreover, the exact mechanisms of action are partially clarified but need further investigation.

ACKNOWLEDGEMENTS: Part of this work was carried out during my time as co-director at the Ludwig Boltzmann Institute for Experimental and Clinical Traumatology in Vienna.

GMP and cell therapies for clinical application

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INTRODUCTION: Production of cell therapies has gone from being performed in the ordinary research lab through the cell and tissue law [1,2] to reaching a state were producing cell therapies for clinical applications require compliance with the regulation for ATMP, advanced therapy medicinal product [3], in several cases. The extent of cell manipulation will determine the applicable legal framework.

METHODS: Compliance with GMP, Good Manufacturing Practice, is a qualification required for the production of cells used for as advanced therapy medicinal products in human application. CGMP are the Current Good Manufacturing Practices followed by the pharmaceutical and biotech firms to ensure that the products produced meet specific requirements for identity, strength, quality, and purity. These guidelines provide minimum requirements that a pharmaceutical or a food product manufacturer must meet to assure that the products are of high quality and do not pose any risk to the consumer or the public.

GMP sets the standard for a wide range of topics: Manufacturing facilities, prevention of cross contamination, defined and controlled manufacturing process, validation of critical processes, trained operators, standard operation procedures *etcetera*. A qualified person is a requirement for releasing the product for use in humans.

RESULTS: Setting up a production of an AMTP is a journey that suitably starts with a quality management system as the foundation for the GMP requirements and then by performing a GAP analysis of all the requirements by using a risk analysis to find the relevant level of production in accordance to the medicinal relevance on risk management. The GAP analysis reveals the difference between the present situation and the required situation.

DISCUSSION & CONCLUSIONS: To act in conformity to the legal framework on advanced therapy medicinal products is a challenge for every producer and even more for state medicine that usually does not produce medicinal products and therefore does not contain that kind of infrastructure. To succeed a close dialogue with the competent medicinal authority is crucial.

Autologous chondrocyte transplantation from bench to clinic

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INTRODUCTION: Autologous chondrocyte transplantation was introduced as a clinical treatment for cartilage injuries over 20 years ago [l]. The treatment was addressing a clinical need in patients and has since been spread worldwide with over 35 000 patients treated.

METHODS: The symposium presentation will deal with the concept of autologous chondrocyte transplantation (ACT), the regulatory surrounding in the 1990s and the long term clinical outcome and development of the treatment over 2 decades.

RESULTS: The ACT procedure was initially an investigator driven study and after the proof of concept paper published in 1994 [l], the methodology was adopted by other researchers as well as industry. During the first decade several startup companies emerged and the methodology was improved with introduction of new biomaterials for cell seeding and implantation and arthroscopic procedures was introduced in 2000s. The cell culture methodology was only undergoing minor changes over the years but several other cell source was published as alternative to autologous hyaline chondrocytes e.g. mesenchymal stem cells, fat stromal stem cells, umbilical cord stem cells, nasal septal cells to mention a few. The indication for treatment widened over time and in addition to cartilage injuries in the knee, injuries in the hip, ankle and shoulder were treated. Several randomized clinical trials have also demonstrated superiority over conventional treatments.

DISCUSSION & CONCLUSIONS: The ACT treatment has helped thousands of patients to a better life and in a majority of the cases restored knee function has been long lasting [2]. The science in cartilage regeneration has resulted in thousands of publications and the knowledge of cartilage regeneration has increased. However, the methodology is expensive and the regulatory environment complicated and has hampered the introduction of improved therapies and we now see a gradual return to a non-cellular regenerative treatment in patients.

Autologous cultured urothelial cells in the clinic

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INTRODUCTION: In Pediatric urogenital reconstructive surgery, lack of tissue can sometimes hamper the surgical repair of severe congenital malformations. We therefore used cultured autologous urothelial cells for the primary repair of the neourethra on six pediatric patients with severe hypospadias during 2000-2002 [1]. Patients were between 1-3 years of age at the time of the repair.

Final evaluation of surgery performed at young age is challenged by the time needed for follow-up and assessment until the patient is fully grown. The aim of this presentation is to present our experience with implementation of tissue engineering techniques to the clinic in a young patient group, including patient selection, ethical considerations, quality controls and follow-ups until early adulthood [2,3].

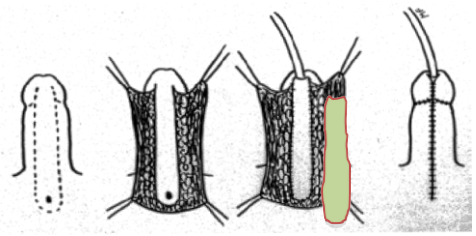
METHODS: Urothelial cells were harvested from bladder washings with saline for cell expansion *in vitro*. The procedure was minimally invasive and in these cases performed at the time of straightening the penile body as part of a 2-staged procedure in severe hypospadias with ventral curvature.

