# The use of large animal models in the pipeline from bench to clinic

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The development of tissue engineering solutions for the repair and regeneration of tissues and organs is a fast moving area of scientific research. However, whilst the development stage of products is relatively straightforward, evaluation of the products produced is not. In this presentation I will discuss the use of large animal models in the development of successful tissue engineering solutions to musculo-skeletal problems.

The bio-compatibility of tissue engineering products is usually evaluated by simple *in vitro* cytotoxicity experiments. These experiments ensure that the materials are capable of supporting cell growth and that at least some of the basic functions of the cell, assessed by the production of key proteins and genes, are preserved. Small animal experiments are then often used to test *in vivo* safety.

The appropriate use of large animal models in musculo-skeletal product development is required for a number of reasons. These include the belief that the rigorous testing of a product in a biologically relevant environment is highly desirable prior to initiating clinical trials. In addition many tissue engineering products are classified as 'Advanced Therapy Medicinal Products' by the European Medicines Agency, and, as such, their licensing includes mandatory regulatory steps that include large animal model trials.

The choice of which large animal to use depends on a number of factors, including the country in which the study is performed, the finances available and the expertise of the group performing the work. For evaluation of chondral and osteochondral tissue repair, commonly used species worldwide include goats, sheep and horses.

Key to the use of large animal models for evaluation of tissue engineering constructs is an appropriate set of agreed clinical output data. This data should include functional evaluation of tissue repair, for example gait analysis and measurements of stiffness of repaired tissue. Once tissue is harvested, industry standard scoring systems, such as the International Cartilage Repair Society Gross Morphology score and the Modified O'Driscoll score should be used to quantitatively assess the quality of the repair tissue. Further assessments can be made by performing immunohistochemical analysis on histological sections. The identification of types I and II collagen is commonly investigated in chondral and osteochondral healing.

The use of an appropriate package of assessment tools for the evaluation of tissue engineering constructs allows the researcher to make an informed judgement on the suitability of a tissue engineering construct in the joint niche. This judgement will inform whether or not the construct proceeds to clinical trials.

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# Influencing the skeletal stem cell niche by targeted liposomal wnt delivery

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**INTRODUCTION:** Bone fracture treatment costs the UK billions of pounds each year. New regenerative medicine therapies for bone tissue are urgently required. Skeletal stem cells (SSCs) of the bone marrow niche are an attractive target for such new technologies, therefore we hypothesise that delivery of growth factors specifically to SSCs may enhance bone regeneration. The Wnt signalling pathway has been shown to regulate SSCs[1], hence we aim to deliver Wnt proteins, entrapped in liposomes, targeted to SSC marker STRO-1[2]. Our previous unpublished data showed that short-term induction of Wnt signalling increased the population of stem cells and promoted generation of early and late osteoprogenitors. However, long-term Wnt induction had the opposite effect, indicating that Wnt signalling has strikingly different effects on bone cells depending on their osteogenic commitment[1]. We think Wnt entrapment in liposomes is a preferable delivery method (Fig. 1) for such a hydrophobic growth factor, and will ensure the specificity and therefore safety and efficacy of our approach.

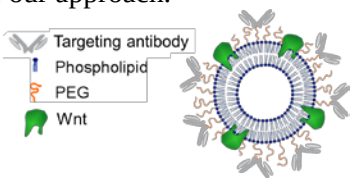


*Fig. 1 Proposed therapeutic liposome design.*

**METHODS:** We characterised the fabricated liposomes' stability and size *via* dynamic light scattering (DLS), nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM). We analysed the lipid content of our preparations by gas chromatography (GC). We also measured by FACS the uptake of these nanoparticles by BMMNCs and the biological activity of liposome-incorporated Wnt3a protein on a reporter cell line. All statistical analysis was conducted using GraphPad Prism software with significance set at $p<0.05$.

**RESULTS:** Nanoparticle preparations were 100±20nm in size and >80% of BMMNCs were able to take up these particles within 1h of incubation (Fig. 2).

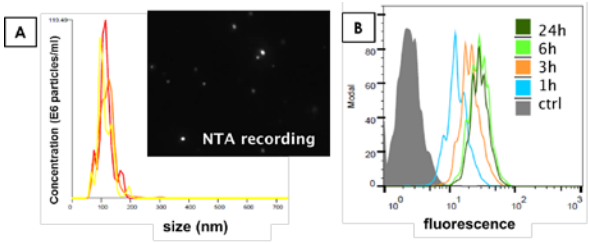


*Fig. 2 Liposomes were 100±20nm in size as measured by NTA (A) and were taken up rapidly by BMMNCs as assessed by FACS (B).*

The biological activity of Wnt3a incorporated in liposomes was dependent on their lipid composition. Liposome preparations consisting only of the DMPC lipid were active and induced a response similar to neat Wnt3a protein. However PEGylation of the DMPC lipid preparations abolished the activity of Wnt protein. This was not the case when the DOPC lipid was used, as both unPEGylated and PEGylated preparations were active.

**DISCUSSION & CONCLUSIONS:** Based on our previous unpublished data on Wnt effects on SSCs and that liposomal Wnt delivery is the method of choice for highly hydrophobic proteins[3], selective targeting of Wnt proteins in liposomes to specific cell populations at a specific differentiation stage might be a safe and efficacious therapeutic approach for promoting bone regeneration. Future experiments must include better characterisation of the chemistry of Wnt protein incorporation into the liposomal bilayer with regards to suitability for the novel targeted delivery approach within the bone marrow stem cell niche.

## REFERENCES:

1. J de Boer et al (2004), *Bone* **34(5)**: 818-826.
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# Dynamic surfaces for modulating stem cell fate

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**INTRODUCTION:** Enhanced control over stem cell fate would be advantageous for supply of stem cells and mature cell types. Some chemistries [1] and topographies have been harnessed to retain stem cell character or stimulate differentiation, but triggering an on demand 'switch' from retention of stemness to maturation would be valuable to promote expansion prior to differentiation.

**METHODS:** Human mesenchymal stem cells (hMSCs) were cultured on peptide modified glass coverslips, presenting adhesive –RGD sequences adjacent to an –FMOC or –PEG blocking group attached to an elastase-cleavable linker. Surfaces could be enzymicatically 'switched' to present the concealed –RGD. Glass, –RGE, -RGD, and –PEG presenting surfaces were controls. Focal adhesions (FAs) were quantified to evaluate cell adhesion, and cells were examined for stem and differentiation markers (Stro-1, adipo- and osteo markers). For metabolomics, solvent-extracted metabolites were analysed using an Orbitrap Exactive mass spectrometer. Ingenuity Pathways Analysis was used to evaluate the functional implications of the differentially abundant metabolites.

**RESULTS:** The stem cell marker Stro1+ was retained more effectively on unswitched surfaces relative to switched surfaces after 2 and 4 weeks of culture. The cells were still multipotent and could be induced to differentiate into other lineages on the unswitched surfaces in the presence of induction medium. On switched surfaces, where greater cytoskeletal tension was promoted and –RGD was exposed, FAs were modulated, and osteogenesis was induced. The metabolic response was distinct, with MSCs on switched surfaces showing a more metabolically active, differentiating phenotype. Unsaturated carbon-carbon double bonds are associated with stem cell plasticity [2]. Cells were depleted of unsaturated lipids on switched substrates, and these were enriched on unswitched surfaces (Fig. 1), consistent with stem cell retention.

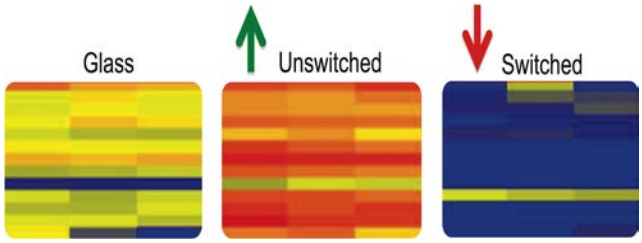


*Fig. 1: Abundance levels of various unsaturated lipid metabolites, generally enriched (orange) on unswitched (-RGD concealed) surfaces and decreased (blue) on switched (-RGD exposed) substrates, relative to controls.*

**DISCUSSION & CONCLUSIONS:** The stem cell phenotype was retained more effectively on unswitched surfaces relative to switched surfaces, and cells retained their multipotency after culture on unswitched substrates. The switched surfaces could modulate FAs and promoted osteogenesis. The system offers potential for the retention of stem cell phenotype and controlled differentiation.

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# Synthesis and *in vitro* biocompatibility of multi-substituted hydroxyapatite for bone tissue engineering applications

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## INTRODUCTION:

Synthetic hydroxyapatite (HA) possesses good biocompatibility, bioactivity and osteoconductivity and closely resembles the mineralised phase of human bone and teeth. For many years the clinical researchers worldwide have been aware that the mineral phase of bone contains a number of substituents (*e.g.* $CO_3$, Si, Zn, Sr, Na, and Mg) and it is not solely calcium phosphate. Carbonate is the major substitute among the trace elements which are present, about 2-8wt% of which varies depending on age. The presence of approximately 2wt% Si is known to be essential in bone formation and calcification[1].

The aim of this study is to synthesise multi-substituted HA with the incorporation of carbonate and silicon ions simultaneously, and to assess the *in vitro* cytotoxicity of the as-synthesised powders in response to human Bone Marrow derived Mesenchymal Stem Cells (hMSCs). It is intended that this novel formulation will improve both osteogenic behaviour and mechanical properties of the resulting scaffolds.

## METHODS:

Multi-substituted HA (SiCHA) powders were synthesised at room temperature using $Ca(NO_3)_2.4H_2O$, $(NH_4)_2HPO_4$, $NH_4HCO_3$ and $Si(CH_3COO)_4$ (Sigma-Aldrich, Gillingham, UK) by nanoemulsion method[2]. The amounts of carbonate ($x$) and silicon ($y$) substituted into the HA structure were calculated based on the stoichiometry empirical formula as shown below:

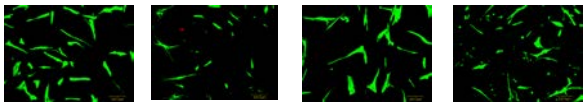
SiCHA: $Ca_{10-x/2}(PO_4)_{6-x-y}(CO_3)_x(SiO_4)_{y\,(OH)2-y}$

The physico-chemical properties of the as-synthesised powders were investigated through XRD, FTIR, CHN and ICP. The biocompatibility of the as-synthesised powders was then tested *in vitro* cell-culture with hMSCs (P2) via direct contact based on ISO10993-5.

## RESULTS:

Both the carbonate and silicon ions successfully substituted into the HA lattice. From the physico-chemical characterisations, 2.0CHA, 0.5SiHA, 2.0:0.3 and 2.0:0.5SiCHA were chosen as the optimum powders.

The powders were non-toxic to hMSC cells. Powders with carbonate encouraged faster cell proliferation and produced relatively higher amounts of total protein as they developed in comparison to the carbonate-free powders.



**2.0CHA** **0.5SiHA** **2.0:0.3 SiCHA** **2.0:0.5 SiCHA**

Fig.1. Live/dead images of the optimum powders after 14 days in culture

## DISCUSSION & CONCLUSIONS:

The multi-substituted HA (SiCHA) powders with different carbonate to silicate molar ratio were successfully synthesised using a nanoemulsion method. No secondary phases were detected to contaminate the multi-substituted HA. *In vitro* cytotoxicity assessment shows that the majority of cells remained viable after 14 days in culture. The results also showed that the presence of carbonate ions, or carbonate and silicon ions in combination, stimulated faster cell proliferation and greater volumes of protein production.

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## ACKNOWLEDGMENTS:

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# Photochemical functionalisation and direct-write patterning of diamond-like-carbon for neural interfaces

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## INTRODUCTION:

Electrodes for direct interface with the brain have the potential to greatly improve prostheses to aid victims of spinal injury or similar disablement. However, the development of such electrodes relies on careful engineering of the biological-electronic interface with control of scarring, cell phenotype and tissue organisation. Diamond-like-carbon (DLC) may be an ideal material for coating electrodes as it has flexible electrical properties, it is extremely stable in vivo and is already used as a coating for orthopaedic implants[1]. Chemical modification is essential, however, to control and promote adhesion, differentiation and organisation of neurons and glia.

## METHODS:

DLC was coated on silicon by chemical vapour deposition. DLC was rendered either non-adhesive by surface initiated atom transfer radical polymerisation of PEG brushes or cell-adhesive by photochemical polymerisation of charged monomers such as acrylic acid or amine functional acrylates. Surfaces were also patterned by direct-write laser induced photopolymerisation using a microchip Nd-YAG nanosecond pulsed laser in order to provide spatial control of cell adhesion. Rat neural stem cells were seeded to the surfaces and allowed to spontaneously differentiate.

## RESULTS:

Modified DLC surfaces were characterised by XPS and contact angle. Hydrogenated DLC and PEG-functionalised DLC were found to be non-adhesive to cells. By modification with surface initiated charged polymers, hydrophilicity and cell adhesion were promoted. Neural progenitors adhered to the modified surfaces and a multipotent phenotype was maintained until withdrawal of growth factors. Spontaneous differentiation occurred on the modified surfaces with both neuronal and glial differentiation observed. Patterned tracks were prepared by direct-write polymerisation of charged monomers and were characterised by AFM.

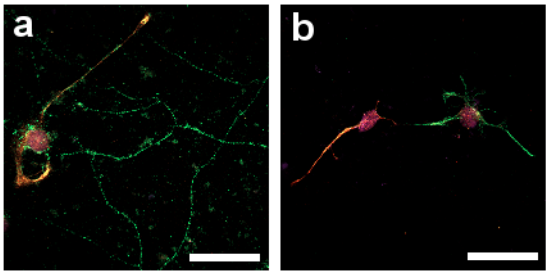


*Fig. 1. Immunofluorescence micrographs of neural progenitors after spontaneous differentiation on polyacrylic acid coated surfaces. Cells were stained for nestin (red), βIII-tubulin (green, c) and GFAP (green, d). Scale = 80 μm.*

## DISCUSSION & CONCLUSIONS:

The surface chemistry of DLC was modified and patterned by facile photochemical methods. Such approaches allow for the tuning of biological properties and the controlled design of the electrode-brain interface. Further studies on the differentiation of neural stem cells and the development of controlled patterning protocols will be carried out.

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## ACKNOWLEDGMENTS:

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# Real time spatial monitoring system for 3d culture models

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**INTRODUCTION:** Current methods available for determining cell behaviour within 3D constructs use end point assays. Such assays destroy samples during the processing stages bringing many problems and limitations. One solution is to develop low cost techniques for non-invasive measurement of samples, providing a real time (RT) read out of 3D tissue constructs and optical approaches are ideal candidates. The Lein CTS2 is an optical measuring device capable of detecting changes in refractive index to denote surface positions in the z plane of delicate, hard to handle hydrogel constructs. Such thickness readings provide quantitative data relating to spatial changes in individual construct layers or the full construct thickness in RT. The aim of the presented work involved the characterisation and evaluation of this approach to test its potential use in compressed collagen models [1].

**METHODS:** Compressed collagen gels with starting collagen volumes 0.5, 1, 1.5 and 2ml were made and used to compare the CTS2 optical thickness readings with the thickness readings observed by traditional histology methods. Serially compressed 0.5ml triple layers and single layer 0.5ml gels were assembled to measure cell free re-swelling after 24h. 1ml collagen gels were compressed with hydroxyapatite particles (HA) increasing in concentration from 0.3-1.2mg total to assess sample thickness and HA concentration. Finally a complex multi-layer construct was analysed. The acellular tri-layer was assembled as a simplified bone fracture model using HA as the test material inserted into the middle collagen layer.

**RESULTS:** Optical RT thickness measurements were compared with those using traditional histology on the same collagen constructs. In all cases unfixed optical thicknesses were greater indicating dehydration shrinkage. 24h Re-swelling tests indicated increased sample thickness. Addition of HA particles to constructs produced a clear increase in thickness by histology. However optical measurements did not correspond to the histology indicating a limitation to this approach. Optical measurements of the tri-layer model showed good predictability of construct thickness (fig 1) and a stable case for how to measure remodelling.

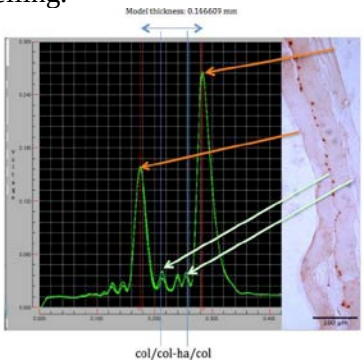


*Fig.1 CTS2 optical trace for tri-layer bone fracture callus model alongside histology image (middle layer with HA particle presence stained with Alizarin Red)*

**DISCUSSION & CONCLUSIONS:** This study has tested the potential of RT optical monitoring using the CTS2 to assess 3D native collagen tissue models. The addition of HA particles indicated a loss in signal due to light scatter leading to loss of thickness accuracy compared to histology findings. However the CTS2 is capable of measuring sample thickness changes, in this case re-swelling; further to this we can provide a non-invasive quantitative method used to analyse and map out multi-layered 3D tissue models.

**REFERENCES:** Alekseeva, T.et al (2011), *ICCE* **30**: 5-6.

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# Investigation of potential osteogenesis influence of carbon based nanomaterials

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**INTRODUCTION:** The unique electronic, mechanical and chemical properties of carbon-based nanomaterials hold great potential in the field of regenerative medicine. Whilst carbon nanotubes (CNTs) have been synthesized using several novel techniques, the methods by which graphene is fabricated have seen dramatic progress in recent years. Whether carbon-based nanomaterials can induce or enhance bone formation and the possible associated mechanisms for such enhancement have not yet been fully investigated. The aims of this study are to assess the potential osteogenic influences of CNTs and two types of graphene oxide on a bone cell line culture and to investigate the possible underlying mechanism.

**METHODS:** CNTs were produced using chemical vapour deposition (CVD) with methane at 700°C on a bio-silica base using $Fe(NO_3)_3.9H_2O$ as the catalyzer. Both types of graphene oxides, A and B respectively, have also been used. The human bone cell line, MG63, was used for all experiments. 20,000 cells per sample were seeded with α-MEM media supplement with only antibiotic and FCS (10%) for 10 days. In one experimental set-up, CNTs and graphene particles were mixed with poly(lactic acid) (PLA) solution in 1% concentration to form a composite film. 0.5x 1x0.0003 cm film sections were inserted into silicone frames for cell culturing. In the second experimental set-up, cells were cultured in 24-well suspension culture plates with above media incubated with the carbon particles (conditioned media, CM). The media was changed every 2 days. The cell morphology was monitored by optical microscopy every other day. After 10 days in culture, the cells were fixed by 10% formalin. The fixed samples were then stained by Alizarin Red (ARS) and von Kossa.

**RESULTS:** Cell aggregates or nodules formed after 2 days in culture in all cases (Fig 1). ARS and von Kossa staining showed stronger minerals deposition (Fıg 2) on both the carbon/PLA composite film samples and the samples cultured using CM when compared to the samples cultured on PLA film or using non-conditioned culture media.

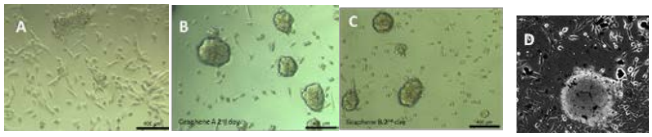


*Fig.1 Microscopic images of live cells; A: in non-conditional culture media; B: in grapheme A CM; C: in CNT CM; D: on PLA-CNT composite.*

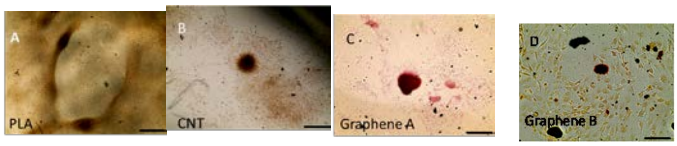


*Fig.2 ARS staining images for MG63 grown on PLA film; B: PLA-CNT; C: PLA-grapheme A; D: PLA-graphene B. Scale bar is 500 µm.*

**DISCUSSION & CONCLUSIONS:** The formation of bone nodules in the presence of carbon particles without osteogenic supplemented media within a short culture period indicates that carbon nanomaterials have the capacity to induce bone formation. The carbon particles are suspected to have become the centers which induce cellular aggregation and promote bone nodule formation, do not promote proliferation.

**ACKNOWLEDGMENTS:**
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# Hydrogels from demineralized and decellularized bone extracellular matrix for bone regeneration

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**INTRODUCTION:** Treatment of bone defects often involves the use of bone graft substitutes, such as demineralized bone matrix (DBM). Current delivery of DBM particles requires incorporation within a carrier liquid. However, differences in osteogenic activity and inflammation have been reported with various carriers. The objectives of this study were to produce hydrogel forms of DBM and ECM from bovine bone, denoted bDBM and bECM, and to determine their suitability for use in bone regeneration applications.

**METHODS:** Bovine femurs were treated with in-house developed protocols[1]; resultant materials were pepsin digested and solubilized. Gelation was induced by salt and pH neutralization followed by warming to 37°C. In vitro cell proliferation of mouse primary calvarial cells (mPCs) on the surface of hydrogels was assessed using the CellTiter 96® MTS colorimetric assay and tested for normality and statistically compared using a Tukey-Kramer multiple comparisons test. A 2% gel solution of low viscosity alginate was utilised to prepare alginate and bECM/alginate hydrogels. These were subcutaneously implanted within MF1 nu/nu male mice. After 28 days, the implants were harvested, fixed in 4% PFA, sectioned and stained with Alcian blue/Sirius red.

**RESULTS:** Enhanced proliferation of mouse primary calvarial cells was achieved on ECM hydrogels, compared to collagen type I and DBM hydrogels (Figure 1A). Alcian blue staining indicated the presence of cartilaginous material in both alginate and alginate/bECM subcutaneous implants (Figure 1B and 1C) however collagen production (labelled by Sirius red) only occurred in the alginate/bECM implant.

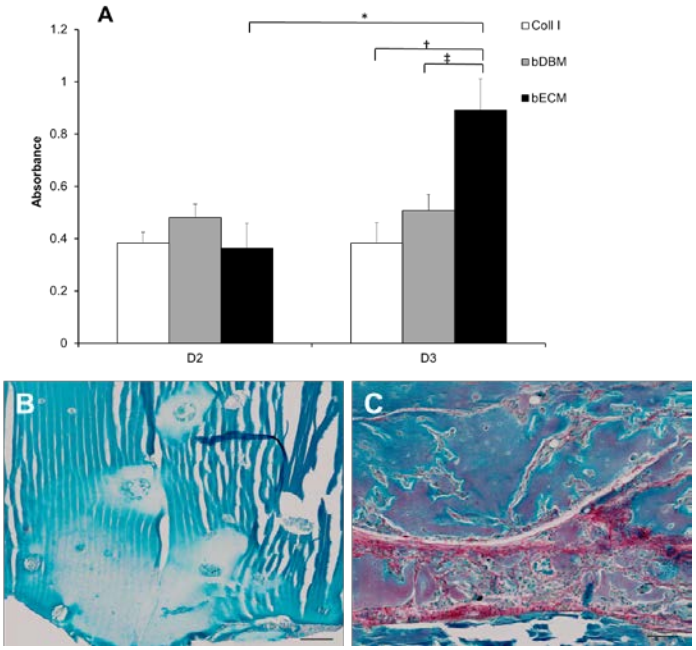


*Fig. 1 Proliferation of mPCs on Collagen 1, bDBM and bECM hydrogels. * denotes significance between D2 and D3 for bECM, † denotes significance between bECM and Coll I, ‡ denotes significance between bECM and bDBM p<0.001, (A); Alcian blue/Sirius red staining of subcutaneous implant of alginate hydrogel (B) and alginate/bECM hydrogel (C).*

**DISCUSSION & CONCLUSIONS:** The bDBM and bECM hydrogels possess distinct structural, mechanical and biological properties, including osteogenic functionality. Limited tissue invasion of the alginate hydrogel occurred in the subcutaneous implant whereas the alginate/bECM hydrogel exhibited extensive host tissue invasion and potential for use in bone regeneration.

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# Clay gels localise and enhance BMP2 induction of osteogenesis

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## INTRODUCTION:

Bone Morphogenic Protein (BMP) is a licensed therapy to enhance bone repair, however delivered BMP rapidly diffuses and degrades requiring supra-physiological doses for efficacy. The synthetic clay Laponite, is non-toxic with a 25 year history of use in cosmetics. We have shown its potential to form hydrogels able to localise biological molecules to induce regenerative responses[1, 2]. Here we demonstrate *in vitro* and *in vivo* the utility of this approach to localise BMP2 to enhance bone graft osteo-induction.

## METHODS:

Clay gels were formed using hydrous suspensions of the synthetic clay Laponite (Rockwood ltd.). The effect of clay gels on BMP-2 localisation and activity was explored *in vitro* using C2C12 cells and staining for alkaline phosphatase activity (APA). Clay gels were spotted on tissue culture plastic (TCP) and BMP-2 was added to the media before or at the time of cell seeding. After 72 hours culture, staining and image analysis for APA was performed. Effect of Laponite, BMP-2 and serum concentration, seeding density and BMP-2 incubation time was assessed. C2C12 BMP-2 response on clay gel coated decellularised bone graft (DBG) was also assessed. *In vivo*, a Laponite-BMP-2 mix perfused into DBG was subcutaneously implanted in mice. New bone formation versus controls was assessed at 28 days using micro-computed tomography (µCT).

## RESULTS:

Induction of APA was significantly ($p<0.001$) enhanced on clay gel surfaces over tissue culture plastic (fig. 1a) in a BMP-2 dose-dependent manner. Enhanced response was localised to clay gels, independent of local cell density and attenuated by increased serum concentration. Enhanced BMP2 induction of

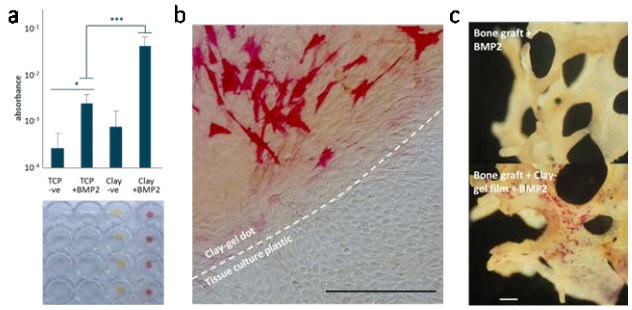


*Figure 1. Laponite gels spotted upon tissue culture plastic localised and enhanced APA in C2C12 cells by BMP2 (a-b). The same approach enhanced BMP2 response in cells seeded upon clay-coated clinical grade bone graft (c). Scale=250 µm.*

APA was also observed in C2C12 cells seeded onto trabecular bone graft pre-coated with a Laponite gel (fig. 1b). Pre- and post- implantation µCT analysis of bone graft + BMP2 with or without clay gels demonstrated a significant increase in bone volume ($p<0.03$) bone volume per tissue volume, ($p<0.02$), and trabecular number ($p<0.03$) in graft material treated with BMP2 + Laponite gels. No significant increase in bone was observed on graft material treated with BMP-2 or Laponite alone.

## DISCUSSION & CONCLUSIONS:

These studies provide *in vitro* and *in vivo* evidence for the utility of Laponite gels to enhance induction of osteogenic responses via the localisation of BMP2.

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# Adapting aligned, stabilised 3d tissues for large-scale neurobiological research

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## INTRODUCTION:

Recreating the 3D environment of the CNS using hydrogel matrices allows neurons and glial cells in vitro to behave similarly to their counterparts in vivo, providing a relevant tool for neurobiological studies[1]. The overall aim is to develop robust 3D CNS tissue models engineered by a process of glial cell self-alignment and subsequently stabilised. Furthermore, these models have been developed for multi-well plate format at a scale suitable for high throughput screening. CNS tissue equivalents can be used to assess numerous aspects of the CNS in a reproducible, controllable and consistent manner.

## METHODS:

Characterisation studies assessed alignment and stabilisation of neurons and glia in collagen gels within a 96-well plate test rig prototype. Their potential for use in neurobiological studies involved identifying neurite growth, neuron-glial interactions and myelination following defined periods in culture. Detection and quantification analysis was conducted via immunohistochemistry and confocal microscopy.

## RESULTS:

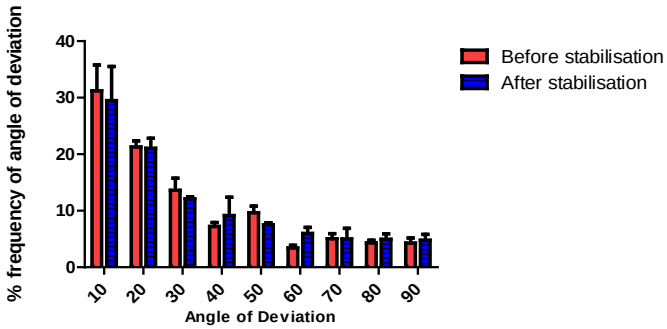


*Fig 1 Alignment of neural cells persists following stabilisation of hydrogels*

Hydrogels constructed within a 96-well plate rig displayed comparable cellular alignment to traditional methods using larger moulds[2], in mid and side regions, before and after stabilisation of constructs.

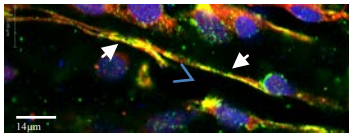


*Fig 2 Confocal micrograph showing cellular alignment and neuron-glial interaction in aligned 96-well plate rig hydrogel after 14 days. Arrowheads indicate immunoreactivity for myelin basic protein adjacent to neuronal structures (Red-β-tubulin, green-MBP, blue-Hoechst).*

Neurite growth was detected and measurable in the aligned tissue equivalents. Markers for myelination were identified in close proximity to neurites.

## DISCUSSION & CONCLUSIONS:

Results suggest that a highly organised, stable hydrogel can be created within the dimensions of a 96-well plate. The aligned nature of the cells and extracellular matrix in this anisotropic system facilitates quantitative analysis of CNS cellular features such as neurite length and the process of myelination. This simple, consistent and physiologically relevant model system, which uses a multi-well plate format can potentially be used at a scale suitable for commercial R&D.

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# Engineering of a functional tendon using load bearing suture technique and tissue engineered collagen graft: an *in vivo* study

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## INTRODUCTION:

Every year in the UK over half million people suffer from tendon related disorder. Surgical management of the ruptured tendon is challenging because treated tendon often retracts and forms a gap. Presently these gaps are filled with auto-, allo- or, synthetic grafts but they all have clinical limitations. To overcome this problem, we have fabricated and tested tendon grafts in vivo which were made up of type 1 collagen which is natural polymer and predominant protein in the tendon.

## METHODS:

Surgical procedure was carried out under ethical approval and home office licence. Thirty NZW rabbits weighing between 3-4.5 kg were used in this study. Acellular and cellular tissue engineered collagen grafts were sutured as previously described by Sawadkar et al. (1)

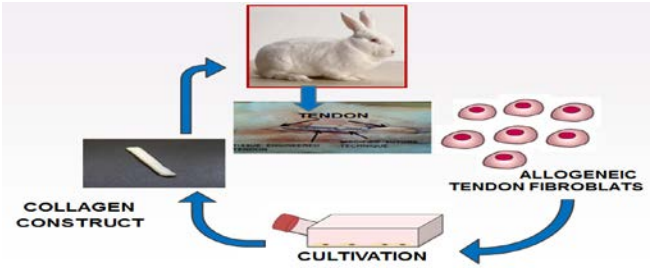


*Figure 1 Showing an in vivo experimental set up*

We have previously reported that this technique partially loads constructs and shown that this load up-regulates tendon remodelling genes *in vitro.* Mechanical tests were performed after 1,3 and 12 weeks along with histological and microscopic analysis of the grafts.

## RESULTS:

Gross observation at 3 and 12 weeks showed bridged integration of the graft without any adhesion (Fig.1) with significant increase in the mechanical properties [35.02 ± 2.1 N ($p < 0.05$) for 12 weeks and [16.26 ± 0.58 N($p < 0.05$)] for 3 weeks as compared to 1 week ( 5.19 ± 0.14 N).

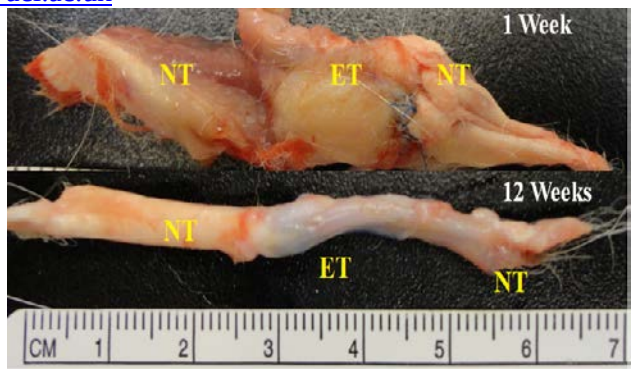


*Figure 2 Showing tissue engineered collagen constructs (ET) incorporated in native tendon (NT)*

Histological analysis showed that tendon fibroblasts from the native tendon were able to migrate to the graft with higher collagen remodelling and graft maturation at 12 weeks as compared to 3 and 1 week.

## DISCUSSION & CONCLUSIONS:

Insertion of tissue engineered collagen graft using a novel load bearing suture technique which partially loads the construct in vivo showed integration, greater mechanical strength and no adhesion formation in the time period tested

## REFERENCES:

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## ACKNOWLEDGMENTS:

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# Identification of distinct niches of functional mesenchymal stem cells in bone marrow and synovial tissues in a mouse model of joint surface injury.

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**INTRODUCTION:** We previously identified functional mesenchymal stem cells (MSCs) in the synovium of mouse knee joints[1]. Here we investigated the presence of a similar population in bone marrow. As nerve growth factor (NGF) has been reported to be a key regulator of non-inflammatory joint pain[2] we investigated its expression in an acute articular cartilage injury model.

**METHODS:** Our double analogue nucleoside labelling scheme1 and joint surface injury murine model[3] were combined in order to label stem cells in vivo. Paraffin sections of knee joints were analysed for the presence of nucleoside-positive cells and expression of MSC markers using double immunofluorescence staining. A time point analysis of NGF expression in articular cartilage was carried out by immunohistochemistry. A Transwell system was used to assess the in vitro chemotactic effect of NGF on MSCs from synovium or bone marrow.

**RESULTS:** Populations of long-term nucleoside-retaining cells were identified in both synovium and bone marrow. These cells proliferated in response to injury with marked accumulation at 4 days post injury (dpi), especially in the synovium where they contributed to hyperplasia of the membrane. Phenotypic analysis showed compatibility with MSCs (CD44+, PGFRα+, LNGFR+, CD105+, vWF-, CD45-). Interestingly, CD105 was differentially expressed by slow-cycling cells; it was detectable in IdU-positive cells in the bone marrow but not in the synovium. In the bone marrow, IdU/CldU double-positive cells co-localized with CD146 in the blood vessel wall. In contrast, perivascular IdU-positive cells in the synovial membrane were distinct from CD146-positive cells.

NGF, observed in uninjured cartilage, showed a decreased staining at 2dpi. At 8dpi the staining was comparable with the uninjured control sample; NGF was also present in the repair tissue. In vitro human BM-derived MSCs migrated in response to NGF as chemotactic agent but synovium-derived MSCs did not.

**DISCUSSION & CONCLUSIONS:** Our results confirmed previous data in the synovium1 and reported the presence of a similar population of functional MSCs in bone marrow. Differences in marker expression highlight heterogeneity in the joint microenvironment which might reflect distinct role of bone marrow and synovium-derived MSCs in joint homeostasis, remodelling and repair. In addition, the differential response to NGF confirmed that tissue of origin is an important source of variability in the phenotype and activity of MSCs[4].

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# Nano-kicking stem cells into making bone

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## INTRODUCTION:

Mesenchymal stem cells (MSCs) have large regenerative potential to replace damaged cells from several tissues along the mesodermal lineage. [1] Controlled differentiation of these cells can be brought about using various physical environmental conditions *in vitro*[2] Here, we present data demonstrating our ability to control these cells at a nano-level, and induce osteoblastogenesis in MSCs using high frequency (1000 Hz, 3000 Hz & 5000 Hz) piezo driven nano-displacements coupled with a nano-topographical (NSq-50) surface.

## METHODS:

MSC were exposed to high frequency nanoscale displacements with and without the presence of a nano-topographical (NSq-50) surface. Using negative and positive controls (osteogenic media) the cells were assessed for the onset of osteogenesis following the flow of biological information from a genomic/transcriptomic level, to the proteomic level and finally at a $Ca_3(PO_4)_2$ mineralisation level. To determine this qRT-PCR (ANOVA), immunostaining, in cell western, Raman spectroscopy and histological stains (Alizarin Red & Von Kossa) were performed at varying time points.

## DISCUSSION & CONCLUSIONS:

High frequency piezo stimulation is a novel way to induce MSC differentiation into osteoblastic cells, giving rise to a high level of efficacy through focal adhesion manipulation at the nano-scale.[2] Having interrogated an optimum condition in 2D it is envisaged that presently ongoing research in 3D gels could be used to develop a vibrational bioreactor. This may in the future have real world practical application to provide a ready source of autologous osteoblastic cells providing a potential therapeutic effect for musculoskeletal conditions.

## RESULTS:

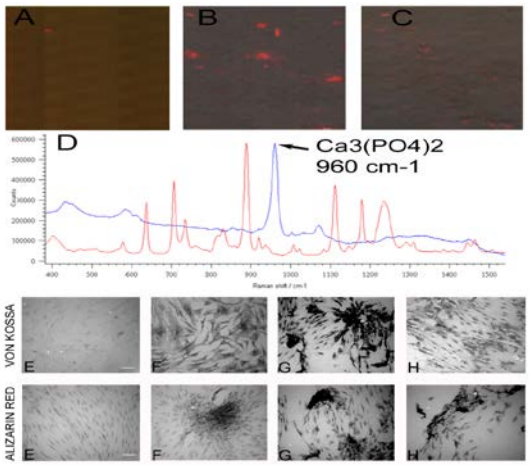


***Fig. 1 : (A-C)*** 25 $mm^2$ *Raman scans at 960* $cm^{-1}$ *, this wavenumber as shown from image* ***(D)*** *is the main peak observed in the fingerprint region of bovine cortical bone.* ***(A)*** *blank surface* ***(B)*** *1000 Hz on a flat surface* ***(C)*** *5000 Hz on NSq-50, these are assessed after 27 days of continuous piezo stimulation.* ***(E-H)*** *Von Kossa & Alizarin stains performed after 34 days of continuous piezo stimulation.****(E)*** *blank surface,* ***(F)*** *NSq-50 surface only,* ***(G)*** *1000 Hz piezo only,* ***(H)*** *5000 Hz and NSq-50 surface. Mag x 10, scale bar = 100 um.*

**A vibrational frequency of 1000 Hz, by all three techniques shows the distinctive deposition of $Ca_3PO_4$ nodules, which confirms osteoblast differentiation.**

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## ACKNOWLEDGMENTS:

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# Hypermethylation in OA - Transactivation of the *COL9A1* proximal promoter region by SOX9 in chondrocytes is regulated by DNA methylation

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## INTRODUCTION:

Methylation of genomic DNA represents a significant mechanism for regulating tissue-specific gene expression. SOX9, a transcription factor pivotal in chondrogenic differentiation[1], activates *COL9A1* gene expression[2]. However, the role of CpG methylation in *COL9A1* transactivation by SOX9 remains unknown.

## METHODS:

The ChIP-IT Express Enzymatic Kit (Active-Motif) was used to perform ChIP assays using C28/I2 chondrocytic cells co-transfected with non-methylated or methylated pCpGfree-Luc-COL9A1 vector (-846/+128bp) as well as the expression vector encoding SOX9 using Fugene HD (Promega). 48 hours after transfection, pre-cleared chromatin was stored as assay input or incubated at 4°C overnight with 5 µg of rabbit anti-SOX9 antibody (Abcam) or normal rabbit IgG (Cell Signalling). After reverse cross-linking and purification, the final DNA preparations were subjected to quantitative PCR analysis using 5µl of the eluted DNA. For real time PCR analysis, the CT of each sample was normalized to the CT of the input sample. Statistical Analysis: Results presented as mean ± S.E. of four independent experiments. Statistical significance was evaluated using Mann-Whitney U test, with $p < 0.05$ considered significant.

## RESULTS:

ChIP assays revealed that methylation treatment significantly reduced SOX9 binding to the *COL9A1* promoter (Fig. 1), specifically two binding sites close to the transcription start site (BS4 and BS5). These results show decreased SOX9-driven *COL9A1* transactivation after methylation treatment of the wild type (WT) *COL9A1* reporter construct.

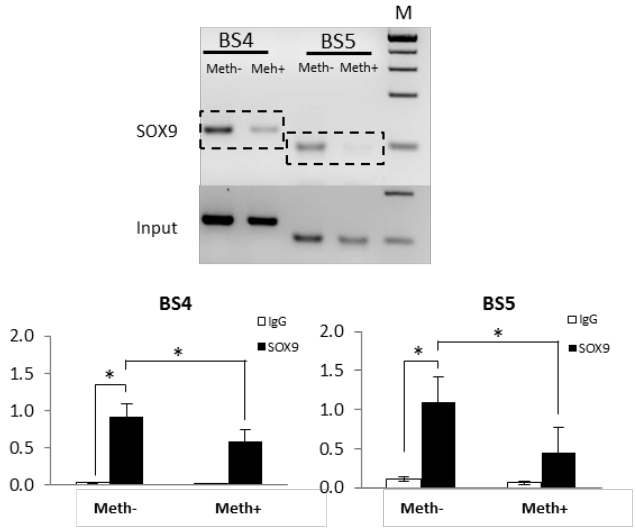


***Fig. 1.*** *ChIP assays were performed using cell lysates of C28/I2 cells transfected with non-methylated (Meth-) or methylated (Meth+) WT -846/+128-bp COL9A1 promoter constructs and the expression vector encoding SOX9. *p < 0.05*

## DISCUSSION & CONCLUSIONS:

We show for the first time that in human chondrocytes CpG methylation of the *COL9A1* proximal promoter specifically impairs SOX9-driven promoter activation by altering SOX9 binding to DNA. Furthermore, transactivation depended on the DNA methylation status of two SOX/Sry binding sites (BS4 and BS5). This is the first demonstration that hypermethylation is associated with down-regulation of *COL9A1* expression in OA indicating the pivotal role of epigenetics in decreased anabolism in OA.

## REFERENCES:

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## ACKNOWLEDGMENTS:

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# The “cryptic” extracellular matrix and its impact on stem cell differentiation

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**INTRODUCTION:**
Important signaling pathways governing cell migration and differentiation are initiated by the extracellular matrix (ECM), which constitutes the initial barrier, the actual tissue boundary for every cell. Migration and differentiation, such as the epithelial-to-mesenchymal transition (EMT), are the main factors in development and interactions between ECM proteins and cells are key players in initiating cellular changes. The proteolytic remodeling of ECM proteins results in the release of ECM fragments hidden within the tertiary structure – so called “cryptic” sites. In recent years, more and more cryptic ECM fragments could be identified and their importance in understanding development has been highlighted (1).

**METHODS:**
Expression patterns of matrix metalloproreinase-2 (MMP2) has been investigated in human and mouse embryonic stem cells (ESCs) by qPCR and protein activity assays. Laminin-111 has been processed with MMP2 and fragments were studied by mass spectrometry and peptide fingerprinting. Impact of the fragment on the differentiation of human and mouse ESCs has been assessed by qPCR and protein expression.

**RESULTS:**
We could identify a biologically active laminin-111 fragment that was exposed through cleavage by MMP2 from the N-terminal region of the β1-chain. This fragment is able to interact with α3β1-integrin/CD147 and to decrease expression of MMP2 and increase expression of E-cadherin – two key players in the EMT. We could show that a cryptic laminin fragment is directly involved in EMT signaling (2).

**DISCUSSION & CONCLUSIONS:**
Here we report a previously unidentified laminin fragment that is released by MMP2 processing. This cryptic fragment induces changes in the EMT of mouse and human ESCs and could be used in various biomedical applications, where the modulation of the EMT is desirable.

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**ACKNOWLEDGMENTS:**
**THE AUTHORS ARE SUPPORTED BY THE MEDICAL RESEARCH COUNCIL UK REGENERATIVE MEDICINE PLATFORM HUB “ACELLULAR APPROACHES FOR THERAPEUTIC DELIVERY” (MR/K026682/1), WELLCOME TRUST SENIOR INVESTIGATOR GRANT 098411/Z/12/Z. THE AUTHORS THANK THE FEDERATION OF EUROPEAN BIOCHEMICAL SOCIETIES FOR FUNDING**

# EGF and PDGF elicit Wnt-independent β-catenin signalling in mesenchymal stromal cells via integrin-linked kinase.

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## INTRODUCTION:

Regenerative medicine therapies targeting osteogenic commitment and differentiation of mesenchymal stromal cells (MSCs) to enhance bone growth and repair are urgently required. Stimulators of the Wnt signaling pathway to act as bone anabolics are receiving considerable attention. On binding to its receptor complex, Wnt acts via Disheveled to inhibit GSK3-β, allowing accumulation and nuclear translocation of active β-catenin to drive expression of Wnt-responsive genes. As GSK3-β may also be inhibited by integrin-linked kinase (ILK), which acts downstream of growth factor receptor and integrin activation, we hypothesized that a Wnt ligand-independent mechanism may also exert control over β-catenin activity in MSCs.

## METHODS:

Primary MSCs were treated with recombinant EGF and PDGF in the presence and absence of an ILK inhibitor, after which protein was harvested at 24 hours and western blotting performed to determine active β-catenin and pERK levels. In addition, primary MSCs were treated with either type I collagen or an RGD agonist to stimulate integrin activation, with and without ILK inhibition, followed by western blot analysis.

## RESULTS:

We demonstrated that both EGF and PDGF increased active β-catenin levels at 24h, mimicking Wnt ligand-induced signaling, and this effect was blocked by ILK inhibition, compared to GAPDH loading controls (Fig. 1A). Direct integrin activation by exposure to type I collagen and RGD peptides also induced stabilization of β-catenin, which again was dependent on ILK activity (Fig. 1 B and C). Furthermore, ILK inhibition prolonged expression of pERK, an early mediator of EGF/PDGF signaling (Fig. 1 A and C).

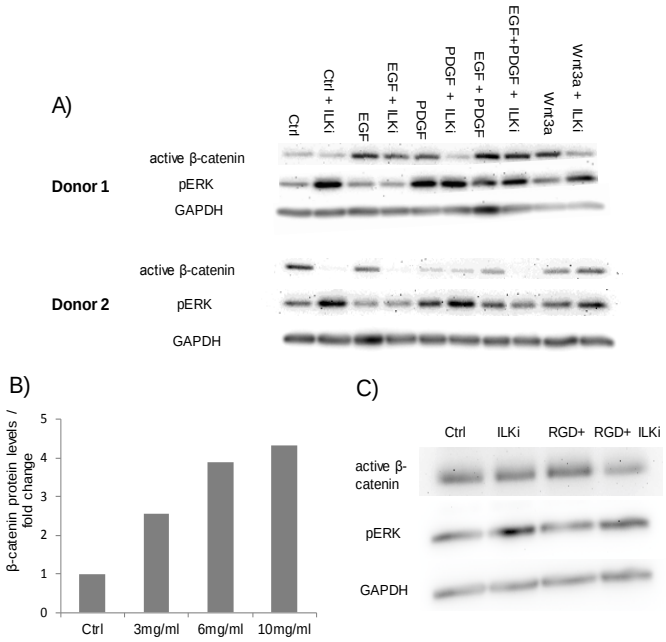


*Fig. 1. Active β-catenin and pERK levels in response to PDGF/EGF stimulation (+/- ILK inhibition, ILKi) (A), integrin activation by type I collagen exposure, densitometry analysis (B) and RGD peptide stimulation (C).*

## DISCUSSION & CONCLUSIONS:

Our evidence indicates that the β-catenin signaling pathway can be independently regulated by growth factor and integrin-mediated adhesive interactions in MSCs, without the requirement for Wnt ligand stimulation. These findings provide a greater understanding of the regulatory cues that control MSC function and reveal previously unrecognized mechanisms to maximize regenerative medicine strategies for skeletal therapeutics.

## ACKNOWLEDGMENTS:

The authors would like to thank Arthritis Research UK and BBSRC for providing financial support for this work.

# Mesenchymal stromal cells organise endothelial networks through pdgf, ilk and fgf signalling

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**INTRODUCTION:** In addition to their capacity for skeletogenic differentiation, there are suggestions that mesenchymal stem/stromal cells (MSCs) have additional roles in organising tissue vasculature through interactions with endothelial cells (ECs). However, suitable experimental models to test these unique MSC activities are lacking and the mechanisms are unclear. Here, we have developed a novel 3D in vitro co-culture spheroid model of MSCs and ECs to track endothelial restructuring and identify the signalling processes involved.

**METHODS:** 3D co-culture spheroids totalling 30,000 cells were produced using a 50:50 mixture of human MSCs and ECs. The cells were labelled using CellTracker™ green and red respectively before being placed in non-adherent U-bottomed 96 well plates in appropriate medium containing 0.25% (w/v) methyl cellulose. The 3D MSC:EC spheroids were cultured in the presence and absence of a range of specific inhibitors of different signalling pathways, ultimately focusing on platelet-derived growth factor receptor (PDGFR), integrin-linked kinase (ILK) and fibroblast growth factor (FGF). Spheroids were imaged by multiphoton and fluorescence microscopy following sectioning.

**RESULTS:** Between day 1 and 2 of culture, dramatic self-organisation was observed in MSC:EC spheroids. ECs formed inter-connected vascular like lattices surrounded by MSCs, which extended from peripheral EC assemblies to internal networks throughout the spheroid (Fig. 1A, left column) compared to the random distribution of MSC:MSC spheroids (Fig1A, right column). All inhibitors of PDGFR, ILK and FGF disrupted spheroid self-organisation by altering the peripheral distribution of ECs and causing them to form enlarged internal cell aggregates without the connected lattices observed in untreated controls (Fig.1A). This was particularly pronounced for FGF inhibition and confirmed by image analysis quantification. Exposure to PDGFR and FGF inhibitors significantly increased the spheroid volume (Fig-1B) and image analysis revealed that ILK inhibition significantly increased the percentage of MSCs at the spheroid periphery (Fig. 1C).

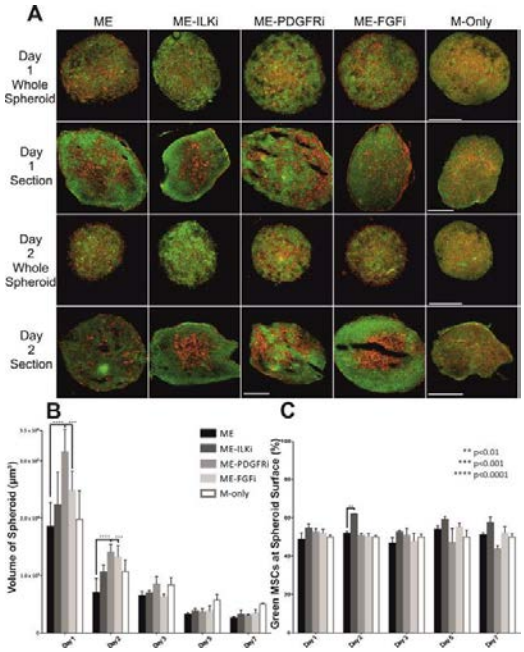


*Fig 1: MSC:EC (ME) spheroids were cultured with and without ILK, PDGFR and FGF inhibitors A – Whole spheroid images and sections show organisation at days 1 and 2 (MSC:EC). Scale bars = 200µm. B – Volume of spheroids over 7 days. C – Percentage of MSCs (green) at the spheroid periphery over 7 days.*

**DISCUSSION & CONCLUSIONS:** This study has showed that MSCs and ECs have an intrinsic capacity to self-organise when co-cultured under defined 3D conditions to form elaborate vascular-like networks in a mechanism that is dependent on PDGF, ILK and FGF-mediated signalling. Our novel 3D co-culture model represents a simplified system to decipher the mechanisms guiding MSC-dependent remodelling of host vasculature, which may be exploited to augment MSC-based tissue repair.

# Development of an acellular xenogeneic nerve graft

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## Introduction

Peripheral nerve injuries affect 1 in 1000 of the population [1]. Injury gaps greater than 1-2cm are bridged using autografts which direct regenerating axons by topographic guidance [2]. Commercially available products are not particularly suitable as they lack architecture similar to that of the native ECM of the nerve. An acellular nerve would address regeneration of axons at the cellular level, encouraging regeneration within a native guidance environment. The present aim is therefore to develop compatible, non-immunogenic, nerve grafts to restore sensory and motor function using a novel technique to decellularise porcine nerve. It will then be used as a scaffold for seeding primary Schwann cells in comparison with a graft alone for early stage evaluation.

## Materials and Methods

The sciatic, tibial, common pereoneal and sural nerves are under investigation as potential conduits. The nerves were decellularised using low concentration sodium dodecyl sulphate and nuclease enzymes. The ECM components of the decellularised nerves have been identified using H&E, Sirius red, Millers Elastin and Alcian blue staining. DAPI staining was used to identify cell nuclei.

## Results

Results show the elimination of cellular components and preservation of native nerve architecture (Figure 1). The ECM components collagen, elastin and glycosaminoglycans remained intact throughout the procedure. Future work includes optimisation of the decellularisation protocol, additional scaffold charaterisation and repopulation with Schwann cells.

## Discussion and Conclusions

Porcine peripheral nerves have been successfully decellularised using a proprietary technique. The acellular model can be used as a basis for the study of perfused flow within the tissue for the introduction of primary Schwann cells. Key questions can additionally be asked such as the influence of a native 3D environment on cell migration and development.

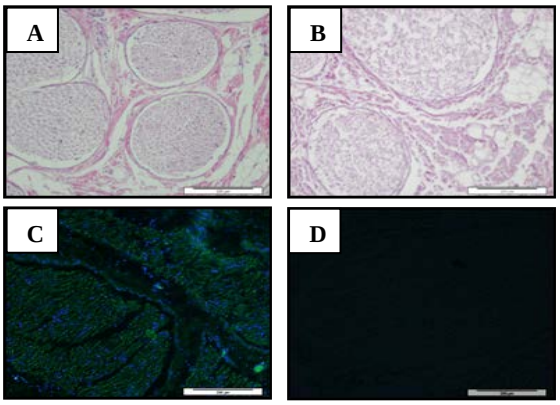


**Figure 1.** Decellularisation of porcine sciatic nerve. H & E of (A) fresh and (B) decellularised nerves. Nuclei staining by DAPI and fluorescence microscopy of (C) fresh and (D) decellularised sciatic nerve. Scale bar = 100μm.

## References

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2. Bell JHA, Haycock JW (2012) Next generation nerve guides - materials, fabrication, growth factors and cell delivery. Tissue Engineering 18(2):116-28.

## Acknowledgments

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## Disclosures

The authors have no competing financial interests.