After cell expansion and freezing, cells were thawed and cultured on acellular donor dermis at the time for 2nd-stage repair and creation of the neourethra 6 months later.

Patients were followed through childhood with control of urinary flows, residual urine, cystoscopies, biopsies, artificial erection and clinical assessment of cosmetic appearance. After puberty history taking also included questions regarding sexuality, erectile function and patient satisfaction.

RESULTS: All patients have been assessed at the outpatient clinic until after puberty (16-19 years of age). Some patients are still pending urethroscopy and biopsy of the neourethra.

At long-term follow-up, no signs of urinary flow obstruction, residual urine or suspicion of megaurethra was found. One patient was planned for correction of curvature noted after puberty. Patient satisfaction and erectile function was good.



A. B. C. D.

Fig. 1: Cartoon of surgical procedure. A. Ventral penis with dotted lines for planned incision. Note urinary opening at penile base. B. Subcutaneous dissection around skin strip (that will be part of the neo-urethra). C. Cultured autologous transplant on top of the skin strip. Catheter placed in between. D. Ventral penis and midline skin sutures.

DISCUSSION & CONCLUSIONS: Autologous *in vitro* expanded urothelial cells can be used for repair of the male urethra. Patient material will be compared with other severe hypospadias patients before including more patients.

Long-term follow-up studies are crucial for evaluation of benefits with the method and to identify late adverse side effects. Standardized quality controls of *in vitro* expanded cells before auto transplantation need to be defined.

ACKNOWLEDGEMENTS: Researchers Prof. A. Nordenskjöld, Prof. G. Kratz and Assoc. prof. J. Svensson. Studies were generously supported by Swedish Society of Medical Research (SSMF), Swedish Society of Medicine, HRH Crown Princess Lovisa's Memorial foundation, Promobilia, Samariten Foundation, Foundation for Pediatric Health Care and Freemason's fund for Children Health.

Role of Mechanics in Chondrogenesis

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INTRODUCTION: Research in the use of mesenchymal stem cells (MSCs) to enhance orthopaedic repair has dramatically increased over the last 20 years. The unique properties of MSCs and their natural presence within the bone marrow make them an attractive source of cells for novel therapeutics. When considering the natural repair environment, it is clear that the microenvironment the cells experience plays a major role in the repair response. Within the musculoskeletal system, one of the major drivers of repair is the mechanical load applied to the cells within the defect.

When developing new therapies in vitro, static culture is the most commonly used method. However, it is clear that due to the critical role mechanics plays in vivo, a more physiological loading regime in vitro would be most appropriate and this can be achieved by the use of bioreactors. Using a multiaxial load bioreactor system1, we have been investigating the effect of mechanical stimulation on human stem cell differentiation. Performing studies in the absence of growth factors, specifically Transforming growth factor β (TGFβ), allows the direct effect of the mechanical strain applied to be elucidated. Our bioreactor system allows for the application of shear, compression or a combination of both stimuli to establish the phenotypic changes induced within MSCs. In particular, the effect of the various mechanical stimuli on chondrogenic differentiation will be discussed and compared to responses seen in chondrocytes.

As a model system, human bone marrow derived MSCs are embedded in a fibrin gel, which is then retained in a macroporous biodegradable polyurethane (PU) scaffold. This system provides a naturally occurring support matrix (fibrin), while allowing for cyclical load to be applied due to the resilience of the PU scaffold. Neither compression alone, nor shear alone can induce a change in MSC phenotype within this system. However, we have demonstrated that a combination of compression and shear is able to induce chondrogenic differentiation and this is due to increased endogenous expression of TGFβ from the loaded cells2, 3. Finite Element modelling of the bioreactor system demonstrated that the degree of principle component strain was the main driving force in this system4.

Using this multiaxial load bioreactor system we are able to investigate novel treatments and therapies in vitro, under physiologically relevant kinematic load. In addition, potential rehabilitation protocols to be used after cell therapy in cartilage repair can also be investigated.

ACKNOWLEDGEMENTS: This study was funded by the AO Foundation and the Swiss National Science Foundation (Grants: SNF 320000-116846/1 and SNF 31003a_146375/1). I would like to M. Glarner and Dr. David Eglin (Musculoskeletal Regeneration Program, AO Foundation Research Institute, Davos) for producing the polyurethane scaffolds and Baxter Biosurgery, Vienna, for providing the fibrin components.