# Control of collagen hydrogel compression for cell rescue

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## INTRODUCTION:

During the compression of collagen hydrogels, water or small molecules can freely travel between inter-fibrillar spaces, whereas large molecules, such as oligomeric collagen species, are thought to block the fluid leaving surface (FLS)[1]. We hypothesize that artificially incorporated large polymers can block the FLS in a similar fashion, allowing control of gel compression rates. This in turn can reduce any cell damage or excessive particle loss due to high fluid flow rates in some (blended collagen) hydrogels.

## METHODS:

Large polymers used within the gel include fibrinogen (340kDa; Sigma-Aldrich) in 0.9% saline, dextran (500kDa; Fisher Bioreagents) or poly(ethylene oxide)(PEG; 400kDa or 1000kDa, Sigma-Aldrich) in deionised water to 0, 2, 5 or 10mg/ml. The polymer solution was mixed 1:9 into neutralised collagen solution (10% 10xMinimum essential media (Gibco, UK) and 80% acid-soluble collagen (Firstlink; UK)) before plating 2.5ml into 24-well plates (37°c incubation, 30 minutes). Hydrogels were compressed using a paper-roll plunger (Whatman grade 1 paper, 95x4cm) at a rate measured as mass (fluid) gained by plungers. To test if cells can be rescued from high fluid shear stress, 10mg/ml PEg was added to gels where 50% of the collagen solution was substituted with polymeric collagen (extracted from calf tendon in 0.5M acetic acid after EDTA treatment)[2], containing 15,000 human dermal fibroblasts per gel. Cell activity analysed with Alamar blue assays.

## RESULTS:

The rate of hydrogel compression is dependent on the concentration and hydrodynamic radius of the added polymer. Polymers <11nm did not affect compression rate.The rate of compression generally decreased with increasing mobile polymer concentration, and 10mg/ml PEG, with largest Stokes radius was most effective in slowing compression rate. Control hydrogels were fully compressed by 23 minutes; whereas gels containing 10mg/ml fibrinogen, dextran or PEG were fully compressed by 24, 36 or 40 minutes respectively. Seeded fibroblasts were rescued from damage with 10% PEG 400 as indicated by a similar cell metabolism reading compared with control gels, 1 day incubation. Cells in all samples continued to proliferate with culture time.

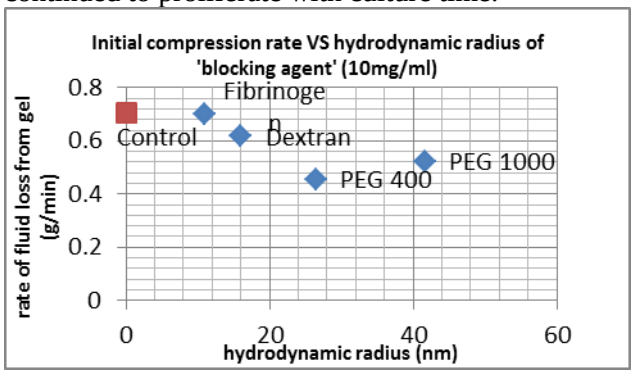


*Fig. 1 initial compression rates against polymer hydrodynamic radius*

## DISCUSSION & CONCLUSIONS:

Compression of blended gels (3 fold increase in modulus) caused significant cell death, but this was reversed by incorporating mobile polymer molecules, such as PEG. This both slowed the fluid flow and spared resident cells. The ability to finely control the extent of plastic compression, hence matrix stiffness, has potential implications for control of cell behavior, and may contribute to increased nanoparticle trapping within gels.

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## ACKNOWLEDGMENTS:

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# Characterisation of distinct regional and immuno-selected cell populations derived from human foetal femurs and their capacity for bone defect repair and regeneration

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## Introduction

Skeletal stem cells (SSCs) isolated from adult human bone marrow (HBM) and utilised for bone tissue engineering strategies remain heterogeneous, exhibiting variable proliferation and differentiation capacity[1]. Here the authors investigate human foetal femurs as an alternative stem cell source[2]. Initial studies investigated regional derivation of cell populations and assessment of their osteochondral differentiation capacity. SSCs were subsequently identified and isolated according to Stro-1 expression. Enriched populations were assessed for osteochondral differentiation and bone defect regeneration.

## Materials and Methods

Femurs were dissected yielding epiphyseal and diaphyseal populations. Stro-1+ cells were immuno-selected from whole femurs by MACS. Proliferation and differentiation capacities within isolated populations were assessed *in vitro* for alkaline phosphatase activity and colony formation. Osteochondral differentiation was assessed though molecular and histological analysis. Tissue formation and regeneration were assessed *in vivo* via subcutaneous diffusion chamber implantation within immunodeficient mice, and *ex vivo* via cell pellet implantation within chick femur drill defects.

## Results

Distinct epiphyseal and diaphyseal populations exhibited robust chondrogenic and osteogenic differentiation potential respectively, according to ALP+ colony formation, osteochondral gene expression, *in vivo* tissue formation, and *ex vivo* bone defect regeneration. Stro-1+ cells isolated from the diaphyseal region of foetal femurs (dependent on developmental stage) exhibited prolonged phenotype stability (Stro-1 form P1-P6), self-renewal, osteo, chondro and adipogenic differentiation, and tissue formation/defect regeneration capacity.

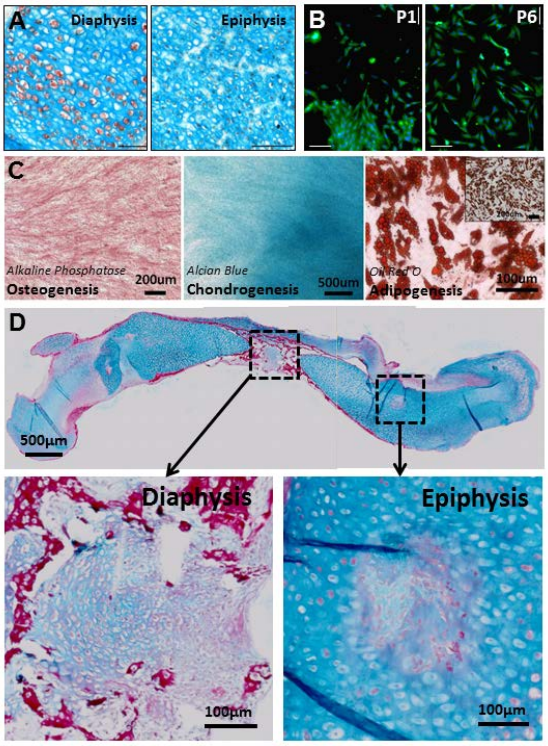


Figure 1: Diaphyseal Stro-1+ cells (**A**) exhibit prolonged Stro-1 expression from passage 1 to 6 (**B**), multi-lineage differentiation (**C**) and defect regeneration (**D**).

## Discussion and Conclusions

Epiphyseal and diaphyseal cells offer robust chondrogenic and osteogenic populations, whilst Stro-1+ cells offer a SSC source for interrogation of bone biology prior to clinical translation.

**References** [1] K. Schallmoser, Haematologica 95 (**6**) 867-874 2010. [2] S. H. Mirmalek-Sani, Stem Cells 24 (**4**) 1042-1053 2006.

## Acknowledgments

We thank surgeons at Southampton General Hospital for provision of foetal tissue. This work was funded by the BBSRC (BB/GO10579/1).

## Disclosures

No conflicts of interest exist.

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# Living biointerfaces to direct cell function

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## INTRODUCTION:

Biomaterials surfaces have been functionalised with a broad variety of proteins and fragments seeking to direct cell response. However, these passive coatings cannot provide the dynamic cues that cells find in the natural ECM. We hypothesised that non-pathogenic bacteria can provide such a role, as they can colonise the surface of a broad range of materials and can be genetically modified to express desired adhesive proteins or factors to a living cell population, upon demand. This work introduces a living interface based on *Lactococcus lactis* expressing a FN fragment ($FNIII_{7-10}$) as a membrane protein.[2]

## METHODS:

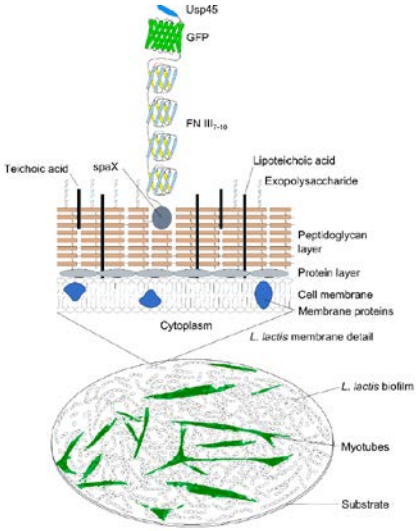
The pGFP-C2 plasmid was used to construct a vector with FNIII7-10 downstream to GFP. FNIII7-10 fragment was amplified and cloned into the PT1NX plasmid. Transformed bacteria were cultured on glass surfaces. The density of $FNIII_{7-10}$ was quantified using ELISA with monoclonal antibodies. C2C12 myoblasts were cultured on the living interface for up to 4 days. Cell adhesion and signalling was investigated by staining focal adhesions (vinculin) and FAK phosphorylation. Cell differentiation was quantified by staining sarcomeric myosin.

## RESULTS:

We engineered food-grade *Lactococcus* to present the $FNIII_{7-10}$ fibronectin fragment as a membrane protein, containing RGD and PHSRN synergy sequences and promotes cell adhesion and differentiation (Fig. 1).

Cells adhere, spread, develop focal adhesions and promote the FAK phosphorylation on the FN-expressing *L. lactis*, in a similar way as when seeded directly on a FN coating. The myogenic differentiation triggered by *L. lactis*-$FNIII_{7-10}$ strain was found higher compared to the non FN-expressing control strain and higher than on standard myogenic differentiation substrates (collagen I and fibronectin coatings).

*Figure 1.*




*Schematic of the living biointerface. C2C12 myoblasts differentiated to myotubes on this system*

## DISCUSSION & CONCLUSIONS:

We have shown that non-pathogenic bacteria based on *L. lactis* expressing the $FNIII_{7-10}$ fragment form a dynamic biointerface between synthetic materials and cells. Overall, this living interface enhances myogenic differentiation. Further genetic modification of this living interface is being done to engineer a dynamic which can be then applied to several strategies to promote tissue repair and regeneration.

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2. A. Saadeddin et al (2013) Adv Health Mater **1213**: 2013

## ACKNOWLEDGMENTS:

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# The Effect of Long-term 3D Culture on Cell Morphology and Behaviour

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## INTRODUCTION:

Conventionally cells are grown and passaged in 2D. However, 2D growth substrates force cells to flatten and lose their characteristic morphology (Fig 1)[1]. Thus, the artificial nature of 2D culture can produce physiologically irrelevant observations of cell behaviour[2]. Using commercially available porous polystyrene scaffolds, a method has been developed to propagate cells continually in 3D, allowing for an observation of the long-term adaptation cells undergo when placed in an *in vivo*-like environment. This is a fundamental question in cell biology and understanding the adaptation process will elucidate tissue formation. The hypothesis behind this work is that changes in focal adhesion signalling networks lead to cytoskeletal reorganisation, and downstream perturbation in gene expression, directly linking a more tissue-like morphology to more physiological cell behaviour.

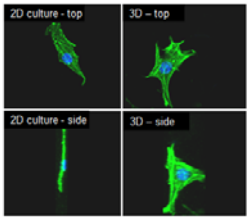


*Fig. 1. Cells show a more realistic tissue-like morphology when grown in 3D culture, as indicated by a change in cytoskeletal staining (Phalloidin).*

## METHODS:

HepG2 cells were seeded onto 6 well Alvetex® Strata inserts at a density of $5x10^5$ and grown for 5 days. Cells were removed, counted, re-suspended and plated onto fresh inserts every 5 days. This process was conducted in parallel with HepG2 cells maintained in 2D culture. The structural phenotype of cells passaged in 2D and 3D was analysed. Statistical significance presented as mean ± SEM.

## RESULTS:

Cells maintained in 3D show rounder morphologies than their 2D counterparts (Fig 2A); this increase in circularity is maintained when cells are re-introduced into 2D, indicating that changes are hard-wired into the cell cytoskeleton. This in term may prime cells in preparation for 3D culture or transplantation. Such adaption was shown by introducing 2D and 3D maintained cells into an aggregate model (Figs 2B and 2C). Cells propagated in either 2D or 3D adapted and behaved differently: 3D primed cells show markedly reduced expression of phosphorylated FAK (pFAK) (a key molecule in cell-ECM signalling and indirectly involved in control of cell shape) (Fig 2D).

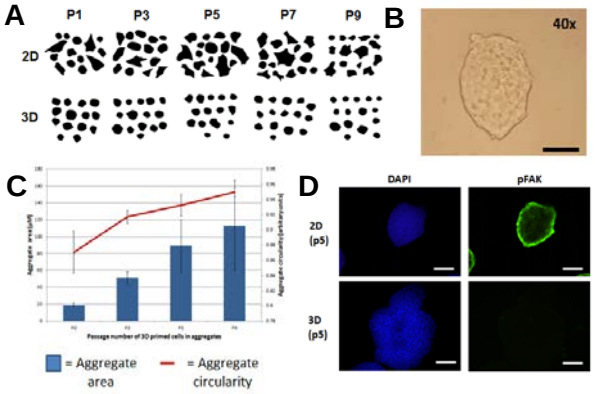


*Fig. 2. Cells on Alvetex® Strata adapt to the 3D micro-environment. Cytoskeletal reorganization over the adaptation period allows cells to acquire a rounder phenotype (A). Cells become primed to 3D culture environments (B&C), and show differences in FAK signaling (D).*

## DISCUSSION & CONCLUSIONS:

Cells primed for 3D growth maintained morphologies more closely resembling cells in primary tissue. A reduction in pFAK in 3D appears to be due to a switch in signalling pathway brought about by the change in physical micro-environment. Differences seen in aggregates grown from 2D and 3D primed cells are possibly due to changes in cell adhesion and cell-ECM signalling, a hypothesis supported by the change in pFAK expression. Further work into this pathway will show how cells adapt to 3D culture. Such mechanisms are fundamental to understanding tissue formation.

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[1] M. Schutte *et al.* (2011) *Assay Drug Dev. Techn.* **9(5)**:475-486. [2] C.S. Chen *et al.* (2005) *MRS Bulletin* **30(3)**:194-20

## ACKNOWLEDGMENTS:

BBSRC and Reinnervate for funding.

# Engineering the corneal stroma: the effect of a three-dimensional environment and growth factor supplementation

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**INTRODUCTION:** A major challenge of producing a tissue-engineered cornea is the recapitulation of the complex environment of the stroma. The resident cells of the stroma are keratocytes; a quiescent, dendritic cell, responsible for maintaining the extracellular matrix (ECM). Upon injury, or during *in vitro* culture in foetal bovine serum (FBS), keratocytes differentiate into a fibroblastic repair phenotype[1]. Differentiating these fibroblasts back to keratocytes may be crucial in stromal tissue-engineering. Methods that have been suggested are three-dimensional environments and serum-free medium[2,3].

**METHODS:** Primary human corneal stromal cells (CSC) were cultured to achieve a fibroblastic cell type reminiscent of an MSC. CSC were seeded in poly(lactic-co-glycolic acid) (PLGA) microfibre scaffolds. Constructs were maintained under 4 media conditions: 20% FBS; 2% FBS; insulin-transferrin-selenium supplementation (ITS); and bFGF and TGF-β3 supplementation (GF). Proliferation, cell phenotype, gene expression and matrix production were assessed.

**RESULTS:** Scaffolds (fibre diameter: 1-3 μm) supported cell adhesion and proliferation in all media types (fig. 1). Gene expression and immunocytochemistry show that scaffolds in ITS and GF media promote the keratocyte phenotype with increased expression of keratocyte markers CD34 and ALDH3A1.

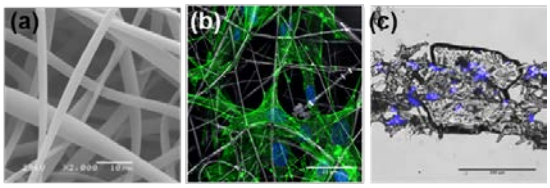


*Figure 1: (a) Scanning electron micrographs of microfibres. (b) F-actin staining shows dendritic morphology of cells. (c) Cross sections of constructs stained with DAPI show cellular infiltration.*

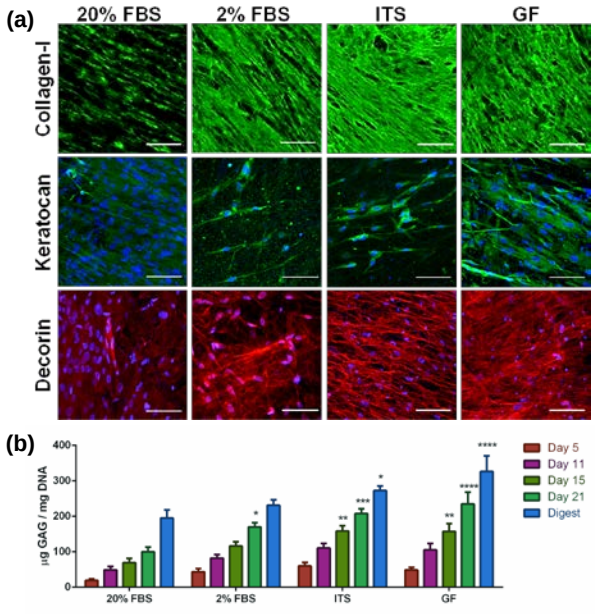


*Figure 2: (a) Immunocytochemistry of collagen-I, keratocan and decorin on scaffolds. (b) Cumulative production of glycosaminoglycans (GAGs) released into medium and when scaffold digested at day 21.*

Collagen-I deposition was seen predominantly in ITS/GF cultures (fig. 2a). Expression of proteoglycans such as keratocan and decorin were greatest in GF. GAG production was highest in GF (fig. 2b).

**DISCUSSION & CONCLUSIONS:** The PLGA microfibre scaffolds allowed infiltration of CSCs and promoted a keratocyte phenotype. This effect was augmented with the use of serum-free medium and growth factor supplementation, which led to improved ECM production and deposition.

## REFERENCES:

1. Poole et al. (1993), *J Cell Sci*, **106**: 685–91.
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3. Wu et al. (2013), *Tissue Eng*, **19**:2063-75.

## ACKNOWLEDGEMENTS:

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# Modulation of human skeletal stem cell fate and function as a consequence of nanotopography and hypoxia

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## INTRODUCTION:

Degenerative skeletal diseases pose a significant socioeconomic burden in aging populations. Skeletal stem cell (SSC) based therapies may enhance current treatment strategies. Essential to this approach is SSC manipulation *in vitro*. Conventional protocols employ chemicals to direct SSC fate. This increases patient risk and reduces the chance of successful clinical translation. Nanotopography and hypoxia are critical components influencing the SSC niche *in vivo* and have been shown, individually, to influence cell behaviour *in vitro*. We have examined the influence of nanotopography and hypoxia to modulate SSC fate and function as alternatives to chemical cues.

## METHODS:

Fetal and adult SSCs were cultured on square (SQ; Fig.1B) and near square (NSQ50; Fig.1C) arrangements of nanopits, 120nm diameter, under 2% and 20% oxygen. Osteogenic and adipogenic differentiation were analysed by qPCR, immunofluorescence and Oil red O staining. Label-free coherent Raman microscopy was used for lipid detection.

## RESULTS:

Cell tracker green staining indicated excellent viability of human fetal SSCs on nanotopographical substrates (Fig.1D)

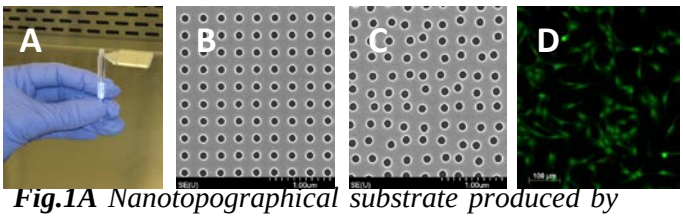


***Fig.1A*** *Nanotopographical substrate produced by electron beam lithography and injection molding.* ***B&C*** *Electron micrographs of SQ and NSQ nanopatterns.* ***1D*** *Viable human fetal SSCs on nanotopography.*

In contrast to adult SSCs[1,2] and ESCs[3], the nanotopographies investigated had no effect on human fetal SSC fate. However hypoxic conditions influenced both fetal and adult SSCs with a decrease in expression of osteogenic genes ALP and OCN and an increase in VEGF expression. On tissue culture plastic Coherent Raman microscopy provided highly sensitive lipid detection compared to conventional Oil Red O Histochemistry. This allowed earlier detection of adipogenic differentiation of fetal and adult SSCs (Fig.2).

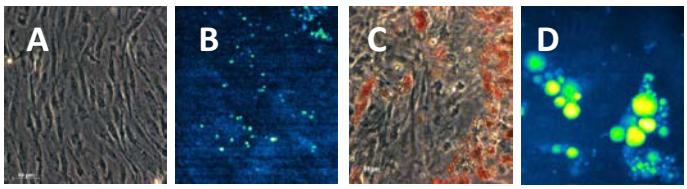


***Fig.2*** *Lipids (yellow) detected by Coherent Raman imaging* ***(2B)*** *but not Oil Red O staining* ***(2A)*** *by day 3 of differentiation.* ***2C&D:*** *Day 14.*

## DISCUSSION & CONCLUSIONS:

The mechanisms underlying the clear differences between adult and fetal SSC response to defined nanotopographies are currently under investigation. Key to tissue regeneration strategies is vascularization of the new tissue. Therefore the increase in VEGF expression under hypoxia is of particular interest and is also currently under examination.

## REFERENCES:

**1.** Dalby M. et al. (2007) Nature Materials. 6:997-1003; **2.** McMurray R. et al. (2011) Nature Materials 10:637-644; **3.** Kingham E. et al. (2013) Small 9(2):2140-2151.

## ACKNOWLEDGMENTS:

The authors would like to thank the Medical Research Council for funding

# Cell and bone donor age influence the osteogenic activity of mscs cultured on an osteoinductive washed human bone scaffold

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**INTRODUCTION:** Bone tissue engineering using native bone scaffolds enables utilization of bones' innate osteogenic abilities, which are not present in synthetic alternatives. However processing techniques required to produce these organic scaffolds (e.g. Gamma irradiation) may weaken there beneficial qualities (e.g osteoinductivity). Therefore new process methods such as washes are required. Having previously demonstrated the efficacy of a novel bone wash to generate a biocompatible and mechanically stable scaffold[1], this current study aims to assess the washed bones' latent osteoinductive ability. Secondly, given that the majority of live donor allograft bone is obtained from the elderly, the study also aims to assess the effect of cell or bone donor age on mesenchymal stem cell (MSC) osteogenic differentiation and activity.

**METHODS:** To assess osteoinductive ability, $1cm^3$ washed human bone cubes were seeded with $5x10^5$ MSCs (N=5), incubated in standard (SM) or osteogenic media (OM) and samples removed at 0, 14 and 28 days for qRT-PCR to assess osteogenic gene expression. Osteogenic activity was assessed by culturing cells from donors <50 and >70 yrs (both N=3) on bone from donors <50 and >70 yrs (both N=3) and analysing gene expression, alkaline phosphatase (ALP) / DNA activity and alamarBlue (AB) metabolic activity.

**RESULTS:** All samples showed significant ($p<0.05$) increases in metabolic activity after 14 and 28 days. Culture of MSCs on washed human bone cubes resulted in significant increases in expression of osteogenic markers runx2, osteopontin (OPN) and osteocalcin (OC) (fig 1) at day 14 and OPN and OC at day 28 for both standard and osteogenic media. Assessment of effect of MSC donor age showed statistically higher gene expression in young cell samples compared to old at day 28 when cultured on bone of varied ages. Interestingly data also showed that old bone induced significantly greater increases in osteogenic gene expression in MSCs compared to young bone; although ALP activity was significantly increased in all samples.

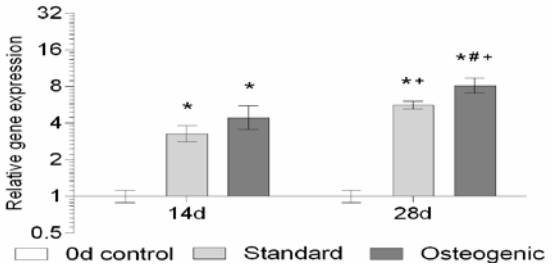


*Fig 1. Relative gene expression of OC at 14 and 28 days compared to day 0 in SM and OM. Significances: * = $p<0.05$ to day 0, + = $p<0.05$ to day 14 and # = $p<0.05$ between conditions.*

**DISCUSSION & CONCLUSIONS:** The results of this study demonstrate that the washed biological bone scaffolds are capable of inducing osteogenic differentiation in MSCs independent of osteogenic media. The study further highlights the influence of cell and tissue donor age, with younger cells better able to osteogenically differentiate on the washed bone, and with older bone better able to promote osteogenic activity. These results suggest that whilst using old donor bone is clinically suitable, old donor cells may require stimulatory factors to boost osteogenic activity.

**REFERENCES:** 1.C.Smith et al (2013), *EurCellMater* **26**:7 p83

**ACKNOWLEDGMENTS:** Funding for this work was provided by the Wrightington Foundation and Joint Action..

# Development of a novel biorheometer for *in vitro* real time monitoring of matrix remodelling

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**INTRODUCTION:** Cells perform their physiological function within a 3D context integrating instruction both from different cells and the extracellular matrix. Currently the majority of systems employed to study cell/matrix interactions are limited in their accuracy and ability to study events in real time. We sought to develop a rheological system capable of detecting biologically induced differences in matrix re-organisation of the most abundant extracellular matrix protein in humans, collagen, between wild type (WT) fibroblasts and fibroblasts lacking the GTPase Rac1 (KO), a protein known to influence cell migration.

**METHODS:** Two TA Instruments ARES-G2 commercial rheometers were modified to include temperature regulation using a peltier plate ($37^0$C) and supplied with 5% $CO_2$/air. Furthermore a stainless steel geometry with regular holes of 2mm diameter was incorporated for media perfusion. The modified geometry showed consistency with unmodified geometries tested with standard viscosity calibration oils (not shown). Rac1 WT and KO phenotype was assessed by Rac1 RT-PCR and functionally with time lapse microscopy. The viability of cell types in 3D (type I collagen lattice) systems was assessed by viability staining (Draq7) and analysed by confocal microscopy. Rac1 WT and KO cells were maintained in DMEM/10% FBS, trypsinised and embedded at 100,000 cells/gel prior to temperature controlled gelation up to $37^0$C. WT and KO cells were run on parallel running rheometers with incubator based cellular controls.

**RESULTS:** Modifications of a commercial rheometer are shown (Fig 1a). Rac1 KO cells were confirmed to lack Rac1 expression by RT-PCR and found to show impaired migration and contraction of collagen gels (Fig 1b/c, 2a) Differences in observable matrix remodelling rates between Rac1 WT and KO cells was first evident after 3 days in culture using traditional assays of gel contraction (Fig 2a, b). Changes in G prime were observable from 3 hours in our customised rheometer (Fig 2d) with both cell populations lacking positivity of the DNA binding dye Draq7 (Fig 2c).

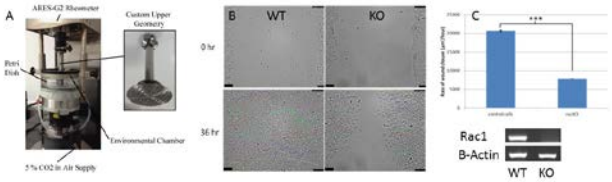


Fig 1: Rheometer customisation (a) with functional and molecular validation of Rac 1 WT and KO cells (b/c). Scale bar bottom left of images, 75µm, ***p<0.001 Mann Whitney U Test.

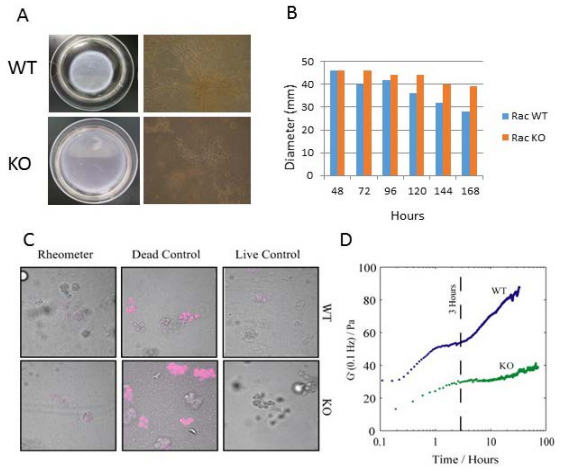


Fig 2: Conventional collagen gel contraction assay after 7 days culture (a) with corresponding daily measurements. Draq7 viability staining of WT and KO cells grown in our customised rheometer with corresponding changes in G prime (d).

**DISCUSSION & CONCLUSIONS:** A novel biorheometer for *in vitro* real time monitoring of matrix remodelling has been developed which detected matrix remodelling at earlier time points than conventional methods.

**ACKNOWLEDGMENTS:** The authors would like to thank the EPSRC (Grant EP/H45848/1) for providing financial support for this project and Prof Klemens Rottner, University of Bonn for donation of Rac1 WT and KO cells.

# Enhanced osteogenesis of hmsc in hydroxyapatite/fibrin gels

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**INTRODUCTION:** Hydroxyapatite (HAp) is a calcium phosphate mineral commonly found in bone[1]. Fibrin is a naturally occurring polymer responsible for clot formation and orchestration of regenerative processes, such as angiogenesis and osteogenesis [2]. Combination of these materials could enhance tissue regeneration rate and seeding efficiency of implantable constructs for bone tissue engineering.

**METHODS:** HAp rod-like, carbonated nanoparticles, Ca/P > 1.67 were synthesised using a controlled sol-gel methodology. In brief, fibrin gels were produced using bovine fibrinogen 0.2mg/ml and thrombin 13.25U/L[3]. Immortalised hMSC were mixed with the fibrinogen to give $2x10^5$ cells/gel. HAp was dispersed in the thrombin solution to give 500µg/ml final gel concentration. Fibrinogen and thrombin solutions were mixed in a sterile cylinder and polymerised for 1 hour in an incubator. Gels were cultured in basal and osteogenic media for 21 days. Histological differences were observed using Masson's trichrome and osteogenic gene expression was analysed using RT-PCR. Statistical analysis was performed and data represented as mean ± SEM.

**RESULTS:** Histology of fibrin sections stained with Masson's trichrome revealed enhanced collagen deposition from cells within the fibrin-HAp matrix. RT-PCR showed an increase in ALP, Runx2, OPN and OC in HAp containing fibrin compared with control.

**ACKNOWLEDGMENTS:** The authors would like to acknowledge the support of Arthritis Research UK (Award 19429) and the FP7 RESTORATION project (Award CP-TP 280575-2).

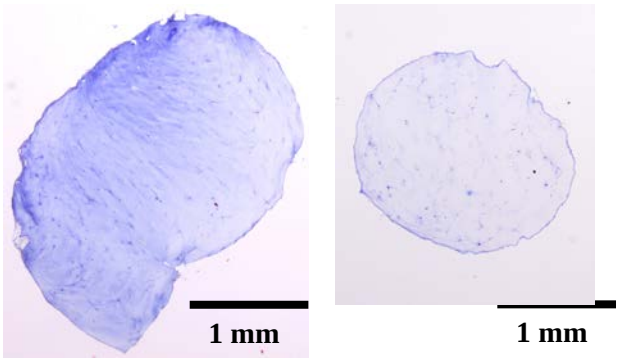


*Fig. 1 Section of fibrin-hMSC following 21 days cultivation with osteogenic medium. A) Fibrin & B) Fibrin-HAp.*

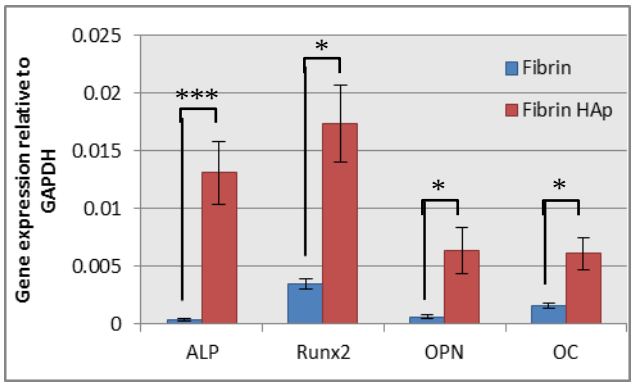


*Fig.2. Relative gene expression of RNA isolated from hMSC following 21 days cultivation in osteogenic medium. *p<0.05 & ***p<0.001.*

**DISCUSSION & CONCLUSIONS:**
The properties of HAp and fibrin alone are well documented and should act synergistically for bone regeneration *in-vivo*. This data indicates that combination of these two materials promote hMSC osteogenic differentiation *in-vitro*.

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1. E. Bouyer et al (2000), J Mater Sci 11:523-531.
2. I. Catelas et al (2006),Tissue Engineering 12: 2385-2396.
3. T. Osathanon et al (2008), Biomaterials 30: 4091-4099.

# Electrostatic stabilisation of bio-ink through the cationic encapsulation of cells for piezo drop on demand inkjet printing

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**INTRODUCTION:** Under the remit of tissue Engineering and regenerative medicine, bioprinting is an emerging tool which, it is hoped, will underpin the fabrication of complex tissue structures. Fundamental to inkjet printing is the ink itself. Researchers have found that cell suspensions were prone to settling and agglomeration and have realised no viable solution[1]. Electrostatic stabilisation using polyelectrolyte coatings is a method of stabilising pigment in common inkjet inks. Many, if not all of these coatings however, are cytotoxic and thus inappropriate for use in a bioink. A cationic polymer, Poly-L-Lyseen (PLL) is known to be both cell compatible[2] and an electrostatic stabiliser[3]. It was therefore hypothesised that encapsulation of cells in a coating of PLL would aide dispersion. The objectives were to ascertain the correct coating thickness as to allow the cell to release from the encapsulatant post print and to visually assess the influence of the coating on print efficacy.

**METHODS:**

*Cell Encapsulation:* Human osteosarcoma cells (U2OS, Sigma-Aldrich, UK) were cultured in completed Alpha-Modified Eagle's Medium. Three concentrations, 200µg.ml$^{-1}$, 400µg.ml$^{-1}$ and 2000µg.ml$^{-1}$, of Cationic PLL (Sigma) were prepared and each added to 2 million U2OS cells suspended in 1 mL of HBSS. After incubation, washes in HBSS were made before centrifugation and re-suspension. 100µl of the preparations were transferred into a 12 well plate and the cell response to encapsulation observed.

*Bioprinting:* 2 milion U2OS cells coated in PLL of concentration 200µg.ml$^{-1}$ were re-suspended in 2ml HBSS, along with 1x10$^{6}$cell.ml$^{-1}$ uncoated U2OS cells in 2ml of HBSS, for printing. The cell specimens were loaded into reservoirs of a piezo based, DOD inkjet printing system (Microfab, USA). 100µl controls were pipetted into 3 wells of a 12 well plate, containing 1ml Eagle's Medium in each well, before the remaining 9 wells received 15k ink drops from a 80µm orifice.

**RESULTS:** Viewed after encapsulation, the cells coated in the 200µg.ml$^{-1}$ PLL exhibited thinner coatings than those cells coated in the 400µg.ml$^{-1}$ and 2000µg.ml$^{-1}$ concentrations. After 7 days the cells encapsulated in the 2000µg.ml$^{-1}$ and 400µg.ml$^{-1}$ concentrations were seen to be unattached. Cells coated at 200µg.ml$^{-1}$ attached to the well plate in the absence of PLL residues. Printing of the unencapsulated cells showed aggregated colonies, from up to 8 colonies in the first well and 0 in the latter. Printing of the encapsulated cells showed well dispersed individuals. Debris was seen in the orifice during printing and, as with the uncoated cells, the first wells to be printed showed higher deposition rates than the latter.

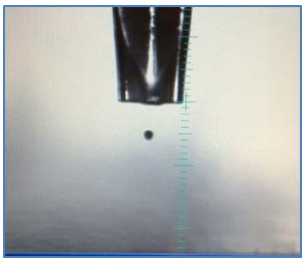

*Fig. 1 a single droplet of bio ink with a diameter of 83µm and 299pl volume*

**DISCUSSION & CONCLUSIONS:**

Preliminary work has suggested that a cationic cell coating aids cell dispersion. Cell deposition rates dropped in both cases. In the instance of unencapsulated cells this was due to cell agglomeration, and in the printing of encapsulated cells was due to fouling of the orifice, presumed to be PLL. These results demonstrate a strong case for further work on the otherwise unexplored concept of printing cells with charged coatings.

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**ACKNOWLEDGMENTS:**

Many thanks to Dr Ana Ferreira-Duarte who co works on this project. This research was funded by the EPSRC

# Corneal stromal cells: a potential cell source for ocular surface regeneration

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## INTRODUCTION:

Keratocytes of the corneal stroma are quiescent, dendritic cells, which maintain the extracellular matrix. Upon injury, or during *in vitro* culture in serum, keratocytes differentiate into a fibroblastic repair phenotype[1]. It is believed that there is a subpopulation of keratocytes that act as multipotent progenitor cells[2]. These cells repopulate the stroma after damage and may play a role in the regeneration of other layers of the cornea, such as the epithelium.

## METHODS:

Primary human corneal stromal cells (hCSC) were extracted from corneal rims. The optimum culture media was determined from a selection available, and the effect on cell phenotype, of passaging and expanding the cells was assessed. *In vitro* differentiation of hCSC into mesenchymal lineages was investigated, alongside the potential for differentiation back into a stromal keratocyte phenotype and transdifferentiation into corneal epithelial cells.

## RESULTS:

The phenotype of hCSC is strongly affected by extraction and passage (figure 1). Culture media used also has an effect on cell phenotype. Under the correct conditions, hCSC show potential as a multipotent stem cell, expressing the indicative markers and differentiating *in vitro* down osteogenic, chondrogenic and adipogenic lineages.

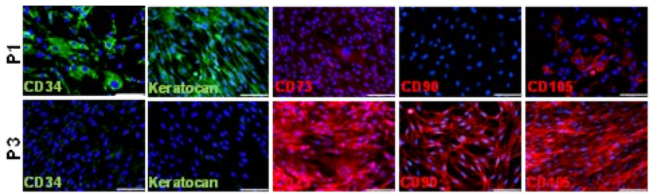


*Figure 1: Passaging of hCSC affects cell phenotype. At passage 1 (P1) cells express high levels of CD34 and keratocan, but low levels of CD73, CD90 and CD105. This is the reverse at passage 3 (P3).*

$CD34^+$ cells can be isolated from hCSC at low passage. This population demonstrates a higher stem cell potential than the unsorted cells. Under the correct conditions, $CD34^+$ hCSC show evidence of differentiation to corneal epithelial cells, showing a rounded morphology and high expression of key markers such as cytokeratin 3 (KRT3), transcription factor HES1, and desmoglein 3 (DSG3) (figure 2).

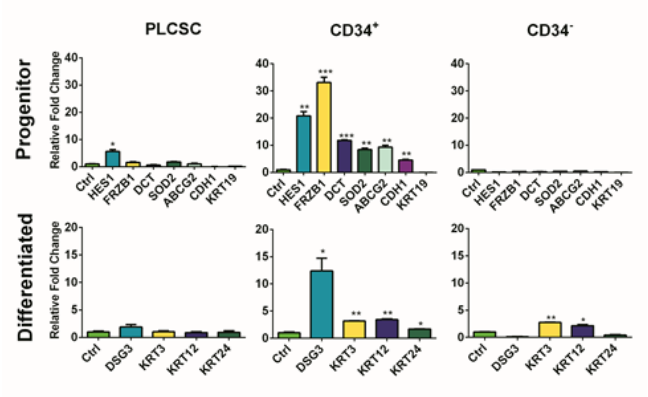


*Figure 2: Differentiation of $CD34^+$hCSC to corneal epithelial cells. RT-qPCR showing significant upregulation of corneal epithelial genes in $CD34^+$ cells.*

## DISCUSSION & CONCLUSIONS:

Corneal stromal cells demonstrate a multipotent stem cell potential, independent of the role they are traditionally associated with in the cornea. *In vitro*, this potential may depend on the culture environment and on isolation of certain subpopulations. In future, these cells show potential in the regeneration of the ocular surface in cases of disease or trauma.

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## ACKNOWLEDGEMENTS:

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# Limbal mesenchymal stem cells; alternative stem cells for transplantation

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**INTRODUCTION:** *Ex vivo* expanded limbal stem cell (LSC) transplantation using amniotic membrane as a carrier system has been used as a cellular-based therapy to reconstruct the ocular surface. Here we look at a population of cells isolated from the limbus region that have the characteristics of mesenchymal stem cells (MSC).

**METHODS:**

Limbal epithelial cells were isolated from cadaveric corneo-scleral rims and the cells resuspended in a MSC growth promotion medium. Immunophenotyping for expression of cell surface antigens known to be expressed by human MSCs (CD44, CD90, CD105, CD106, CD146 and CD166), hematopoietic lineage markers CD19 and CD45 and expression of MHC Class I and Class II were performed. The ability for these cells to undergo tri-lineage differentiation (chondrogenic, adipogenic and osteogenic) was determined and lineage differentiation confirmed with histological staining and mRNA expression. Chemotaxis transwell assays were performed to study cell migration. Cells were also plated on to cryopreserved amniotic membrane to determine if they would adhere, and proliferate.

**RESULTS:**

Limbal MSC were adherent, rapidly proliferated on plastics and were positive for antibodies specific to human MSC, and negative for markers of lineage committed haematopoietic cells. They stained positive for mineralisation (Alizarin Red), cartilaginous deposition (Safranin O) and oil droplets (Oil Red O) on histology and expressed markers of differentiation to the three lineages at mRNA level. Limbal Mesenchymal stem cells (LMSC) showed high expression of CXCR4. Transwell migration analysis demonstrated LMSC migrated at higher rates than human corneal epithelial cells and breast cancer cells MDAMB231 ($P<0.05$). LMSC were also able to grow and proliferate on amniotic membrane.

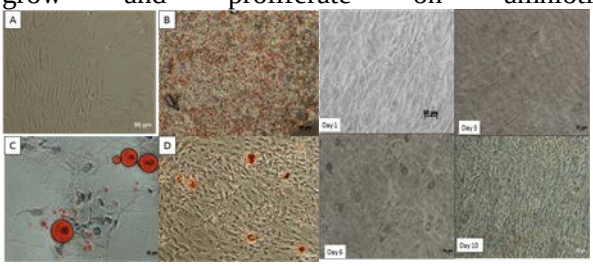


***Figure 1**. Histology of LMSCs. A) undifferentiated B) Cartilaginous deposition stained by Safranin O for chondogenic differentiation C) Osteogenic differentiated cells stained with Oil Red O. D) Osteogenic differentiated cells show evidence of mineralisation by Alizarin Red staining.*

***Figure 2**. Growth and proliferation of LMSC on cryopreserved amniotic membrane over ten days*

**DISCUSSION & CONCLUSIONS:**

Limbal mesenchymal stem cells can be successfully isolated from cadaveric corneo-scleral rings and grown initially in low oxygen (5%) in mesenchymal growth promotion media. These cells showed classic expression of human MSC markers. Using our tissue culture protocols, limbal MSC show tri-lineage differentiation as confirmed by histology and mRNA expression. Amniotic membrane (AM) as a niche for limbal stem cell transplantation is a widely acceptable practice. LMSC adhere to the AM and proliferated rapidly on this tissue substrate. The potential of these cells to contribute to improved outcomes in tissue transplantation bears further investigation.

**REFERENCES:**

1. Pellegrini, G et al (1997),*The Lancet*, **349**(9057), pp. 990-993.
2. Sun, T.T et al (2010), *Nature*, **463**(7284), pp. E10-E11.
3. Knuth, C et al (2013), *Stem Cell Reviews and Reports*, **9**(5), pp. 599-608.

# Supplementing the corneal donor pool using human decellularised corneas

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**INTRODUCTION:** There is a clinical need for reliable & quality biomimetic corneas that are as effective, preferably superior to cadaveric donor tissue[1]. Decellularised matrices are advantageous compared to synthetic or semi-synthetic engineered tissues in that the complex native milieu is preserved to retain intrinsic biological cues including growth factors, cytokines & glycosaminoglycans (GAGs)[2]. However, there is currently no effective, standardised, decellularisation protocol suitable for human corneal tissue[1]. Therefore, the purpose of this work was to provide a systematic evaluation of existing decellularisation methods in terms of their applicability to human corneal tissue.

**METHODS:** Corneal eye-bank tissue unsuitable for transplantation was utilised. NaCl, SDS, Triton-X100 & mechanical agitation, followed by nuclease treatments were investigated. Removal of detectable cellular/immunoreactive material was evidenced by immunofluorescence & Picogreen® analysis. Macroscopic preservation of optical properties & light transmittance was evaluated. Retention of corneal architecture & GAGs was assessed *via* histological, immunofluorescence & quantitative analysis.

**RESULTS:**

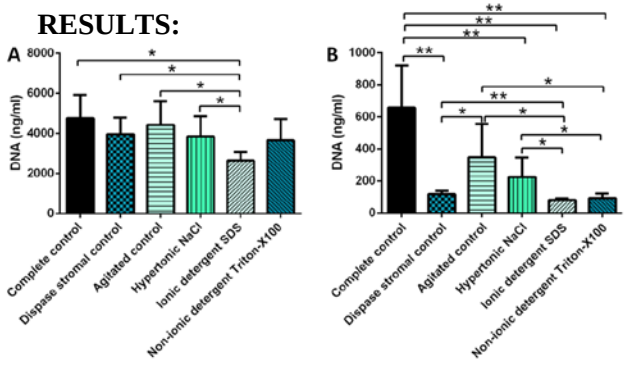


*Fig. 1: Picogreen® quantification of residual DNA following decellularisation treatments (**A**) & decellularisation with an additional nuclease treatment (**B**), n = 5, * $p \leq 0.05$, ** $p \leq 0.01$.*

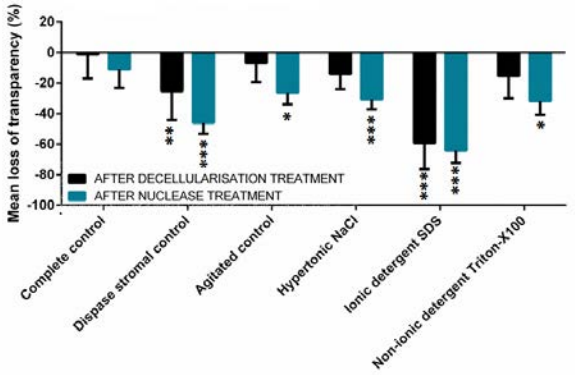


*Fig 2: Quantitative analysis of reduction of light transmittance following decellularisation & nuclease treatments, n = 5, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, when compared to corneas prior to any treatment.*

None of the employed decellularisation techniques successfully removed 100% of the residual cellular material (Fig. 1). The techniques which had the least residual DNA were the most structurally compromised, with the greatest loss of transparency (Fig. 2). GAG analysis demonstrated the stripping effects of the different decellularisation treatments.

**DISCUSSION & CONCLUSIONS:** The ability to utilise, reprocess & regenerate tissues deemed "unsuitable" for transplantation allows us to salvage valuable tissue. Reprocessing the tissue has the potential to have a considerable impact on addressing the problems associated with cadaveric donor shortage, which would have a significant economic benefit. Patients would directly benefit by having access to greater numbers of corneal grafts & health authorities would fulfill their responsibility for the delivery of effective corneal reconstruction to alleviate corneal blindness.

**REFERENCES:** [1]S.L. Wilson et al (2013) *J. Funct. Biomater.* **4**:114-161; [2]S.F. Badylak (2004) *Transpl. Immunol.* **12**:367-277.

**ACKNOWLEDGMENTS:** The authors would like to thank the EPSRC (EP/I01701/1) for funding this research.

# Limbal mesenchymal stem cells; alternative stem cells for transplantation

Bakiah Shaharuddin[1,2] Charles Osei-Bempong[1] Sajjad Ahmad[3,4] Paul Rooney[5] Simi Ali[6] Rachel Oldershaw[7] Annette Meeson[1]
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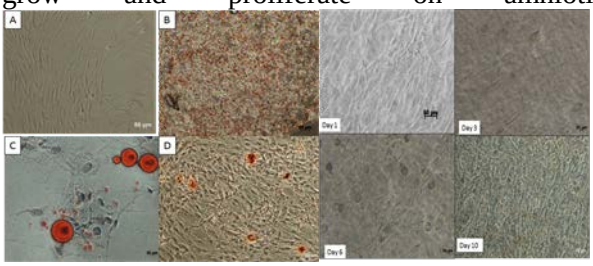


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***Figure 2****. Growth and proliferation of LMSC on cryopreserved amniotic membrane over ten days*

**DISCUSSION & CONCLUSIONS:**

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3. Knuth, C et al (2013), *Stem Cell Reviews and Reports*, **9**(5), pp. 599-608.

# The use of polymersomes in stem-cell specific targeting for bone regeneration

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## INTRODUCTION:

Musculoskeletal disorders in the UK cost ~£5billion/year, and new therapies are urgently required[1]. Mesenchymal stem cells (MSCs) are fundamental in bone regeneration given their intrinsic capacity to differentiate into osteoblasts. Wnt signalling plays an essential role in the osteoblastic differentiation of MSCs[2], but can have stimulatory or inhibitory effects depending on the timing of delivery. Polymersomes (PMs) are nanosized carriers composed of amphiphilic block copolymers that can be loaded with hydrophilic and hydrophobic compounds, and can be engineered for specific targeting. Our hypothesis is that PMs can deliver Wnt agonists specifically to populations of MSCs to induce bone regeneration.

## METHODS:

PMs were produced via nanoprecipitation using transactivator of transcription (TAT) conjugated polyethylene glycol 5.8K (PEG)-b-poly-ε-caprolactone 19k (PCL) block copolymers. PMs were loaded with 0.1 M sodium fluorescein; at this concentration the dye inside PMs is self-quenched, but fluoresces upon release. Loaded PMs were sized by dynamic light scattering or added to a murine fibroblast cell line (L929) for evaluation of cellular uptake and cytotoxicity using live imaging and FACS.

## RESULTS:

PMs were produced with an average diameter of 85.90±33.24nm. FACS analysis demonstrated that after 3hrs of incubation, 92.53% of cells had internalised loaded PMs. Cytotoxicity, measured by propidium iodide (PI) staining demonstrated only 3.27% dead cells after 24 hrs.

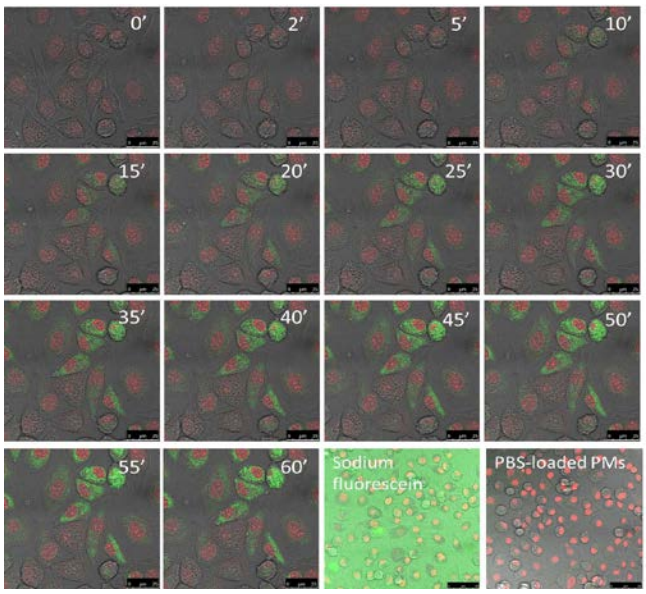


*Fig. 1 Live imaging of L929 cells incubated with 1 mg/ml of sodium fluorescein-loaded TAT-PMs*

## DISCUSSION & CONCLUSIONS:

These results demonstrate that PEG-PCL PMs can be loaded with a hydrophilic cargo, are quickly internalised into cells and are able to release their payload without cytotoxic effects. These characteristics make polymersomes a promising candidate for targeted delivery in bone regeneration.

## REFERENCES:

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## ACKNOWLEDGMENTS:

Authors wish to thank the Institute for Life Sciences (Ifls) Southampton and the Wessex Medical Research charity for funding.

# Surface functionalization of electro-spun poly(L)Lactic acid scaffolds with heparin to induce angiogenesis

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## INTRODUCTION:

Delays in new blood vessel formation after implantation of a tissue-engineered construct within the host is a major limiting factor for the long survival of the constructs [1]. Smarter biomaterials are needed to be able to induce angiogenesis post-implantation and keep the newly formed tissue alive. Hence, biomaterials can be designed to be specifically functionalised for this purpose. The aim of this study is to develop a versatile approach to modifying scaffolds to induce angiogenesis once implanted.

## METHODS:

Electrospun PLA scaffolds were plasma polymerized with PolyAcrylic Acid (PAA) and coated with alternative layers of PolyEthyleneImine (PEI) and PAA or PEI and Heparin for a total of seven layers, in a layer-by-layer (LBL) coating approach. Coated scaffolds were then dipped in heparin solution, dried and immersed in Vascular Endothelial Growth Factor (VEGF) solution. Surface chemistry was verified by X-Ray Photon Electron Spectroscopy. An ELISA was used to quantify the amount of VEGF bound to the scaffolds. The Chick Chorionic Allantoic Membrane (CAM) assay was used to assess *in-vivo* the angiogenic potential of the scaffolds over a period of 7 days.

## RESULTS:

XPS showed that plasma polymerization of the scaffolds with PAA was successful as the presence of S indicates Heparin is present. Heparin bound well to LBL-coated scaffolds, compared to non-functionalised scaffolds, and showed an increase in VEGF binding. The CAM assay showed that the functionalized scaffolds induced blood vessel formation compared to non-functionalised scaffolds.

| | Atomic Ratios | | |
|---|---|---|---|
| **Type of Scaffold** | **O/C** | **N/C** | **S/C** |
| Not-functionalized PLLA | 0.375 | 0.011 | 0.000 |
| 1 layer functionalized PLLA | 0.468 | 0.128 | 0.052 |
| 5 layers functionalized PLLA | 0.542 | 0.155 | 0.076 |
| 7 layers functionalized PLLA | 0.546 | 0.149 | 0.082 |

Table 1. Atomic ratios of elements present on the surface of different scaffolds, normalized by the amount of carbon atoms present (C= carbon, N=Nitrogen, S=Sulphur, O=Oxygen).

## DISCUSSION & CONCLUSIONS:

The LBL functionalization we describe allows heparin to be bound to the surface of the scaffold which can in turn bind VEGF. The CAM assay demonstrated that this bound VEGF is functional inducing directed neovascularisation. We conclude that this protocol ( which can be applied to a range of scaffolds) offers a new approach to tackling the problems of delayed angiogenesis for tissue engineering .

## REFERENCES:

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## ACKNOWLEDGMENTS:

X-ray photoelectron spectra were obtained at the National EPSRC XPS User's Service (NEXUS) at Newcastle University, an EPSRC Mid-Range Facility.

# Indirect three dimensional printing (3dp)of apatite-wollastonite (a-w) scaffolds

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## INTRODUCTION:

The 3D printing (3DP) technique based on powder-binder system is an excellent method for the fabrication of porous structure scaffolds. Sintered apatite-wollastonite (A-W) of suitable porosity has been known to show similar mechanical properties to natural bone during controlled manufacturing. This research has investigated the potential of using the indirect 3DP process to create 3D porous A-W scaffolds.

## METHODS:

A-W as defined by Kokubo et al.1 with weight ratio of 4.6MgO, 44.7CaO, 34SiO2, 16.2P2O5, 0.5CaF2 was used in this study. The approach taken to indirect 3DP was: (i) Blending of the A-W with maltodextrin (MD) powder. A range of formulations was used as shown in Table 1. (ii) Using a Z Corp Z310plus 3D printer to selectively print binder into sequentially deposited thin layers of the blended powders in order to build up a 3D structure. Two binder solution were used: 98% distilled water and 2% glycerol (DW); and zb®60 (from Z Corp). (iii) The 3D printed parts were then heat treated up to 1150ºC to burn off the MD and sinter the A-W to create a consolidated 3D structure.

**Table 1 Powder blends**

| Blend | Composition (wt %) |
|---|---|
| PB1 | 70% 54-90µm A-W<br>30% 0-53 µm MD |
| PB2 | 85% 0-53 µm A-W<br>15% 0-53 µm MD |
| PB3 | 80% 0-53 µm A-W<br>20% 0-53 µm MD |
| PB4 | 70% 0-53 µm A-W<br>30% 0-53 µm MD |
| PB5 | 55% 54-90µm A-W<br>15% 0-53 µm A-W<br>30% 0-53 µm MD |

## RESULTS:

Table 2 presents a summary of the porosity, shrinkage and strengths measured on the various samples which were approx. 40 x 4 x 3 mm. 30% MD was required for sufficient strength to develop. Table 2 also shows that PBs 1, 4 and 5 develop the highest strengths with sintering. Figure 1 and 2 shows the macro and micro porosity structures of PB4 and PB5.

**Table 2 Summary of Strength, Shrinkage and Porosity Results**

| Samples | Average Volume Shinkage in Sintering (%) | Average Total Porosity (%) | Average Open Porosity (%) | Average Bend Strength (MPa) |
|---|---|---|---|---|
| **PB1/DW** | 34.38 | 51.76 | 33.01 | 8.95 |
| **PB1/zb®60** | - | 47.95 | 15.43 | 23.65 |
| **PB2/DW** | 47.6 | 53.67 | 39.48 | 11.56 |
| **PB2/zb®60** | 34.96 | - | - | 8.23 |
| **PB3/DW** | 50.32 | 52.24 | 39.08 | 8.09 |
| **PB3/zb®60** | 15.77 | - | - | 6.13 |
| **PB4/zb®60** | 48.56 | 35.27 | 11.62 | 35.64 |
| **PB5/zb®60** | 41.30 | 41.85 | 12.4 | 25.68 |

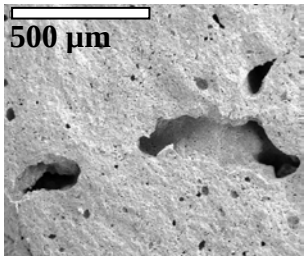


**Fig. 1 SEM of the fractured surface of the sintered part of PB4**

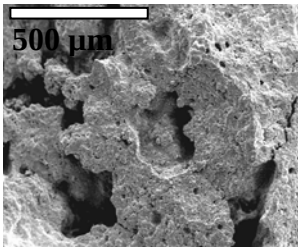


**Fig. 2 SEM of the fractured surface for sintered part of PB5**

## DISCUSSION & CONCLUSION:

In all cases the increase in strengths is as a result of the increased consolidation during sintering, as indicated by the reduced porosity.

Indirect 3D printing of A-W structures can be used to create strong, highly porous structures, but care must be taken to appropriately select binder and processing parameters.

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## ACKNOWLEDGMENTS:

I would like to thank the interior ministry of Saudi Arabia for providing financial support to my study.

# Optimising 405 nm hins-light technology for patient safe decontamination during arthroplasty surgery

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## INTRODUCTION:

Infection rates following orthopaedic arthroplasty surgery are as high as 4%, while the infection rates are even higher after revision surgery [1]. The duration of routine arthroplasty surgeries is typically between 1 and 2 hours. 405nm High-Intensity Narrow-Spectrum Light (HINS-light) has bactericidal activity against Hospital Acquired Infection (HAI) related bacterial pathogens including MRSA [2] and hence may aid in reducing the incidence of infections that arise from environmental contamination during arthroplasty surgery.

## METHODS:

Immortalised rat osteoblast (OST 5) cells were exposed to 405 nm light at an irradiance of 5mW/cm$^2$ in Dulbecco's Phosphate Buffered Saline (DPBS) at different dose rates (18, 27, 36 and 45J/cm$^2$) at 37˚C and 5% $CO_2$. Unexposed controls were treated in the same way. After 48 hours post treatment, cell viability (MTT assay), cell function (ALP assay)and cell proliferation rate (BrdU assay) were measured. Live/Dead cell staining was carried out using Acridine Orange/ Propidium Iodide (AO/PI) dyes after 48 hours post light treatment. Statistical analysis was performed using unpaired Student t-test and differences considered significant when $p<0.05$.

## RESULTS:

After 48 hours post light treatment, no significant difference was observed between the unexposed and 405 nm treated samples for up to a dose rate of 36J/cm$^2$ in cell viability, function and proliferation rate (fig 1.a). More apoptotic and dead cells were observed for the 45J/cm$^2$ exposed samples compared to the 36J/cm$^2$ exposed samples (fig. 1.b).

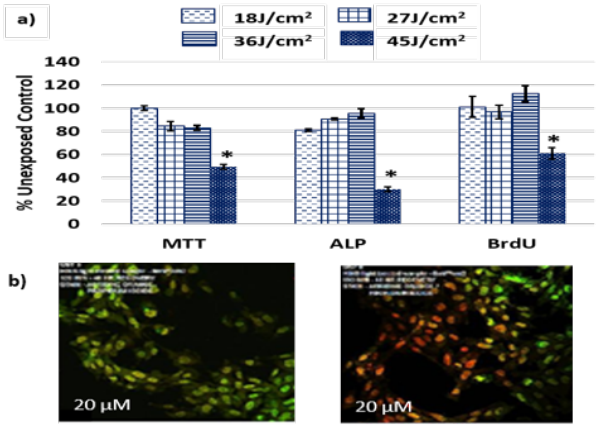


*Fig 1. (a) Effect of 405 nm light treatment at 5mW/cm$^2$ on OST 5 cell response parameters after 48 hours incubation, (b) AO/PI staining. Left – 36J/cm$^2$ and Right – 45J/cm$^2$ of 405 nm light treatment after 48 hours incubation. AO (live - green, apoptotic- orange), PI (dead -red).*

## DISCUSSION & CONCLUSIONS:

From the quantitative and qualitative studies, it is found that the cells were healthy for up to a dose rate of 36J/cm$^2$ (5mW/cm$^2$ for 2 hours) whilst cell death became evident with doses of 45J/cm$^2$. These results suggest that exposure to a dose of 36J/cm$^2$ may be suitable for use for continuous decontamination during orthopaedic surgery whilst being safe for tissue exposure.

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## ACKNOWLEDGMENTS:

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# Population kinetics of MSCs in the development of bio-artificial bone scaffolds

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**INTRODUCTION:**

Our group is assessing the potential for Mesenchymal Stem Cells (MSCs) to populate synthetic biomaterials for manufacturing scaffolds to support the growth and differentiation of MSCs into osteocytes for bone repair in osteoarthritis. We compared the use of non-haematopoietic medium with MSC basal medium across three cultures of bone marrow derived MSCs taken from the femoral heads of osteoarthritis patients. No difference was found in doubling potential, and growth rate using either growth media.

**METHODS:**

Selecting the use of MSC growth media to promote growth of undifferentiated, plastic adherent MSCs, we cultured MSCs through six passages, testing their tri-lineage potential to differentiate into osteocytes, chondrocytes and adipocytes at early, mid and late time points. We also assessed the surface phenotype by flow cytometry of undifferentiated MSCs against the ISCT standard classification guidelines (>95% expression of CD73, CD90 & CD105 with <2% CD14, CD19, CD34, CD45 and HLA-DR).

**RESULTS:**

Results showed the MSCs cultured could be differentiated into the three specific lineages and demonstrated conformance with the ISCT classification, except for CD34. Data showed no presence of CD34 in early cultures, but in two of three experiments, by day 50 in culture CD34 positivity was 3% and 8% - exceeding the classification criteria by this single parameter. By day 89 in culture, CD34 expression advanced to 15% and 46%. Those CD34+ cells remained >95% positive for CD73, CD90 & CD105.

**DISCUSSION & CONCLUSIONS:**

The data show that in our culture conditions, there is potential for MSCs to upregulate CD34 expression, whilst retaining all the other surface markers identifying them as MSCs. The retention of CD73 expression and the preferential culture medium used mitigates against the suggestion that CD34+ cells are haematopoietic cells, and instead suggests expansion of CD34+ MSC-like cells. The impact of this occurrence in manufacturing cells for regenerative medicine therapies is currently being assessed.

**ACKNOWLEDGMENTS:**

The authors would like to thank Arthritis Research UK for providing financial support to this project.

# Effect of nano- and microscale roughness on cellular behaviour

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## INTRODUCTION:

Apatite/Wollastonite (A/W) is a glass-ceramic that combines biocompatibility, bioactivity, osteoconductivity and bioresorbability[1] that can be used for bone scaffolds. The objectives of this study were to investigate how the difference of micro and nano- roughness affect the human mesenchymal stem cell behaviour.

## METHODS:

A/W glass powder (GTS, Sheffield) was milled and then sieved to separate the particle fractions of <20μm, 20-53μm and 54-90μm. A/W powder was mixed with water resulting in a slurry and sintered at 1150ºC for 1 hour. Two different groups were fabricated with particle range 20-53μm (AW1) and combination of 54-90μm:<20μm (80:20) (AW2) to vary the surface roughness. Surface profile was determined using interferometry and morphology through SEM. Immortalised human MSCs were used to evaluate cell number and alkaline phosphatase (ALP) activity. Gene expression was quantified using real- time PCR. Statistical analysis was performed using a student's t-test.

## RESULTS:

Profilometry revealed a significant difference in surface roughness between AW1 scaffolds (Ra=397nm) and AW2 (Ra=2.2μm).
Alkaline phosphatase activity showed lower levels on A/W scaffolds compared with control surfaces. Scaffolds with smoother surfaces have elevated alkaline phosphatase levels in both basal and osteogenic media in comparison to rougher surfaces (Figure 1).

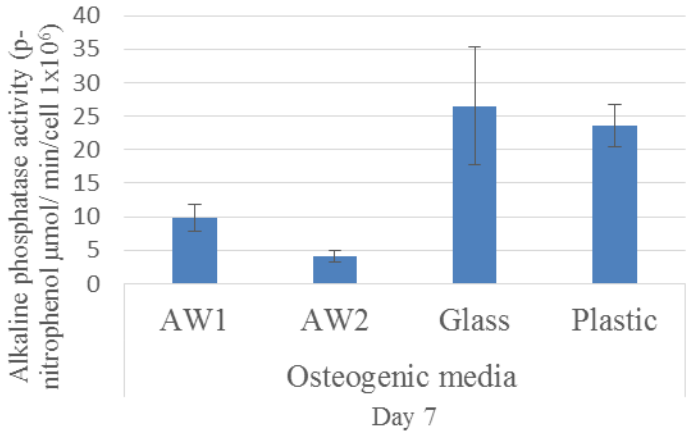


Fig 1: Normalised ALP activity of immortalised human MSCs cultured on various surfaces for 7 days.

## DISCUSSION & CONCLUSIONS:

According to previous research, roughness stimulates osteogenesis[3]. However, the increase of ALP activity on the smoother surface can be explained by variations in cell number that could be due to primary cellular adhesion.

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## ACKNOWLEDGMENTS:

The authors would like to acknowledge the support of Arthritis Research UK (Award 19429), the EPSRC, and the FP7 RESTORATION project (Award CP-TP 280575-2)

# Analysis of osteoclastogenesis/osteoblastogenesis using human bone marrow derived co-cultures on nanotopographical titania surfaces.

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**INTRODUCTION:** Titanium (Ti) is currently used for orthopaedic applications due to its excellent load bearing properties. However, Ti is bioinert, and this can affect osseointegration and outcomes of implants.

Nanopatterning of implant surfaces could be the solution to this hurdle. We have previously shown that 15nm high nanopillars are bioactive [1,2] using human mesenchymal stem cells (MSC). We have also developed an osteoblast/osteoclast co-culture system using nanopits on polycarbonate[3]. Here we have used these osteoblast/osteoclast co-cultures as they are believed to give the most accurate representation of the *in vivo* environment, allowing assessment of bone remodeling related to biomaterials. Under co-culture conditions 15nm high nanopillars on titanium, will induce significantly increased levels of osteoblastogenesis and reduce osteoclast activity producing a method of enhancing secondary implant fixation.

**METHODS:** Co-culture of osteoblast and osteoclast progenitors on polished titanium and titanium patterned with 15nm nanopillars fabricated by the block copolymer technique was performed. Time points of 14 and 28 days were selected for analysis as a reasonable timescale for osteointegration *in vivo*. Histochemical staining was performed to identify and quantify osteoclasts and bone nodule formation. Scanning electron microscopy (SEM) was conducted to morphologically examine the effect on differentiation of untreated and nanopatterned titanium substrates on osteoprogenitors and osteoclasts. Real-Time, quantitative reverse-transcription polymerase chain reaction is currently being utilised to quantify expression of osteoblast, osteoclast and inflammatory response related genes.

**RESULTS:** On SEM nanopillar titanium substrates were shown to be less inductive of osteoclastogenesis, with decreased maturity and decreased activity with time visualised when compared to flat titanium substrates (Fig. 1a). This was supported by TRAP staining, which showed macrophages present on flat titanium substrates only (Fig.1b). Increased osteogenesis on the 15nm pillars was indicated by Alizarin red staining (Fig.1c). qPCR revealed a time related-decrease in osteo-clastogenesis related genes on the nanopillars with an associated increase in osteoclast inhibitors.

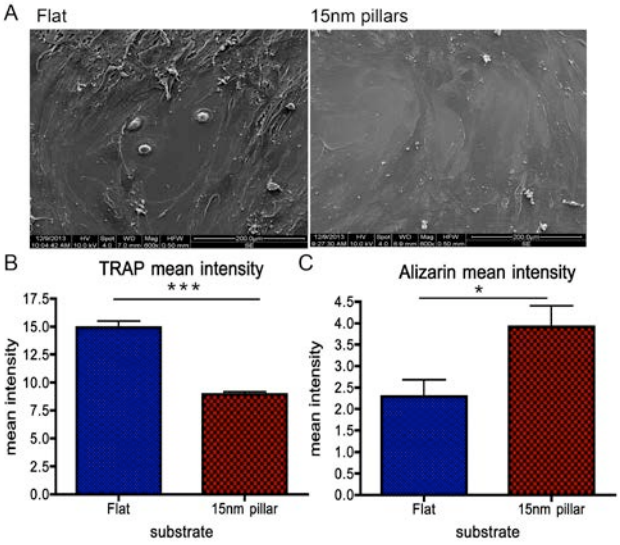


**Fig.1** Showing a) SEM, b) TRAP and c) Alizarin staining results (n=3, T-test, *p<0.05, ***p<0.001).

**DISCUSSION & CONCLUSIONS:** Dramatic reduction in number of osteoclast progenitor cells on nanopillar substrates versus flat was noted. Alizarin red staining demonstrated osteoblast proliferation on both surfaces, with no difference in osteoblastogenesis. Genomic data on osteoclast and osteoblast-related genes will be presented.

**REFERENCES:**

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**ACKNOWLEDGMENTS:**

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# TOWARDS THE SMART PROSTHESIS: JOINING MATERIALS AND BIOLOGY

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## INTRODUCTION:

Prosthetics aimed at restoring natural human functions are becoming very popular forms of limb replacement. Newer prostheses are looking at the integration of different components (i.e. electrical components [(1)]), but real functionality of a robotic limb can be achieved only if the user can control it as part of their own body. This requisite could be accomplished by developing an interface between the natural and the artificial systems so the user can interact with the artificial device in a natural manner. [(2)]

We are focused on this interface between the artificial device and the remaining limb. By joining musculoskeletal biology and materials which can be chemically modified, we will find the optimum conditions compatible with the biological needs, whilst also optimising the prosthesis in terms of mechanical properties: structural integrity, flexibility etc.

## METHODS:

Glass slides were chemically modified by attaching different polymers chains to the surface. The chemical modifications were performed attending to the different functionality of the end groups: PHEMA (-OH), PMMA ($-CH_3$), APTES ($-NH_2$) BIBB (-Br), PMETAC ($-N^+CH_3)_3Cl^-$), PKSPMA ($-SO_3^-K^+$), plastic (polystyrene treated) and glass coverslips (glass no coated). 50,000 cells per well of SHSY-5Y Neuroblastoma cells were seeded onto each surface. Cells were cultured in DMEM media, 10% FBS, and 1% P/S until a 50% of confluence was reached. Neurite length was measured at this stage as pre–RA (pre-retinoic acid); surfaces were subsequently cultured in similar media containing 10 μM retinoic acid (RA). Neurite length was measured at day 1, 3, and 5 in RA media (DM1, DM3, and DM5).

## RESULTS:

Neurite length increased up to 73μm in SHSY-5Y cells cultured on the BIBB surface after 1 day in RA. Differences in the neurite length could be observed between coated and non coated glass.

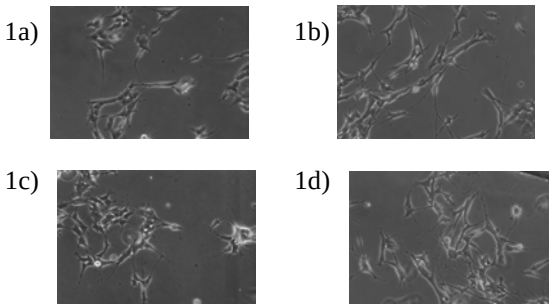


*Figure 1. a)BIBB surface PRE-RA b)BIBB surface at DM1 c)Glass no coated PRE-RA d)Glass no coated at DM1 Scale bar = 50μm.*

## DISCUSSION & CONCLUSIONS:

The mechanisms of cell interaction with foreign surfaces are known to be influenced by various factors such as hydrophilic/hydrophobic balance in the surface [(3)]. Here we show that neurite length is at least partly dependent on the interaction of the cell and the surface to which it adheres, inducing their differentiation, and/or influencing their survival. Gene expression analysis will subsequently be undertaken to identify the specific cell phenotype (e.g. motoneuron, sensory neuron) induced by each chemical modification. Furthermore, different polymers (e.g. block copolymers, different monomers with different end groups) will be used to determine which specific conditions influence cell behaviour, helping us to obtain the requirements for cellular survival and differentiation in an adequate material.

## REFERENCES:

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## ACKNOWLEDGMENTS:

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# Preclinical Musculoskeletal Junction Testbed: Optimisation of a Reproducible Skeletal Muscle Construct

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**INTRODUCTION**: Prior to new material or drug use in humans, a series of preclinical and clinical tests to determine compatibility with human subjects must be passed1. Tissue engineered constructs allow for high throughput testing in a tightly controlled environment whilst maintaining strong mimicry of in vivo architecture. However, this is often costly in terms of resources and cell numbers. Therefore, it was sought to optimise the manufacturing variables to limit use of resources whilst maintaining characteristics of the in vivo tissue. This work proposes that variation in cell density (2, 3 and 4 million cells/mL) and alterations in attachment forces governed by gel width and surface area of attachment will regulate the formation and maturation of tissue engineered skeletal muscle constructs.

METHODS: 3D type-1 rat-tail collagen (2.20mg/mL) neutralised constructs were seeded with C2C12 murine myoblast cells as previously described3. The constructs were tethered at either end by bespoke polythene mesh floatation bars to create longitudinal lines of isometric tension (Fig 1). Constructs were placed in 20% FBS high glucose DMEM for 4 days and then cultured in 2% horse serum high glucose DMEM to induce differentiation. Preclinical work relies on high numbers of replicates; therefore, this study is conducted in a scaled down model (0.3mL-0.8mL) to reduce resource use whilst maintaining basic characteristics of the previous model3 (3mL total volume)

**RESULTS:** Variation between 2, 3 and 4 million cells/mL of collagen and construct designs (volume and attachment area variation) had no significant effect on contraction time or width reduction (ANOVA $P>0.05$). Preliminary analysis indicates that the width of the construct has an effect on alignment and higher cell densities produce greater alignment. Physical success of the muscle construct with an extension past the anchor point is 30% greater than without [~100% (n=6) vs. ~70% (n=6)]. High cell densities displayed greater number of unattached cells. Immunohistochemical staining for the intermediate filament protein Desmin showed the capacity for alignment and differentiation of C2C12 myoblasts within the collagen system (Fig. 2).

Fig. 1 Muscle construct variables: Observed effects of initial width past anchor point and cell density. (left) Day 0 (right) Day 14 (top), 4million cells/mL (0.8ml, ~15mm initial width) (bottom) 2million cells/mL (0.3mL ~5mm initial width)

Fig. 2. Immunohistochemistry shows Desmin intermediate filament (red) and DAPI nuclear stain (blue) (40x magnification).

DISCUSSION & CONCLUSIONS:

The optimum extension lies between 0%-50% of the anchor point width based on alignment and physical success of the construct. This data replicates published work in a similar model3 whilst expanding previous alignment data4. Further phenotypic and genotypic analysis is to be completed.

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# Development of mesenchymal stem cell niche *in vitro* using magnetic nanoparticles

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## INTRODUCTION:

Stem cells are able to self renew and differentiate into other cell types.[1] In the body, stem cells reside within a special microenvironment, termed the 'niche'.[2] The niche protects the stem cells from over stimulation and apoptosis, whist maintaining quiescence and homeostasis.[2] Understanding the regulatory mechanisms within the niche allows the *in vitro* expansion of stem cells and desired differentiation for regenerating damaged tissue. This project aimed to mimick the bone marrow mesenchymal stem cell (MSCs) niche *in vitro.* The development of a 3D spheroid culture system 'niche' in collagen gels, demonstrated excellent cell viability, maintenance of STRO-1/nestin expression and quiescence compared to parallel MSCs cultured in monolayer.

## METHODS:

Human MSCs (hMSCs) and magnetic nanoparticles (mNPs) were purchased from Promocell and Chemicell respectively. Magnets comprised of a NdFeB core (13 mm diameter, 350 mT).

***MSC Labelling***. The MSCs were labelled with mNPs (PEA coated, 200 nm diameter, $Fe_3O_4$) at 0.1 mg/mL mNPs (30 minutes + magnetic field). ***Spheroid Culture***. Labelled MSCs were suspended in media. A single magnet was placed on *the top of the culture well and the plate was incubated for up to 2 weeks.* ***Collagen gel Culture.*** *MSC spheroids were implanted into collagen gels for up to 2 weeks.* ***Analysis.*** *Monolayer, spheroid and collagen gel culture systems were assessed for viability (calcein/ethidium homodimer), cell architecture (actin), phenotype (STRO-1/nestin) and quiescence (BrdU) using fluorescence microscopy.*

## RESULTS:

MSCs were successfully labelled with mNPs and were able to form a 3D spheroid culture system within hours. MSCs are adherent cells and previous studies have shown the formation of similar spheroids, which have very strong cell-cell interactions have provided a better representation of the natural niche environment.[3] The cultured MSCs remained strongly viable and quiescent over 2 weeks, with strong MSC marker expression, whilst the collagen gels provided a similar environment to bone marrow *in vivo.*

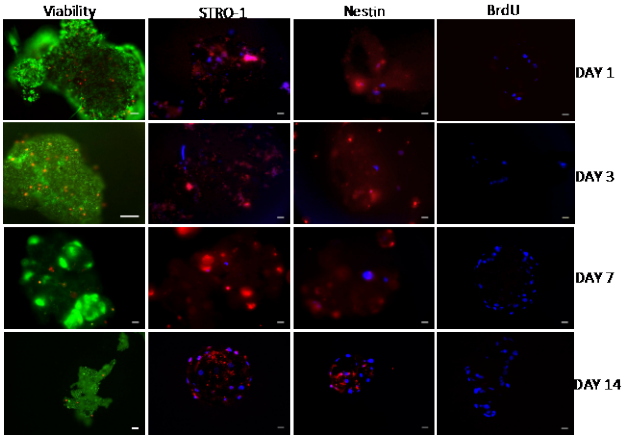


*Fig. 1 Schematic diagram of a spheroid culture system. Fluorescence images of spheroid MSCs cultured for 14 days assessing cell viability (green: live cells, red: dead cells), phenotype (red: STRO-1/nestin, blue: nucleus) and quiescence (purple: proliferating cells, blue: quiescent cells). Scale bar: 20 μm.*

## DISCUSSION & CONCLUSIONS:

A simple, quick and highly efficient method has been developed to use mNP-labelled MSCs to form spheroid 3D niche cultures. The NPs allow for simple cell tracking and imaging *in vitro*, with a view to potential clinical use. The system was novel in mimicking the *in vivo* environment by creating a realistic 3D *in vitro* model. The spheroids remained viable and quiescent, whilst retaining their multipotency potential and exhibiting functional responses (results not shown here) over 2 weeks compared to conventional 2D cultures.

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# Increasing osterix expression by blocking mir-31 with rna conjugated gold nanoparticles

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**INTRODUCTION:** Controlled differentiation of stem cells into defined cell types is a major aim of tissue engineering. Mir-31a expression is involved in tumorigenesis, adipogenesis and skeletal muscle development, however a recent report has also indicated mir-31a's role in osteogenesis as a regulator of osterix. Osterix is a transcription factor and master regulator of bone differentiation. In this project we propose to increase osterix expression by blocking mir-31a, with antagonists to mir-31 (antagomirs) delivered to cells via gold nanoparticles (AuNPs). This hypothesis is tested initially with the pre-osteoblast like bone cancer cell line MG63 (known to have low osterix levels) and subsequently with primary human mesenchymal stem cells (hMSC) with a view towards influencing osteogenesis.

**METHODS:** Nanoparticles were synthesised (~12nm), protected with PEG and tagged with antagomirs. The AuNP toxicity was assessed by MTT; cellular uptake of AuNPs was visualized by TEM and quantified *via* ICP-MS. Osterix expression was assessed by qPCR (gene level) and in-cell westerns (protein level).

***In-Cell Westerns***. Briefly, cells ($1x10^4$ cells/ml) were seeded for 24 hours in DMEM/10% FBS (antibiotics & L-glutamine), and subsequently incubated in 50nM AuNPs. Following a further 48 hours, cells were fixed, permeabilised and blocked prior to incubation with an osterix primary antibody (Abcam, UK). Samples were washed and co-incubated with secondary antibodies (CellTag700 & donkey anti-rabbit IR800CW; Licor, UK). The plates were scanned by an Odyssey SA.

**RESULTS:** Osterix expression was significantly increased in MG63 cells incubated with the antagomirs, as determined by PCR, ICW and immunofluorescence. In addition, the antagomirs were found to produce an increase in osterix expression in MSCs, with particular enhancement via the 3' antagimir (figure 1).

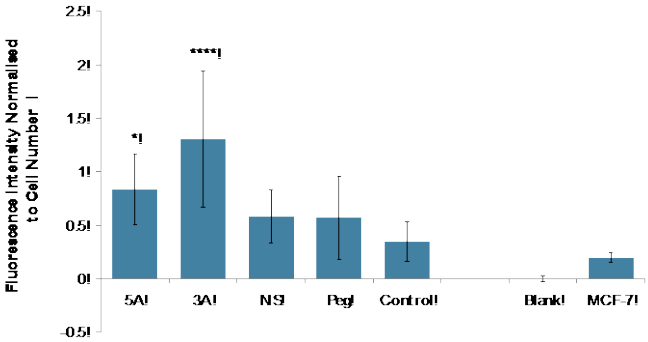


Figure 1. In-cell western showing osterix expression in MSCs. Cells were treated for 48 hours. The treatments included four AuNP species (5A:antagonist to 5' end of mir-31, 3A:antagonist to 3'end of mir-31, NS:nonsense RNA, PEG:PEG only. Control:MG63 cells (low osterix), MCF-7:osterix negative cell line (error bar: St. dev, n=6).

**DISCUSSION & CONCLUSIONS:** This study demonstrates the highly novel use of delivering small functional antagonists of miRNAs to cells *via* nanoparticles, and the potential capability of using such NPs to drive osteogenesis. Such studies highlight translational roles in clinical treatments including osteoporosis and osteogenesis imperfecta.

**REFERENCES:** 1. Baglìo et al (2013). Gene, 527 (1), 321–331

# *In vitro* modelling of human gastrointestinal peritoneal carcinomatosis: a pre-clinical photodiagnostic & photodynamic therapy treatment tool

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## INTRODUCTION:

Gastrointestinal peritoneal carcinomatosis (PC) is a debilitating late stage disease that is characterized by multiple tumour lesions in the abdominal cavity. These tumours are associated with the peritoneal tissue, this is the thin tissue which covers organs & lines the abdominal cavity. Our aim is to develop an *in vitro* model system of PC for use in a feasibility study to use protoporphyrin IX (PpIX) fluorescence as a diagnostic aid, and photodynamic therapy (PDT) as a potential adjuvant focal treatment with surgical debulking.

## METHODS:

Monolayer cultures and 3D hydrogels are being investigated for use in the construction of an *in vitro* PC-model. Human colon cancer cell lines were investigated, e.g. T84 & HT29. PC-models were constructed based on tumour to non-tumour cell ratios seeded at: 1 in 10, 1 in 50 & 1 in 100 and cultured for 3, 7 & 14 days. A range of 5-ALA concentrations (0-1mM) and incubation times (0-6h) were investigated for levels of PpIX fluorescence emitted using a spectrofluoreometer. Optimal doses were tested in our developing PC-model in order to estimate its future tumour diagnostic potential. In addition, a selection of antibodies was screened for tumour cell specificity in order to support our finding.

## RESULTS:

1 in 10 ratio of tumour to non-tumour cells cultured for ~7 days showed promising results for use in constructing a PC-model. 6 h 5-ALA incubation gave the brightest PpIX fluorescence of the times tested, with HT29 cells showing the greatest fluorescence of the cell lines screened (Fig 1).

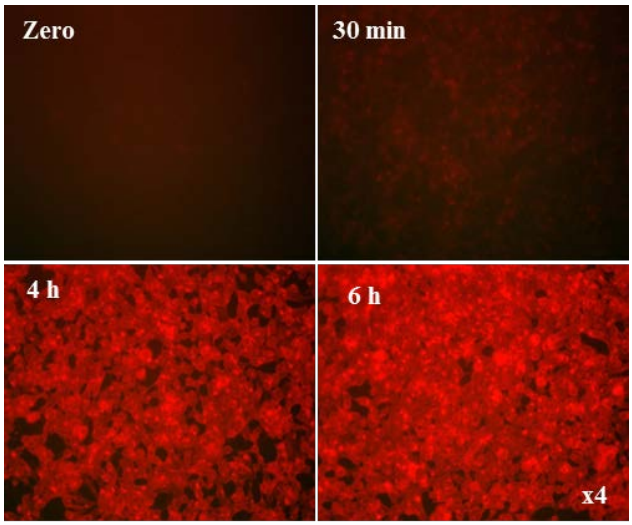


*Fig. 1 Epi-fluorescence micrograph of PpIX fluorescence in HT29 cell line monolayers after incubation with 1 mM 5-ALA for 0, 30 min, 4h & 6 h.*

## DISCUSSION & CONCLUSIONS:

To be able to distinguish tumour from non-tumour cells & tissues, in a viable culture system would be a valuable research tool in the development of new diagnostic & therapeutic treatments such as PDT for treating local recurrence & metastatic diseases such as gastrointestinal-PC. We surmised, administering exogenously administered 5-ALA to a mixed-culture system show greater accumulation of intracellular localized PpIX fluorescence in tumour cell lines, such as HT29, to levels that are distinguishable from non-tumour cells.

## ACKNOWLEDGMENTS:

The authors would like to thank NIHR and the CRUK Leeds Centre-Development Fund (Grant no: C37059/A16369) for providing financial support to this project.

# The effect of physical microenvironment on the shape of pluripotent stem cells and their developmental potential

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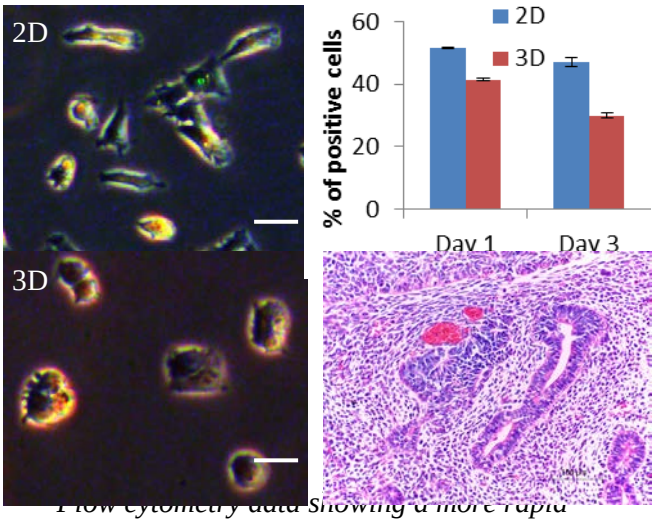
**INTRODUCTION:** Intercellular signalling between stem cells is vital for cell fate determination. Cells cultured on 2D (two dimensional) and 3D (three dimensional) substrates have different morphologies – cells grown in 3D possess a more *in vivo*-like and spherical structure rather than a flattened out shape as occurs in 2D monolayer cultures[1].
Differences in cell shape markedly affect gene and protein expression. This in turn changes the developmental potential of 2D flattened cells[1]. Cells cultured in 3D show enhanced differentiation compared with 2D monolayer counterparts[2]. It is proposed that changes to cytoskeletal structure and subsequently gene/protein expression profiles enabled by 3D culture enhance the pluripotent phenotype of stem cells *in vitro* and their ability to differentiate.

**METHODS:** Using Alvetex® Strata, a non-biodegradable commercially available porous polystyrene membrane, we established routine protocols for long term propagation of TERA2.cl.SP12 cells in 3D which maintain a stem cell phenotype[3]. Cells were continually maintained on Alvetex® Strata membranes and passaged every 4 days. Cell shape was assessed and flow cytometry performed on 2D and 3D propagated cells cultured and then differentiated as 2D monolayers in response to 1μM ATRA. Teratomas were produced in nude mice.

**RESULTS:** There was a noticeable difference in the shape of cells after 2D and 3D culture: 3D passaged cells maintained a spherical shape whilst 2D passaged cells rapidly flattened as normal. Compared to 2D monolayer culture, levels of SSEA-3 expression in 3D passaged cells rose as the passage number increased (p4<p6<p10) indicating enhancement of the stem cell phenotype. During differentiation, the stem cell marker SSEA-3 decreased more rapidly in 3D passaged cells than cells propagated in 2D. Teratomas produced from 3D propagated cells were significantly larger and more differentiated than tumours derived from 2D passaged cells.




*Flow cytometry data showing a more rapid reduction in SSEA-3 during ATRA induced differentiation; Phase microscopy images of passage 10 2D and 3D cells put back into 2D culture. Scale bar: 50μm; Example teratoma generated from 3D propagated stem cells.*

**DISCUSSION & CONCLUSIONS:**
We demonstrate the ability to propagate stem cells continually in 3D using a novel porous scaffold. We show that 3D passaged cells alter their shape, which in turn appears to affect the stem cell phenotype. Altering the shape of the cells can also influence their ability to differentiate *in vitro* and produce teratomas *in vivo*. Maintenance of 3D shape therefore has important implications on stem cell culture.

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# Fabrication & functionalisation of nerve guidance conduits

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## INTRODUCTION:

Nerve Guidance Conduits (NGC) are increasingly used in surgical peripheral nerve repair. Autografts give superior results, but require additional surgery at a donor site with resultant morbidity.

Improving NGC performance is challenging as existing FDA approved materials are bio-inert. Microtopographical features ameliorate neuroregeneration on such materials[1], but surface functionalisation may provide greater enhancement[2]. As biological coatings face significant challenges in clinical acceptance, synthetic coatings are an attractive option.

The aims of this work are to investigate: i) whether the plasma polymer surface coating of NGCs and ii) the inclusion of organised micro geometries improves their effectiveness at stimulating nerve regeneration.

## METHODS:

NGC were produced from photocurable polymers by microstereolithography (µSL). Plasma chambers were used to etch and coat substrates with either Acrylic Acid (AAc), Allylamine (AAm) or Maleic Anhydride (MA).

Surfaces were characterised by X-ray Photoelectron Spectroscopy (XPS) to determine element composition and confirm consistent coating. The hydrophilic/hydrophobic nature was determined by Water Contact Angle (WCA) analysis.

NG108-15 neuronal cells were cultured on surfaces in serum-free DMEM to induce differentiation. MTT assay was used to confirm cell viability. After 72 hours, cells were fixed and stained with Phalloidin-TRITC and DAPI. Cells were imaged by confocal microscopy and neurite growth analysed using ImageJ software.

## RESULTS:

Guidance features with dimensions less than 100 µm were created on the inner surfaces of NGC (figure 1).

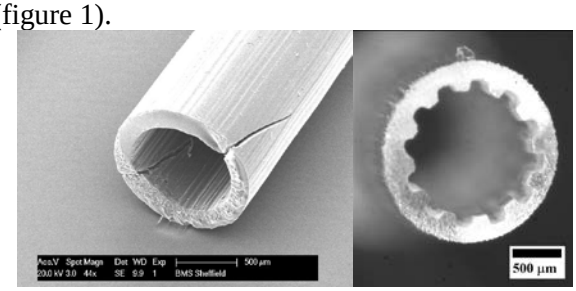


*Fig. 1 NGC produced by µSPL*

Plasma coating was achieved evenly on the inner surfaces of NGC <1 mm internal diameter with a length of 5 mm.

Preliminary results show increased metabolism and neurite growth on AAc coated surfaces. Cells adhered well to other coated surfaces but neurite growth was less extensive.

## DISCUSSION & CONCLUSIONS:

We have produced NGC with user-defined topography and surface chemistry. Plasma treatment may be used to enhance neuronal cell response to bio-inert materials.

## REFERENCES:

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## ACKNOWLEDGMENTS:

The authors would like to thank the EPSRC for DTA funding and the National EPSRC XPS User's Service (NEXUS) at Newcastle University. Confocal microscopy was performed at the Kroto Research Institute Imaging Facility.

# Bioactive scaffolds as bone templates for *in-vitro* testing

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## INTRODUCTION:

Bone tissue engineering (TE) aims at improving musculoskeletal health. Our aim is to develop a tissue engineered bone construct that could be used for studying *in-vitro* bone formation around prototype implant materials. In this study, we investigated polymer composite materials fabricated with electrospinning and particulate leaching (PL) as a support for tissue engineered bone matrix. The scaffold therefore needs to have good mechanical strength and resilience with a high porosity, low degradation rate and be osteoconductive.

## METHODS:

Polyurethane (PU) and Polyurethane-Hydroxyapatite (PU-HA) composite solutions were electrospun to attain scaffolds with either aligned or random fibres, using 15%wt PU dissolved in 70/30 DMF/THF solvent for PU scaffolds. To create composite scaffolds, PU solutions were doped with either micro or nano-sized HA particles in a ratio of 3 PU: 1 HA. For particulate leached scaffolds, solutions were combined with NaCl particles (~250 µm). MLO-A5 and hES-MP cells were used for biocompatibility studies over a 28-day culture period. Mechanical testing of scaffolds; and assays of calcium and collagen deposition were also undertaken as part of this study.

## RESULTS:

FTIR and Raman characterization confirmed the presence of HA in all composite scaffolds, whilst µ-CT confirmed good pore interconnectivity. Although all scaffolds supported proliferation of both cell types and the deposition of calcified matrix, random polyurethane scaffolds with nano-HA enabled the highest cell viability amongst electrospun composite scaffolds. Interestingly, within the PU-only group, aligned fibres allowed better cell support than random fibres.

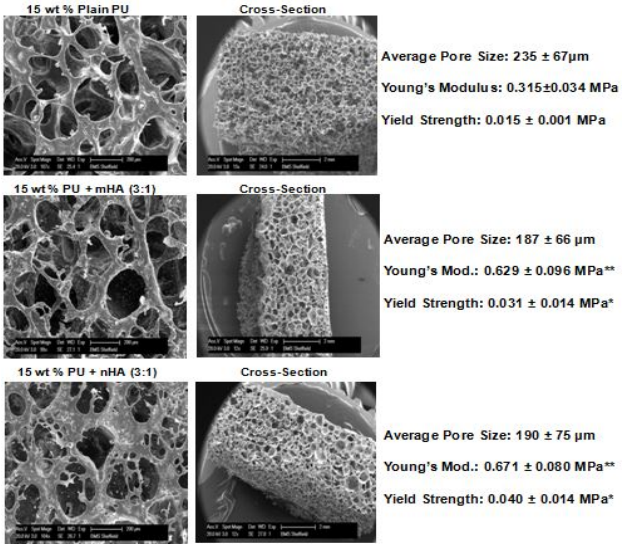


*Fig 1: SEM images of particulate leached scaffolds; left: top view (scale bar=200µm) and right: cross-sectional view (scale bar=2mm) Text: Mean ± standard deviation; (n=6) one way Anova **= $p<0.001$ and *= $p<0.05$*

## DISCUSSION & CONCLUSIONS:

These particulate leached scaffolds composed of highly interconnected macro and micro porous network (>60% porosity) with pore sizes previously described as optimum for bone regeneration (>180µm)[1], and electrospun scaffolds support bone matrix formation and have good mechanical properties in relation to their good porosity. We therefore propose the use of these autoclavable scaffolds as a support for tissue engineered constructs, and for in-vitro studies of bone formation including testing of implant materials.

## REFERENCE

[1] J. Klawitter, S. Hulbert, (1971) *JBMR* **5:** 161-229.

## ACKNOWLEDGMENTS

The authors would like to thank the Ghana Education Trust Fund for providing financial support to the project.

# Development of a three-dimensional model for ameloblastoma

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## INTRODUCTION:

Ameloblastoma is a benign, locally invasive neoplasm originating from epithelial cells of the tooth-forming apparatus. It is associated with extensive local bone destruction and high recurrence rate after surgical resection. Little is known of the molecular mechanisms regarding tissue invasion and bone resorption, recent studies suggest osteoclast activation by ameloblastoma cells as the main mechanism[1]. This study aims to develop *in vitro* organotypic models of the tumour to better detail the molecular mechanisms of tumour tissue invasion and destruction.

## METHODS:

AM-1 cells[2] were cultured in flasks with keratinocyte serum-free media (KSFM) with 50ng/ml b-FGF. They were incorporated into collagen gels neutralised with NaOH, compressed[3] and incubated in KSFM for up to two weeks. Cells were then examined with confocal microscopy using phalloidin as a cytoplasmic stain and propidium iodide as a nuclear stain. In order to create cell spheres to accurately model the tumour, cells were placed on 6-well low-attachment plates (Corning) for 3 days in KSFM and imaged at regular intervals. As a control, HOS cells were cultured on low-attachment plates in DMEM.

## RESULTS:

AM-1 cells form large cell aggregates within the 3D collagen scaffolds (fig.1), however no spheres are formed in 2D culture (fig.2).

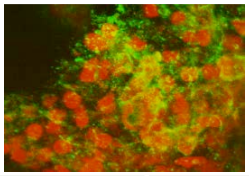

*Figure 1: 40x confocal microscope image of AM-1 cells in compressed collagen. Nuclei – red, cytoplasm - green.*

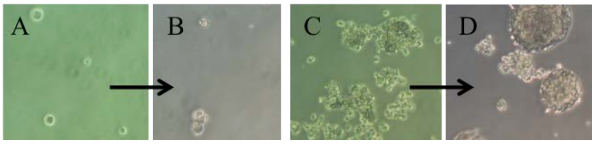


*Figure 2: Low-attachment plate culture of AM-1 cells (A,B) and HOS cells (C,D) at 1 (A,C) and 3 days (B,D). HOS cells form large spheres.*

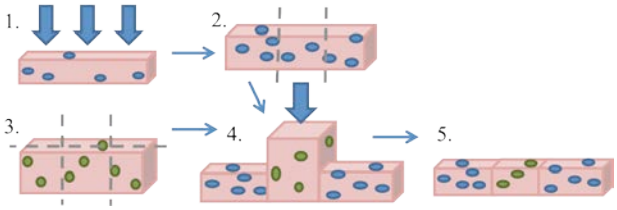


*Figure 3. Creating co-culture model with AM-1 cells: 1. Compressed collagen gel with bone cells or fibroblasts, 2. Remove some of the gel, 3. Collagen gel with AM-1 cells, 4. Compress to combine gels, 5. Incubate and analyse.*

## DISCUSSION & CONCLUSIONS:

Despite being an invasive tumour cell line, AM-1 cells displayed attachment-dependence and did not form spheres. For further development of the 3D model, soft tissue scaffolds of compressed collagen and Matrigel (BD Biosciences) with gingival fibroblasts and bone models using Bio-Oss (Geistlich Biomaterials), compressed collagen and bone cells have been developed. AM-1 cells will be incorporated into these scaffolds as detailed in fig. 3.

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## ACKNOWLEDGMENTS:

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# Optimisation of a novel neurite outgrowth model to study the role of neurite inhibition in human stem cell derived neurons

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**INTRODUCTION:** Spinal cord injury results in activation of molecules that suppress reinnervation of broken nerves and inhibit growing neurites. To fully understand the molecular mechanisms involved in this process, we are developing a novel *in vitro* cell-based model. This involves a combination of human stem cell-derived neurones and a highly efficient process of neuritogenesis within a 3D culture system. Initially, we are focused on the optimisation of neuritogenesis using novel synthetic retinoids to produce a robust model of neurite outgrowth.

All-*trans* retinoic acid (ATRA) has important roles *in vitro* during neural development.[1] It can be utilised *in vitro* to promote differentiation of pluripotent cells, [1] but is limited due to its ability to undergo photoisomerisation and degradation.[2] Synthetic analogues of ATRA such as EC23 and AH61 do not readily breakdown when exposed to light[3] therefore, are more stable for use *in vitro*. The plutipotent embryonal carcinoma cell line, TERA2.cl.SP12 differentiate into neural subtypes when treated with retinoids.[4] The purpose of this aspect of the study is to optimise the use of retinoids in neurite outgrowth from TERA2.cl.SP12 cell aggregates.

**METHODS:** TERA2.cl.SP12 cells were maintained as 2D monolayers with passaging every 3 days. Cells were seeded at a density of $1.5x10^6$ per sterile Petri dish and allowed to form cell aggregates in suspension for 24 hours prior to retinoid treatment. Aggregates were maintained in suspension for 21 days and were subsequently placed in a 48 well tissue culture plate coated in poly-D-lysine and laminin to promote neurite outgrowth in the presence of mitotic inhibitors. After 10 days, cells were fixed with 4% PFA and prepared for immunocytochemistry.

**RESULTS:** The stem cells differentiated into neurons and formed multiple TUJ-1 positive neurites that projected radially from the aggregate of neural perikarya. Quantification of the number and lengths of neurites was determined using Image J. Each retinoid tested induced the formation of neurites with synthetic retinoids (AH61 and EC23) inducing significantly more and longer neurites compared to ATRA. Neurite outgrowth appeared to be maximal at 1μM AH61 and 0.01μM EC23, whereas lower concentrations of ATRA resulted in less neurites and migration of cells from the aggregate, (see Figure).

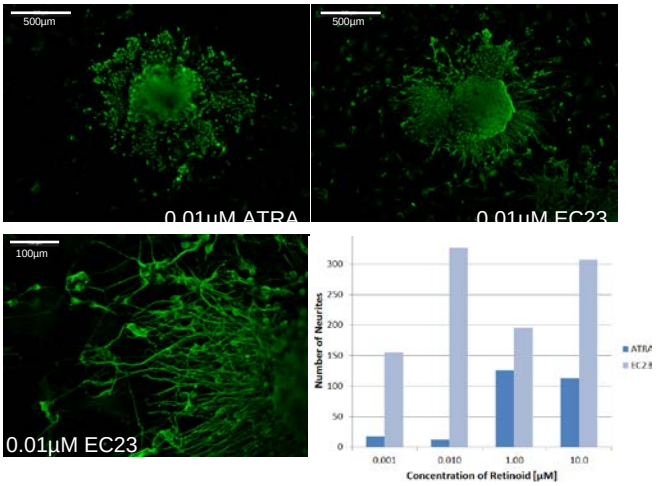


*Fig. TUJ-1 expression for aggregates differentiated with 0.01μM ATRA and 0.01μM EC23 and a plot showing the relationship between neurite number and retinoid concentration for both ATRA and EC23.*

**DISCUSSION & CONCLUSIONS:** The data demonstrate that synthetic retinoids can be used to efficiently induce neural differentiation from human stem cells, resulting in the formation of significant levels of neuritogenesis. Synthetic molecules EC23 and AH61 provide the added benefit of compound stability and greater reproducibility. This technology is being further developed to create a novel 3D cell culture system to investigate neurite inhibition in response to the glial scar that forms after spinal cord injury.

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**ACKNOWLEDGMENTS:** This research is funded by a BBSRC CASE studentship in collaboration with Reinnervate Ltd.

# Nutrient transport properties of tissue engineering membranes and scaffolds

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## INTRODUCTION:

The idea of growing artificial tissues in bioreactors such as hollow fibre membrane bioreactors (HFMBs) has started some time ago and preparation of biocompatible porous membranes and scaffolds has been attempted extensively. There have also been a number of studies on modelling glucose transport processes in HFMB. However, there is little information available that discusses specifically the glucose diffusivity across tissue engineering membranes or scaffolds and, importantly, its dependence on the properties of the materials (i.e., membrane and, scaffold)[1]. In this study, we construct a simple diffusion cell to determine scale dependent glucose transport processes for a number of porous membranes and scaffolds of different pore size and shapes, saturated with water and cell culture media (CCM). Porosity and tortuosity of the used materials are determined and consequently correlated to the glucose diffusion coefficient values.

## METHODS:

Five different membranes and scaffolds were employed, which include cellulose nitrate membrane (CN), polyvinylidene fluoride membrane (PVDF), poly(L-lactide) scaffold (PLLA), poly(caprolactone) scaffold (PCL) and collagen scaffold (see Table 1).

## DIFFUSION EXPERIMENT:

The diffusion cell consisted of two half chambers with identical volumes, namely donor and receptor phase. The membrane/scaffold was fixed in between. The donor phase was filled with glucose solution while the receptor phase contained pure water or CCM. The whole apparatus was placed in a thermostated water bath at either 27 or 37 ± 1°C. Samples were taken from both the donor and receptor phase at intervals of 1 h until equilibrium was established. A UV spectrophotometer was used to monitor the change in glucose concentration over time for materials saturated with water while an YSI glucose analyser was used for experiments in CCM.

## RESULTS:

Data revealed an increase in the diffusion coefficient at a larger pore size, indicating least resistance of glucose molecules diffusing through the pores. Data also showed a significant reduction of glucose diffusion coefficient through materials saturated with CCM at a given temperature. For instance, the glucose diffusion coefficient of PVDF membrane was reduced from 1.87 x $10^{-10}$ $m^2/s$ to 7.68 x $10^{-11}$ $m^2/s$ when the membrane was saturated in water and CCM at 37°C, respectively.

*Table 1 Characteristics of the materials studied*

| Material | Thickness (µm) | Pore size (µm) |
|---|---|---|
| PVDF | 125 | 0.1 |
| CN | 122.5 | 0.45 |
| PLLA | 50 | 12-18 |
| PCL | 50 | 20-30 |
| Collagen | 1500 | 80 |

## DISCUSSION & CONCLUSIONS:

In this study, the significance of the increase of diffusion coefficient with increasing pore size of the materials was derived. It was also observed that glucose diffusion coefficients through membrane and scaffold pores saturated with CCM are significantly reduced at a given temperature which is contrary to what have been assumed in the previous studies on glucose transport processes in HFMB or similar bioreactors.

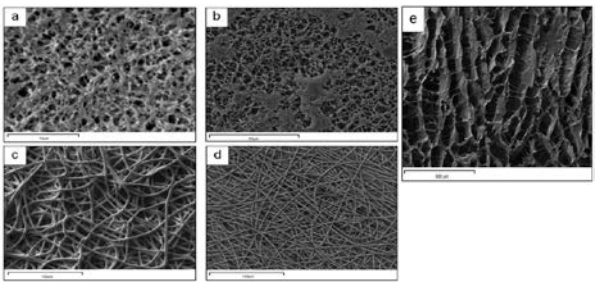


*Fig. 1 SEM micrographs showing morphology of: (a) PVDF membrane, (b) CN membrane, (c) PCL scaffold, (d) PLLA scaffold and (e) Collagen scaffold*

## REFERENCES:

[1]Liu et al (2013), Journal of Tissue Engineering and Regenerative Medicine, DOI: 10.1002/term.1654

## ACKNOWLEDGMENTS:

The authors gratefully acknowledge Brunei Government for funding this work.

# Development of pre-clinical musculoskeletal models to investigate the onset and degeneration of osteo-arthritis

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## INTRODUCTION:

Osteoarthritis (OA) is a musculoskeletal disease characterised by the degeneration of hyaline cartilage. Recent reports suggest that the onset and degeneration of OA may reflect pathology across the whole joint, as an integrated 'organ'[1]. *In vitro*-based tissue engineered models allow for highly controlled investigations of potential factors involved in the onset and degeneration of OA, including biomechanics and inflammation.

## METHODS:

The well characterised type-1 collagen-based skeletal muscle model[2], has been used to investigate the acute cyto-mechanical responses of both myoblasts and myotubes to unloading. We also have recently established a tissue engineered cartilage model, to investigate the response of chondrocytes to different modes of acute mechanical compression.

## RESULTS:

Utilising an established model of tissue engineered skeletal muscle, we have been able to demonstrate that myoblasts and myotubes respond differently to acute mechanical unloading. The force change is reduced in myoblast cultures, whereas in myotubes there is a degree of compensatory reloading to maintain tissue tensional homeostasis.

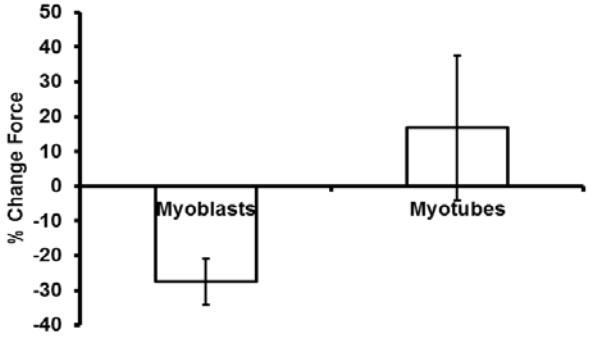


**Figure 1.** Response of myoblast and myotube cultures to -10% mechanical unloading.

Acute mechanical compression of tissue engineered cartilage, resulted in diverse chondrocyte morphology. Static loading contributed to the development of extensive actin networks, whereas dynamic loading reduced the number of actin positive processes compared to control.

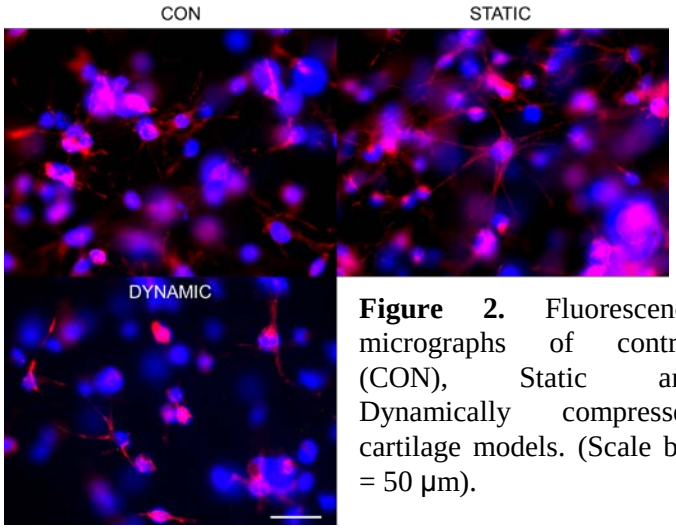


**Figure 2.** Fluorescence micrographs of control (CON), Static and Dynamically compressed cartilage models. (Scale bar = 50 μm).

## DISCUSSION & CONCLUSIONS:

The experiments presented herein, demonstrate a programme of research to identify mechanisms that contribute to the onset and degeneration of OA. The mechanisms by which compensatory tension exists in myotube cultures are yet to be elucidated, however it is thought to be mediated by both active (actin-myosin) and passive cytoskeletal-extracellular matrix (desmin, titin, integrins etc.) components. The mode-dependent response evident in the cartilage mechanical compression model, will allow future experiments to investigate potential cartilage-muscle cross-talk mechanisms.

## REFERENCES:

1. Loeser et al (2012), *Arthritis Rheum.* 64(6):1697-707. 2. Player et al (2014), Biotech. Letters. DOI 10.1007/s10529-014-1464-y.

**ACKNOWLEDGMENTS:** This work is funded as part of the Arthritis Research UK Centre for Sport, Exercise and Osteoarthritis.

# Development of Novel 3D Skin Models to Study Barrier Function

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**INTRODUCTION:** The skin is the largest organ in the body and has evolved to perform many specialised functions including as a barrier to the environment and toxins, preventing water loss, acting in immune response and resisting mechanical stresses. Barrier function is of huge importance in maintaining homeostasis of the organism. Many disease states such as Netherton syndrome, icthyosis vulgaris and different types of xerosis are due to a loss of barrier function[1]. By studying how the skin is able to develop and maintain this barrier, will lead to insight into the pathways and controls of skin function. This knowledge will aid in the development of new treatments for such skin conditions.

This project aims to use novel three-dimensional (3D) skin models to investigate the roles of Vitamin A (retinol) in skin homeostasis. In particular, the development of barrier function in relation to tight junction (TJ) complexes. These 3D models will lead to a greater homology to *in vivo* samples over existing methods

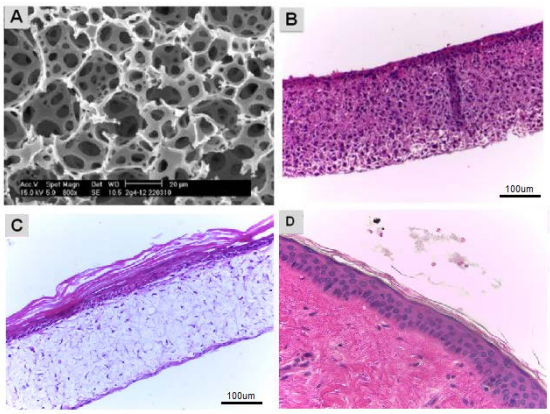



*Fig 1A: SEM image of Alvetex®Scaffold and H&E images for B: HaCaT cells grown in Alvetex®, C: Primary human keratinocytes grown on top of Alvetex®Scaffold filled with human embryonic fibroblasts and D: human skin sample.*

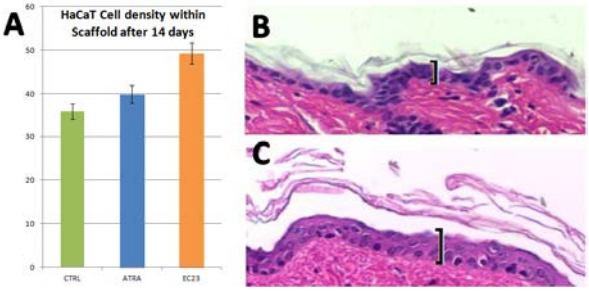
**METHODS:** Two 3D models of skin have been developed using Alvetex®Scaffolds. The first uses the established keratinocyte cell line (HaCaT) and the second utilises primary human keratinocytes isolated from tissue samples which are grown in co-culture with human embryonic fibroblasts. Growing the cells in 3D allows for the cells to develop and stratify which closely mimics structures that are seen *in vivo*. This allows more accurate assessment of the changes in barrier formation and TJ development by vitamin A. Results from these model systems are then collated back to data collected from *in vivo* samples from humans and mice, to further validate the models and findings collected from them.

**RESULTS:** Our data shows that when the HaCaT cells are grown within the scaffold they are able to grow in dense layers (Fig 1B) and have much greater contact with surrounding cells than is seen in traditional 2D culture. The cells also begin to differentiate at the top surface of the culture into the beginnings of a cornified layer, this mimics what is seen *in vivo*. The tightly packed nature of the cells also allows for the formation of more cell-cell junctional complexes (Fig 2), which are limited in standard 2D culture.

Co-culture of primary human keratinocytes on top of human embryonic fibroblasts grown in the scaffold (Fig 1C) shows even greater similarities to the structure of mammalian skin (Fig 1D). The cells show distinct stratification and cornification at the air-liquid interface.

*Fig 2: A; Graph detailing changes in cell density of HaCaT cells grown in Alvetex®Scaffold when treated with retinoid compounds. B; Mouse epidermis without treatment, C; mouse epidermis 10 days after topical retinoid treatment.*

Treatment of keratinocytes with retinoid compounds, ATRA and ec23, causes notable changes to the cells behaviour and




morphology in all model systems. The HaCaT model shows increases in cell density with application of retinoids (Fig 2A), which is correlated to an increase in epidermal thickness and more retention of the stratum corneum in mouse studies (Fig 2B/C). Treatment of the primary model with retinoid compounds is ongoing.

**DISCUSSION & FUTURE WORK:** Our models show great potential for studying cell morphology and protein expression during treatment with exogenous compounds. The models show close homology with *in vivo* skin samples. Ongoing work involving treatment with retinoid compounds will allow us to investigate the effects of these molecules on the structure and barrier properties of our skin models.

**REFERENCES:** 1 Ishida-Yamamoto et al (2011) Journal of Dermatology **38,** 645–654 .2 Simpson et al (2011) Nature Rev. Mol. Cell Biol. **12,** 565-580

**ACKNOWLEDGEMENTS:** This work was funded by a CASE studentship from BBSRC, with industrial support from Reinnervate Ltd.

# Demethylation of a crucial nf-κb enhancer element orchestrates *inos* induction in osteoarthritis

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## INTRODUCTION:

Osteoarthritis (OA) is a complex disease of the joint, characterized by progressive degradation of the cartilage matrix by aggrecanases and collagenases. Nitric oxide (NO), the product of inducible nitric oxide synthase (*iNOS*), not only suppresses the synthesis of cartilage matrix, but also increases expression of proteases in OA. DNA methylation is an epigenetic mechanism implicated in the induction of *iNOS* in OA. We have examined the methylation profile of the NF-κB enhancer region at -5.8 kb of this gene, important in OA[1] to determine the role in *iNOS* induction of OA.

## METHODS:

Methylation was determined in human articular chondrocytes and the chondrocytic cell line C28/I2. Specifically, percentage methylation was determined by pyrosequencing and gene expression by qPCR. Cell proliferation was determined using the MTT assay.

## RESULTS:

*In vitro* de-methylation of the CpG sites localised at -5.8 kb showed decreased levels of DNA methylation in control chondrocytes (Fig1), which correlated with higher levels of *iNOS* expression. *In vitro* methylation of the NF-κB enhancer region at -5.8 kb showed high levels of apoptosis and G0/G1 arrest (Fig2).

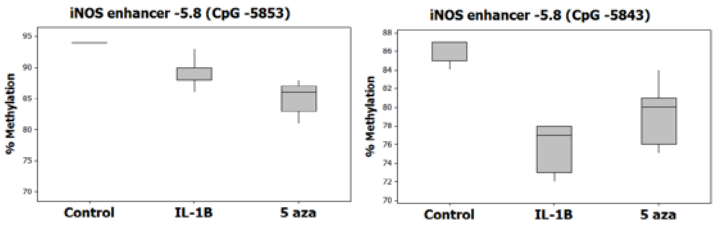


*Fig. 1 Methylation of CpG sites in the -5.8 kb enhancer element of iNOS is affected by IL-1β and in vitro demethylation by 5-aza-dC.*

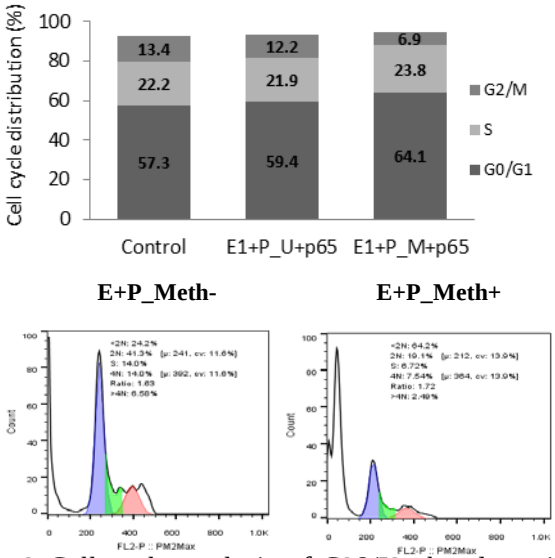


*Fig. 2 Cell cycle analysis of C28/I2 chondrocytic cells transfected with the crucial NF-κB enhancer element before (Meth-) and after (Meth+) in vitro methylation treatment with a CpG methyltransferase. Results show the mean ± SD of three independent experiments.*

## DISCUSSION & CONCLUSIONS:

The NF-κB enhancer element localised at -5.8 kb upstream of the transcription start site is important for *iNOS* induction. Loss of methylation of this region correlated with lower levels of apoptosis, enhanced proliferation and increased cells at G2/M phase. The loss of methylation and consequent higher *iNOS* expression observed therefore, could be transmitted to daughter cells during cell division and, critically, contribute to OA pathology.

## REFERENCES:

1. de Andrés et al (2013), *A&R* **65**: 732-42.

## ACKNOWLEDGMENTS:

The authors would like to thank the Leverhulme Trust for financial support for this project.

# Novel polymer topography to influence mesenchymal stem cell behaviour

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## INTRODUCTION:

A cells response to the physical environment can regulate gene expression and differentiation[1]. Tailored architecture and topography of implants can regulate cell activity to augment the use of autologous progenitor cell populations to rebuild a diseased tissue more successfully. Adaptation of implant surfaces with appropriate temporal and spatial cues, possibly in combination with biomolecules and/or biomolecular motifs has the potential to influence multipotent cell activity influencing cell orientation, organisation and potentially cell differentiation leading to control of tissue formation and structure.

The aims of this project are to explore the influence of polymer surfaces on mesenchymal stem cell (MSC) biology and exploit different polymer chemistries to functionalize surfaces with biomolecules. These studies will assist scaffold design and fabrication, to aid regenerative applications.

## METHODS:

Thin films of polymers were spread onto a flat substrate by spin coating at high speeds. Using a combination of immiscible polymers to generate a micro-scale landscape of opposing structures. Addition of water induces breath figure patterns, creating pores and crators to further accentuate the topographical pattern. Immiscible polystyrene (PS) and poly(methyl methacrylate) (PMMA) solutions were demixed at various ratio's (v/v) and spun at speeds > 8,000rpm under humid conditions[2] . Topographical surfaces were assessed using atomic force microscopy (AFM) and scanning electron microscopy. Primary adult human MSCs, isolated from bone marrow were characterized by flow cytometry and tri-lineage differentiation. Biological responses to surfaces were evaluated using immunofluorescece, histological staining and qPCR.

## RESULTS:

After evaluation of a range of demixed ratios/ concentrations/ solvents a selection of surfaces [PS:PMMA 40:60/50:50/60:40] 3%w/v in toluene] were further characterized. These generated opposing raised PMMA and low-lying PS islands. Breath figure patterns generated crater-like features ranging from 0.5-1µm in height and depth (Fig. 1A). The raised *caldera* attracted increased cell interactions, where cells concentrate focal adhesion plaques (Fig1.B) to the raised features; altering their morphology (Fig.1 C) and gene expression (Fig.1D). Adaptation of demixed polymer ratios altered the spatial distribution of *Caldera*.

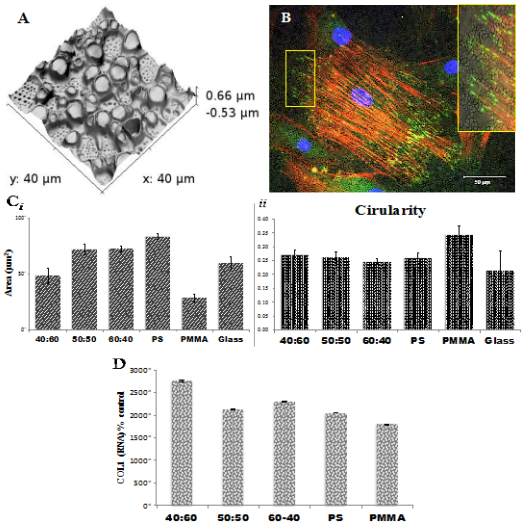


**Figure 1.** Demixed PS:PMMA [50:50] **A)** AFM 3D image **B)** Immunofluorescent staining of hMSC (P-2) 72hr. Focal adhesion plaques [*Vinculin*-FITC; green]; F-Actin cytoskeleton [*Rhodamine;* red]; Nucleus [Dapi; blue]. **C)** Quantification of cell morphology; ***i)*** Cell area and ***ii)*** Circularity. **D)** qPCR: COL1 expression (ΔΔCt) % control (glass) after 14days culturing on surfaces.

## DISCUSSION & CONCLUSIONS:

Demixed polymer blends provide tuneable topographies for the stimulation of various cell responses through regulation of adherence dynamics. Utilizing the immiscible polymer chemistries we can restrictively alter the dispersion pattern of humidity induced pores and *Caldera,* to induce a biological response. Tailoring these surfaces to influence specific cell types has the potential to manipulate the tissue formation and hence tissue function.

## REFERENCES:

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2. Park, M.S. and Kim, J.K. *Langmir* 20, 5347-5352 (2004)

## ACKNOWLEDGMENTS:

Authors would like to acknowledge support from Arthritis Research UK (Award 19429).

# Demethylation of a crucial nf-κb enhancer element orchestrates *inos* induction in osteoarthritis

María C. de Andrés[1], Atsushi Takahashi[1], Karl Alvarez[1], Richard O.C. Oreffo[1]
[1]Bone and Joint Research Group, University of Southampton, UK
mdag1d08@soton.ac.uk

## INTRODUCTION:

Osteoarthritis (OA) is a complex disease of the joint, characterized by progressive degradation of the cartilage matrix by aggrecanases and collagenases. Nitric oxide (NO), the product of inducible nitric oxide synthase (*iNOS*), not only suppresses the synthesis of cartilage matrix, but also increases expression of proteases in OA. DNA methylation is an epigenetic mechanism implicated in the induction of *iNOS* in OA. We have examined the methylation profile of the NF-κB enhancer region at -5.8 kb of this gene, important in OA[1] to determine the role in *iNOS* induction of OA.

## METHODS:

Methylation was determined in human articular chondrocytes and the chondrocytic cell line C28/I2. Specifically, percentage methylation was determined by pyrosequencing and gene expression by qPCR. Cell proliferation was determined using the MTT assay.

## RESULTS:

*In vitro* de-methylation of the CpG sites localised at -5.8 kb showed decreased levels of DNA methylation in control chondrocytes (Fig1), which correlated with higher levels of *iNOS* expression. *In vitro* methylation of the NF-κB enhancer region at -5.8 kb showed high levels of apoptosis and G0/G1 arrest (Fig2).

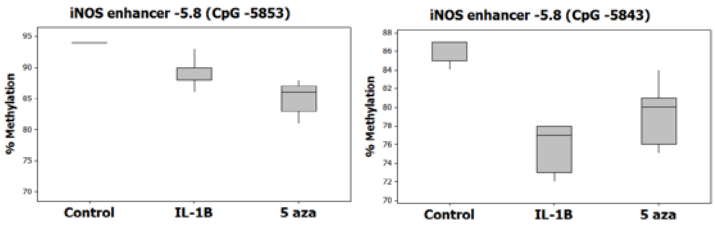


*Fig. 1 Methylation of CpG sites in the -5.8 kb enhancer element of iNOS is affected by IL-1β and in vitro demethylation by 5-aza-dC.*

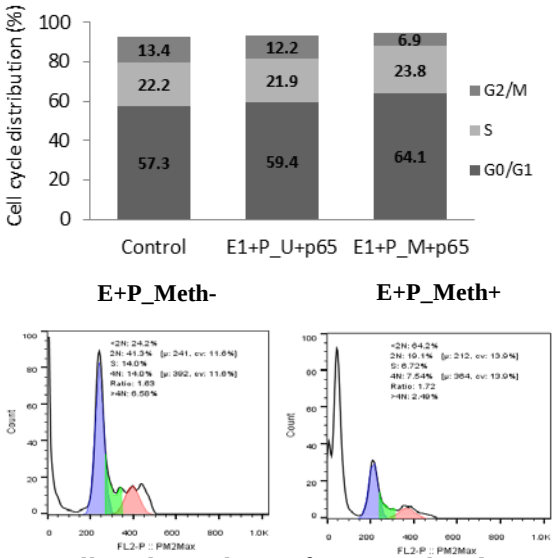


*Fig. 2 Cell cycle analysis of C28/I2 chondrocytic cells transfected with the crucial NF-κB enhancer element before (Meth-) and after (Meth+) in vitro methylation treatment with a CpG methyltransferase. Results show the mean ± SD of three independent experiments.*

## DISCUSSION & CONCLUSIONS:

The NF-κB enhancer element localised at -5.8 kb upstream of the transcription start site is important for *iNOS* induction. Loss of methylation of this region correlated with lower levels of apoptosis, enhanced proliferation and increased cells at G2/M phase. The loss of methylation and consequent higher *iNOS* expression observed therefore, could be transmitted to daughter cells during cell division and, critically, contribute to OA pathology.

## REFERENCES:

1. de Andrés et al (2013), *A&R* **65**: 732-42.

## ACKNOWLEDGMENTS:

The authors would like to thank the Leverhulme Trust for financial support for this project.

# Surface modification of injectable microspheres for cell therapy applications

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## INTRODUCTION:

Injectable microspheres offer a minimally invasive approach to deliver cells for tissue repair. As substrate elasticity can direct stem cell fate[1], the aim of this study was to develop a method to alter the elasticity of PLGA microspheres with methacrylated gelatin (GelMA) using surface entrapment. Cross-linked GelMA supports cell growth[2] and has controllable elasticity[3]. Developing a technique to modify the surface of injectable microspheres with GelMA presents the opportunity to tune microsphere surface elasticity and thus influence cell fate for cell therapy applications.

## METHODS:

GelMA was prepared by methacrylating porcine and fish gelatin. GelMA hydrogels were formed by UV crosslinking with photo-initiator (Ergacure 2959). Elasticity was assessed with a TA.HD+ texture analyzer. PLGA microspheres were surface modified with TFE/GelMA solution and immobilized GelMA was UV cross-linked. Microspheres were visualized using microscopy. Bicinchoninic acid (BCA) protein assay was used to quantify surface immobilized GelMA.

## RESULTS:

Fit-C labelled GelMA was observed on the surface of microspheres using fluorescent microscopy (Fig.1A). Differences in surface morphology between non-modified (blank) and surface modified microspheres were visualized by SEM (Fig.1B). BCA assay results demonstrated the presence of protein (GelMA) on modified microspheres (Fig.2). Increased protein levels were observed with higher concentrations of (TFE) used in the surface entrapment process.

## DISCUSSION & CONCLUSIONS:

A promising approach for modifying the surface of PLGA microspheres with GelMA was developed. Future work will focus on altering GelMA elasticity to create a tuneable delivery system for cell therapy applications.

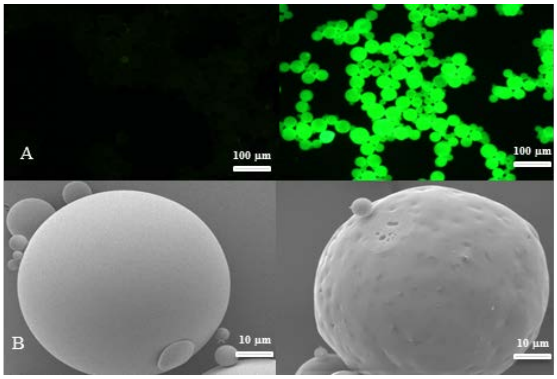


*Figure 1 Images of blank PLGA microspheres (Left) and gelMA modified microspheres (Right) as shown in (A) fluorescent microscope, and (B) scanning electron microscope SEM*

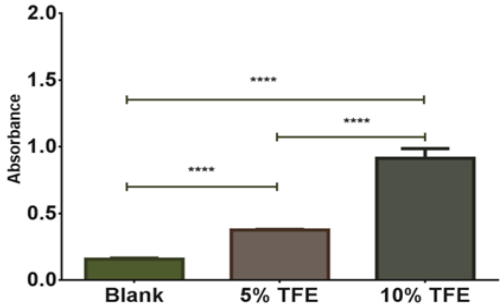


*Figure 2 BCA assay results showing the change in gelMA concentration on modified PLGA microspheres.*

## REFERENCES:

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2. Nichol et al., Biomaterials, 31(21): 2010.
3. Van Den Bulcke et al., Biomacromolecules, 1(1):2000.
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## ACKNOWLEDGMENTS:

Authors would like to thank the UK RMP Hub for support in this research.

# Dynamic patterned electrospun fibres for 3d cell growth

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**INTRODUCTION:** Cell based therapies offer potentially revolutionary treatments for a number of diseases, but are dependent on the culture and supply of defined cell types in appropriate numbers. In turn, this supply requires culture environment that mimic the 3D structure *in vivo* and maintain the integrity of the cultured cells. The project was devised following the work published by Dey *et al.*, with the vision to take this work previously completed in 2D to a 3D matrix [1]. Here we demonstrate the use of co-electrospun poly (lactic-co-glycolic acid) (PLGA) and poly (ethylene terephthalate) (PET) with poly (poly (ethylene glycol) methacrylate) Poly (PEGMA) to produce three dimensional, non-woven and thermoresponsive fibres on which to culture red fluorescent protein expressing 3T3 fibroblast cells.

**METHODS:** Poly (PEGMA) was prepared by free radical polymerization [2], then mixed with 18.5% PLGA or 30% PET in six different concentrations (0%, 2%, 4%, 6%, 8%, 10% (*w/v*)) to form thermoresponsive scaffolds by a blend-electrospinning technique. Then, their thermoresponsive behaviors were characterized by $^{1}$H-NMR, XPS and WCA measurements. 3T3 fibroblast cells were seeded on these fibres and cultured for five days. Subsequent cell viability tests (Almar Blue assay) were performed to measure the difference in cell populations while changing the culture temperature.

**RESULTS:** This study has demonstrated that blend-electrospinning is a promising way to create fibres with thermoresponsive surfaces, with poly (PEGMA) presence confirmed by XPS, $^{1}$H-NMR and WCA measurements. These responsive scaffolds were able to support 3T3 fibroblast cells adhesion and proliferation at 37 °C. Also, it was possible to detach the cells from the scaffolds by decreasing the temperature to 15 °C, (Fig. 1), such that the poly (PEGMA) chains were strongly hydrated. Irrespective of the concentration of poly (PEGMA) used, all scaffolds exhibit thermoresponsive proprieties and cells were viable and proliferated in a similar manner to those cultured on control surfaces (PLGA or PET scaffolds) except with 10% concentration cell proliferation were less.

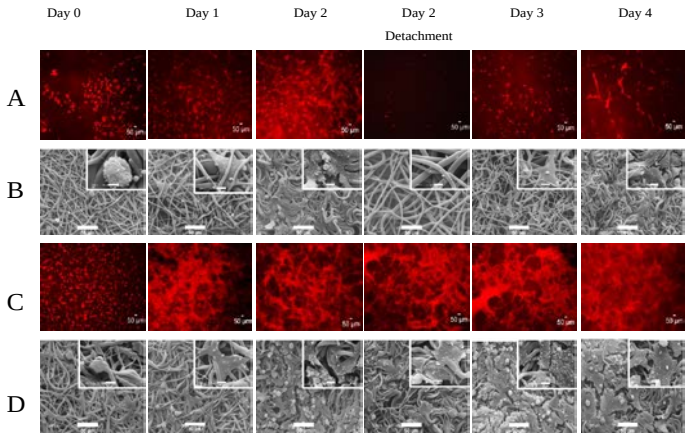


Fig. 1 Representative fluorescence microscopy and SEM images showing 3T3 cells attachment to (A-B) PLGA- 4% poly (PEGMA) and (C-D) PLGA scaffolds surfaces after 6 hrs at varying temperature–time regimes.

**DISCUSSION & CONCLUSIONS:** These thermoresponsive, non-woven, and bead-less co-electrospun fibres were found to be suitable for 3T3 fibroblast culture by supporting their attachment, proliferation and detachment. We are currently further investigating the use of this thermoresponsive system for prolonged cell culture and passaging experiments.

**REFERENCES:**

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2. Al Ghanami, R.C., et al. (2010), Soft Matter 6: 5037-5044.

**ACKNOWLEDGMENTS:** Funded by government of Saudi Arabia.

# Directional cues for bone regeneration in the avian growth plate

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## INTRODUCTION:

Enhancement and application of our understanding of skeletal developmental biology is critical to developing new tissue engineering approaches to skeletal repair. We have previously developed a 3D *ex vivo* culture system of embryonic chick femora[1], used within this study to analyse critical directional osteogenic and chondrogenic cues present within the developing growth plate.

## METHODS:

Central segmental defects were created in embryonic day 11 (E11) chick femurs. Regions of growth plate (resting and proliferative) were excised from separate E11 chick femurs, and implanted into the segmental defects. Orientation was either maintained the same, or reversed. In addition, to assess whether cues were matrix- or cell-based, resting and proliferative cells were isolated into matrix-dissociated cell pellets, and either placed into femoral defects or assessed *in vitro* for markers of bone (collagen I), cartilage (collagen II, collagen X, proteoglycan), and proliferation (PCNA). Femurs were cultured for 10 days in basal media, and assessed histologically for proteoglycan (Alcian blue), collagen (Sirius red) cell proliferation (PCNA) and the presence of skeletal stem cells (STRO-1).

## RESULTS:

Isolated growth plate cells cultured *in vitro* demonstrated unique morphologies dependent on the regions from which they were isolated. Resting zone cells displayed a fibroblastic morphology, whereas cells from the proliferative zone displayed a small, rounded morphology with increased proteoglycan expression. PCNA and collagen II was expressed in both cell types, and no hypertrophy was detected (collagen X). Implanted whole growth plate regions initiated host tissue integration on only one side of the defect, depending on the orientation of the implanted growth plate region. Removal of growth plate cells from within their natural extracellular matrix disrupted this directional response with host tissue integration at both regions and a strong host periosteal induction (Fig. 1).

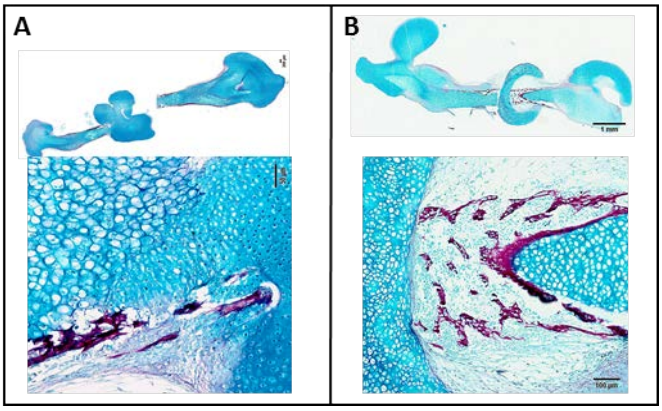


*Fig. 1: Implanted growth plate regions integrate into host tissue dependent on orientation (A), which could be disrupted by removal of cells from their ECM (B).*

## DISCUSSION & CONCLUSIONS:

Implantation of whole growth plate regions into a chick femur defect model indicates the presence of directional cues within the growth plate that are critical for ordered directional bone growth *in vivo*. These studies provide new approaches to understanding construction of cartilage structures & chondrocyte organisation.

## REFERENCES:

1. J.M. Kanczler, E.L. Smith et al. (2012) *Tissue Eng Part C: Methods* **18**(10): 747-60.

## ACKNOWLEDGMENTS:

This work was supported by the strategic longer and larger grant (LOLA) from the BBSRC, UK-grant number BB/G010579/1.

# Do chondrogenic atdc5 cells in dense collagen make a simple cartilage?

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## INTRODUCTION:

Current *in vitro* models rely on flat monolayers of cells because they offer a quick, cost-effective and easy analysis. Nonetheless, in the body, cells are not attached flat, on one side to an ultra-stiff surface; they are embedded into a compliant, viscoelastic fibrous mesh of extracellular matrix (ECM) rich in 3D gradients of environmental cues. It is now widely understood that in vitro 3D tissue models are needed for more reliable, physiological test and screening systems. Our aim was to develop and characterise a living 3D model of cartilage using dense collagen (type I) and a chondrogenic cell line (ATDC5).

## METHODS:

Different cellular concentrations of living 3D constructs were fabricated using the method known as Plastic Compression (PC). Constructs cultured for 1, 3 and 7 days were used to assess cell viability (Live/Dead staining) and aerobic metabolism (Alamar Blue). We used a novel optical instrument to quantify structural properties using in real time (CTS-2; Lein Diagnostics Ltd.). Matrix structure and composition was qualitatively assessed using basic H&E and Alcian Blue histology.

## RESULTS:

Our findings showed that the PC did not alter viability-activity of ATDC5 cells (fig.1), though degradation/remodelling of the collagen network (data not shown) and deposition of cartilage specific matrix components by ATDC5 cells was identified (fig.2).

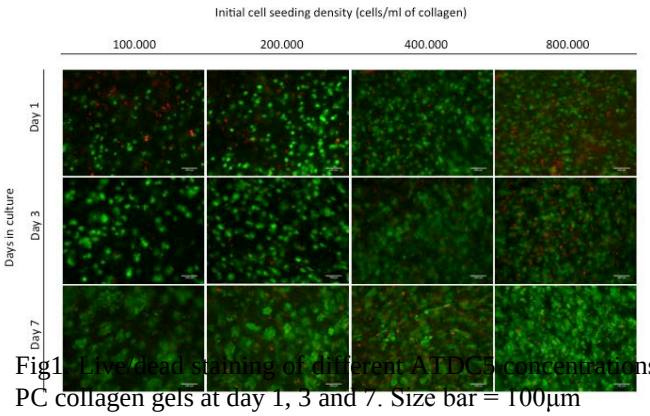
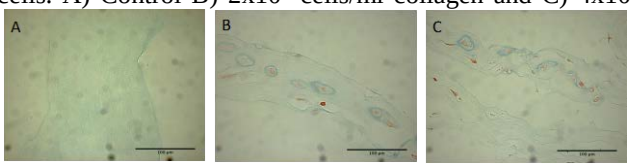



Fig1. Live/dead staining of different ATDC5 concentrations PC collagen gels at day 1, 3 and 7. Size bar = 100µm

Fig. 2. Alcian Blue staining at day 14 of ATDC5 seeded PC collagen gels denoting accumulation of PG surrounding the cells. A) Control B) $2x10^4$ cells/ml collagen and C) $4x10^4$ cells/ml collagen. Size bar = 100µm.




Thickness measurements showed that constructs swelled in culture and that this effect was enhanced slightly by the presence of cells and their products, consistent with the idea that proteoglycans deposition of swell the mesh.

## DISCUSSION & CONCLUSIONS:

We have showed that it was possible to culture ATDC5 cells in dense PC matrices with cartilage-specific matrix deposition with promise as a possible 3D model tissue of cartilage.

## REFERENCES:

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## ACKNOWLEDGEMENTS:

I would like to acknowledge the invaluable work of Prof. Robert A. Brown and the assistance of Dr. Vehid Salih.

# Discrimination between high and low proliferative/regenerative dental pulp progenitor cell clones for tissue engineering by raman spectroscopy

Amr Alraies[1,2], Elisabetta Canetta[3] Rachel Waddington[1,2], Ryan Moseley[1,2] and Alastair Sloan[1,2]
*[1]Tissue Engineering & Reparative Dentistry; Cardiff University, UK; [2]Cardiff Institute for Tissue Engineering and Repair, Cardiff University, UK, [3]School of Applied Sciences, St Mary's University College, London, UK*

**INTRODUCTION:** Distinct dental pulp stem cell (DPSC) niches exist within dental pulp, with contrasting proliferative /regenerative potential[1]. Characterisation of DPSCs is a key consideration for tissue engineering, in terms of isolated clones undergoing sufficient *in vitro* expansion, whilst maintaining their regenerative potential. However, current methods are time consuming, which may be destructive for the such cells. Our recent work has confirmed clonal differences in proliferation and differentiation are partly due to contrasting telomere lengths. However, it is unknown if non-invasive techniques, such as Raman Spectroscopy, are also capable of distinguishing between DPSC clones and this initial study used Raman Spectroscopy to characterize the signatures of high and low proliferative/regenerative DPPC clones.

**METHODS:** Individual DPSC clones were isolated from the wisdom teeth of two patients, by fibronectin adhesion. Clones were previously identified as being high and low proliferative/regenerative by population doubling levels (PDs), telomere length and differentiation studies. Clonal suspensions of highly proliferative (A31 at 18PDs and 60PDs) and low proliferative (A11 at 8PDs, B11 at 7PDs) were fixed and analysed using an iHR550 Raman Spectrometer, equipped with a camera and linked to an Eclipse Ti-U Inverted Microscope. Single cell spectra were obtained for 20 cells for each clone over 600-1800cm$^{-1}$. Ten spectra were obtained for the nuclear and cytoplasmic regions from each cell and average spectra obtained. Spectra were analysed by Origin v7 and peaks assigned by comparison to references. Raman spectra were studied using principal component analysis to achieve clonal classification.

**RESULTS:** Raman spectra contained the typical peaks for DNA, proteins and RNA. Highly proliferative clone A31 (18PDs) possessed higher spectral band intensities that A31 (60PDs) or low proliferative clones, A11 (8PDs) and B11 (7PDs). This was particularly evident for DNA (729, 789, 1092, 1402cm$^{-1}$), RNA (1054cm$^{-1}$) and amino acids/protein peaks, such as phenylalanine, tyrosine and serine (958, 1167, 1245 cm$^{-1}$). PC3 and PC4 scatter plots demonstrated overlapping signatures between low proliferative clones, A11 and B11. In contrast, A31 at 18PDs and 60PDs did not overlap with each other, or with A11 and B11.

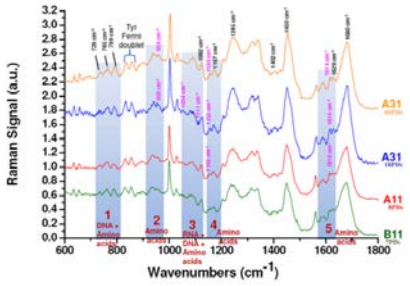


*Fig.1*
*Mean Raman spectra obtained from three clones at early PDs and A31 at later PD, displayed in the fingerprint range 600 to 1800cm$^{-1}$.*

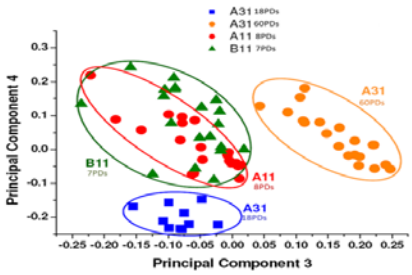


*Fig 2*
*Principal component analysis.*

**DISCUSSION & CONCLUSIONS:** This initial study demonstrates that Raman Spectroscopy may be capable of non-invasively discriminating between high and low proliferative DPSCs. As most spectral band differences identified between clone A31 and A11/B11 are implicated in the cell cycle, findings concur with our previous data that clone A31 is highly proliferative in nature. This study advocates the potential use of Raman Spectroscopy as a selective screening tool for the identification and isolation of highly proliferative/regenerative DPSCs from whole dental pulp for clinical use.

**REFERENCES:** [1]Waddington RJ, *et al.* (2009). *Cells Tiss. Organs* 189:268-74.

**ACKNOWLEDGEMENTS:** Albawani Company for their financial support.

# Introducing complexity in biomedical devices throughout the incorporation of artificial stem cell microenvironments

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## INTRODUCTION:

Stem cells are crucial for tissue regeneration and the ability of these unique cells to self-renew is thought to be at least partially due to their location within enclosed protective niches. Our aim is to design and manufacture biomaterial devices containing artificial niches for exploring the regeneration of soft tissues such as cornea and ultimately other tissues such as bone. In this study we have designed two types of microfabricated corneal outer rings (one biodegradable and the other non-biodegradable; fig.1D, 1G) and we have studied the effect of including microfeatures within the constructs.

## METHODS:

PEGDA (polyethylene glycol diacrylate) non-degradable scaffolds were manufactured using microstereolithography[1]. PLGA (polylactide-co-glycolide) biodegradable scaffolds were manufactured using electrospinning[2]. The potential use of the microstructured rings as cell delivery devices was evaluated in a 3D rabbit cornea model using both limbal tissue explants and rabbit limbal epithelial cells.

## RESULTS:

We specifically located cells in the artificial micropockets and we obtained promising results regarding epithelial cell transfer and re-epithalisation for both kinds of constructs (Fig.1). We also observed that the inclusion of microfeatures within the constructs affected epithelial cell migration and morphology. Moreover, a population of slow-cycling cells (positive for P63) was identified when tissue explants were cultured inside the artificial pockets and placed on 3D models; this was not observed when using cultured limbal epithelial cells suggesting that the enclosed niches together with the presence of native stromal cells within the explants play an important role in stem cell maintenance.

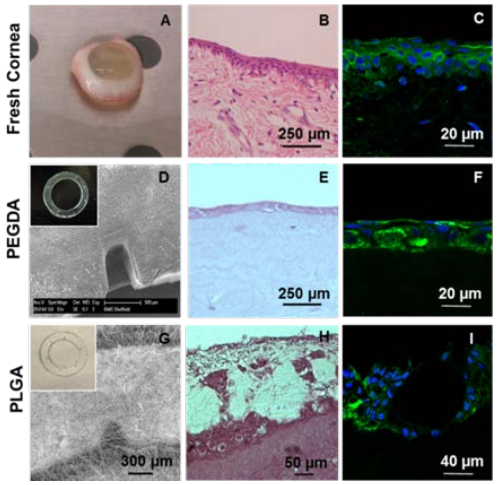


*Fig 1. Rabbit cornea model (A); H &E and CK3 staining of fresh rabbit cornea (B, C); H &E and CK3 of tissue engineered cornea after 6 weeks organ culture with a RLE loaded PEGDA ring (E, F); H &E and CK3 of tissue engineered cornea after 4 weeks organ culture with a PLGA membrane with limbal explants (H, I); SEM images of PEGDA and PLGA microfeatured outer rings (D, G).*

## DISCUSSION & CONCLUSIONS:

This work provides techniques for producing artificial niches for studying stem cell behaviour using *in vitro* and *ex vivo* models. Using corneal ring models we demonstrated that the presence of microfeatures has a direct effect on parameters such as migration, cell morphology and stemness. Future work will explore the use of similar models for the regeneration of other tissues (e.g.bone).

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1. Ortega I. et al (2013), Biofabrication **5**: 025008;
2. Ortega I. et al (2013), Acta Biomaterialia **9**: 5511

## ACKNOWLEDGMENTS:

We thank the EPSRC Landscape Fellowship Scheme and the Wellcome Trust Foundation.

# Silk-based microparticles as an osteogenic scaffold for tissue engineering

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## INTRODUCTION:

Silk fibroin (SF) has long been considered a material with potential for tissue engineering as it is biocompatible, strong, light-weight and can be processed into a number of formats[1]. However, it is not highly cell-adhesive; hence it is frequently blended with other materials such as gelatin. SF has previously been investigated as a scaffold material for bone repair in the form of porous sponges and hydrogels. The work described here investigates microparticles produced from SF/gelatin (SF/G) as osteogenic cell scaffolds. The microparticles described will allow for the creation of larger tissue constructs by the process of moulding.

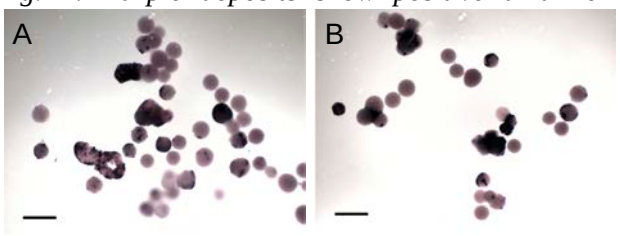
## METHODS:

An aqueous solution of SF was extracted from the cocoons of *Bombyx mori*. Gelatin was porcine derived (Type A). Blends of SF and gelatin were processed into homogenous microcarriers by axisymmetric flow focussing[2]. Mesenchymal stem cells (MSCs), harvested from the bone marrow of juvenile Wistar rats, were maintained in MEM with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin, at 37°C in a 5% $CO_2$ atmosphere. Osteogenic differentiation medium (ODM) was supplemented with 0.1 µM dexamethasone, 0.2 µM ascorbic acid 2-phosphate and 10 mM glycerol 2-phosphate. Cells seeded onto microparticles were cultured in either basal medium or ODM for a minimum of 21 days. Osteogenic differentiation was confirmed by positive alkaline phosphatase (AP) activity (BCIP/NBT assay).

## RESULTS:

MSCs were successfully seeded onto all of the blended SF/G microparticles with high efficiencies. Seeding efficiencies of the blended microparticles were significantly higher than for SF alone. Cell seeding and viability was confirmed using LIVE/DEAD stain with confocal microscopy. A high proportion of cells remained viable after culture on the SF/G microparticles. MSCs showed evidence of differentiation in both medium types (Figure 1), suggesting the scaffold itself supports differentiation in non-supplemented medium.

*Fig. 1: Purple deposits show positive alkaline phosphatase activity in MSCs cultured on SF/G 25:75 microparticles in basal media (A) or ODM (B). Scale bar represents 500 µm.*




## DISCUSSION & CONCLUSIONS:

MSCs were successfully seeded onto novel microparticles composed of SF/G blends. The microparticle scaffolds supported osteogenic differentiation in both basal and supplemented media. The results described here suggest that the production of a macroscopic bone construct could be achieved using SF/G blended materials under appropriate growth conditions.

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1. Wang, Y., et al., Biomaterials, 2006. 27(36): p. 6064-6082.
2. Terray, A. and S.J. Hart, Lab on a Chip, 2010. **10**(13): p. 1729-1731.

## ACKNOWLEDGMENTS:

Funding from the MRC is gratefully acknowledged.

# Get: glycosaminoglycan (gag)-binding enhanced transduction of functional proteins

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## INTRODUCTION:

Protein transduction domains (PTDs) are powerful non-genetic tools that allow intracellular delivery of conjugated cargoes to modify cell behaviour. Their use in biomedicine has been hampered by inefficient delivery to nuclear and cytoplasmic targets.

## METHODS:

mRFP1, Cre Recombinase, transcription factors, antibiotic resistance proteins and PTDs were cloned, fused and expressed in/purified from *E.coli* using GST affinity chromatography. Cells were as described[1]. Flow cytometry used a MoFlo$^{TM}$ DP (DAKO) using a 488nm green laser. Cre assays used NIH3t3: LSL-eGFP cells created in-house.

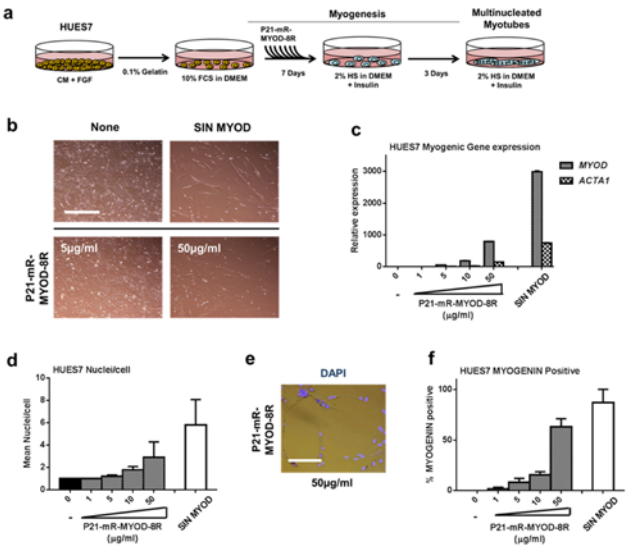
## RESULTS:

Here we overcame PTD deficiencies by developing a novel fusion protein that couples a membrane docking peptide to heparan sulfate glycosaminoglycans (GAGs) with a PTD. We showed this GET (GAG-binding enhanced transduction) system could deliver fluorescent reporters (mRFP1), functional enzymes (Cre, neomycin phosphotransferase) and transcription factors (NANOG, MYOD) at efficiencies of up to two-orders of magnitude higher than previously reported in cell types considered hard to transduce, such as mouse embryonic stem cells (mESCs), human ESCs (hESCs) and induced pluripotent stem cells (hiPSCs).

## DISCUSSION & CONCLUSIONS:

This technology represents an efficient strategy for controlling cell behaviour and directing cell fate that has broad applicability for basic research, disease modelling and clinical application.

***Figure 1. GET of MYOD promotes Myogenic differentiation of human embryonic stem cells. (a)*** *Scheme of testing the differentiation activity of transduced MYOD in HUES7 cells.* ***(b-f)*** *P21-mR-MYOD-8R drives myogenic differentiation of HUES7 cells to multinucleated Myotubes.* ***(b)*** *Light microscopy of HUES7 cells cultured under the myogenic regime. bar, 100µm.* ***(c)*** *Relative gene expression analyses of HUES7 cultures using quantitative PCR (QPCR). Error bars indicate s.e.* ***(d-f)*** *P21-mR-MYOD-8R differentiated cells are multinucleated and MYOGENIN positive. Error bars indicate s.d.*




## REFERENCES:

1. Dixon et al. (2014). PNAS. 10.1073

## ACKNOWLEDGMENTS:

We would like to thank Dr. Andrew D. Johnson (University of Nottingham) and Dr. Catherine Merry (University of Manchester) for helpful discussions. We thank the European Research Council under the European Community's Seventh Framework Programme (FP7/2007-2013)/ERC grant agreement 227845 for funding. KMS acknowledges the support of the UK Regenerative Medicine Platform.

# Nanoindentation of hydrogel composites determined by a flat punch tip

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## INTRODUCTION:

As a non-destructive approach, nanoindentation is effective to determine the materials properties of heterogeneous materials in tissue engineering with high-resolution[1]. This is important because cells sense local stiffness instead of bulk stiffness[2]. Nanoindentation with a flat punch tip is particularly good for indenting very soft biopolymer like hydrogels[3]. However, the relation between the nanoindentation elastic responses of the two-phase hydrogel composite and the stiffness ratio of these two individual hydrogels remains elusive. Therefore, we will use finite element (FE) simulations and mathematical model to explore such correlation.

## METHODS:

An axisymmetric FE model is adopted to study the nanoindentation of the two-phase hydrogel via a flat punch with radius 0.5 μm and a fillet radius of 0.01 μm (see Fig.1). For phase 1, its elastic modulus is fixed at 10kPa and for phase 2 it is varied from 2 to 100kPa which covers a range of typical hydrogels for tissue engineering. Their viscoelastic behaviour is captured by Prony series model.

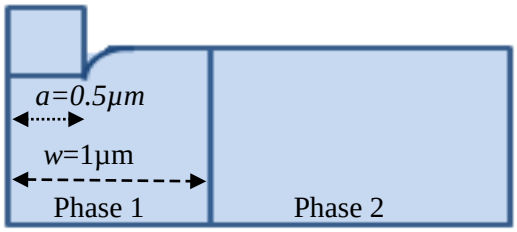


Fig. 1 Schematic of indentation of a two-phase hydrogel with a flat punch tip.

## RESULTS:

Fig.2 displays some typical force-displacement curves during nanoindentation. Fig.3 shows the relation between the elastic responses of the two-phase hydrogel composite and the stiffness ratio of these two individual hydrogels based on the Clifford model[4].

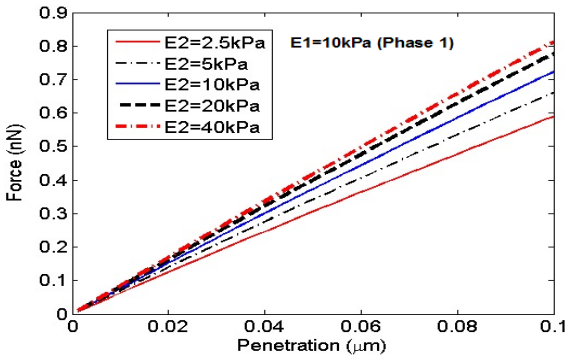


**Fig. 2** Typical force-displacement curves.

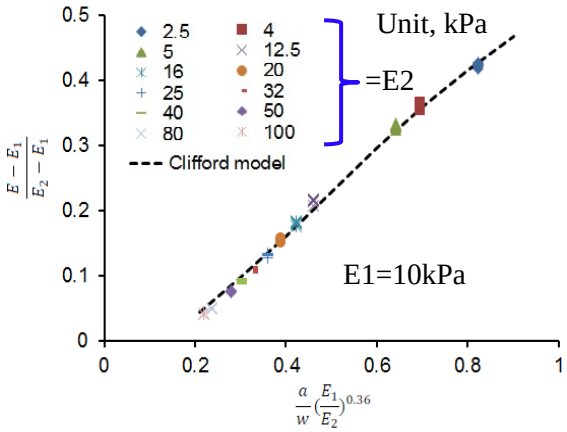


Fig.3 Relation between the elastic responses of the composite and the stiffness ratio of these two individual hydrogels.

## DISCUSSION & CONCLUSIONS:

It has revealed the surrounding matrix has significant contribution to the measured modulus of the inclusion phase. The Clifford model originally developed for indenting elastic materials via a spherical tip can be modified to predict such an influence for indenting viscoelastic materials with a flat punch indenter.

## REFERENCES:

1. E.T. Herruzo et al (2014) *Nat Commun* **5**: 3126/1-8.
2. J. Chen et al (2014) *Acta Mech. Sin.***30:**2-6
3. J. Chen (2014) *Interface Focus* **4**: 20130055/1-17
4. C.A. Clifford et al (2012) *Nanotechnology* **23:** 165704/1-7

# Nanomechanical responses of biopolymer composites determined by nanoindentation with a conical tip

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## INTRODUCTION:

Nanoindentation is particularly useful to determine the elastic moduli of individual phases in composite materials (e.g. biopolymer composites for tissue engineering) [1]. Indeed, the engineered tissues are also composite materials. When performing nanoindentation on these composite materials, it is essential to define the influence of surrounding second phase. In this study, we will use finite element analysis (FEA) and mathematical model to investigate such an influence.

## METHODS:

An axisymmetric FE model is established to study the nanomechanical responses of the two-phase biopolymer composites indented by a conical tip (see Fig.1). The equilibrium elastic modulus for inclusion (Ei) is assumed to be 10MPa. The modulus of the matrix (Em) varies from 2 to 50MPa which covers a wide range of typical hydrogel biopolymers. Their viscoelastic behaviour is described by Prony series model[2].

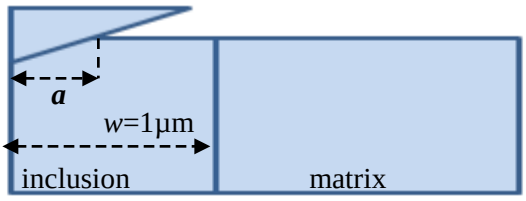


Fig. 1 Schematic of the indentation of two-phase biopolymer composites.

## RESULTS:

Fig.2 displays some representative force-displacement curves and the corresponding spatial dependent elastic moduli when indenting this composite. Fig.3 shows the prediction about how the surrounding matrix contributes to the measured composite modulus based on the similar concept in Clifford model[3].

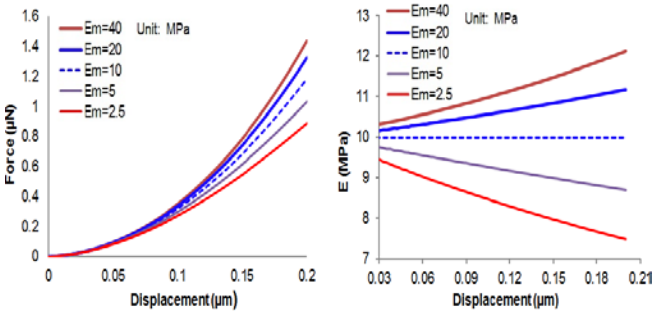


**Fig. 2** The force-displacement curves and corresponding spatial dependent elastic moduli.

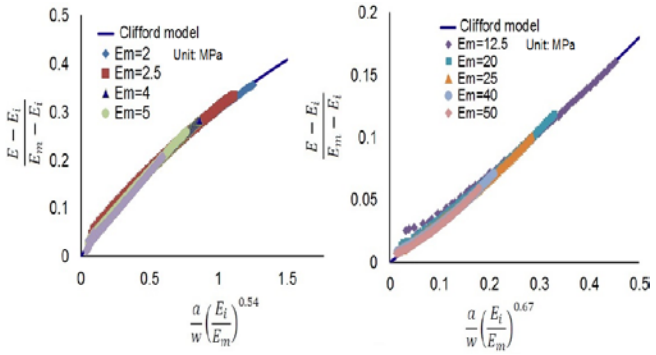


Fig.3 The contribution of the surrounding matrix to the measured composite modulus.

## DISCUSSION & CONCLUSIONS:

It revealed that measured elastic modulus of the inclusion can be significantly affected by the surrounding matrix well before the indenter touches the matrix. The model presented here enables modelling such an influence, which will give guideline for modulus mapping when indenting various biopolymer composites.

## REFERENCES:

1. E.T. Herruzo et al (2014) *Nat Commun* **5**: 3126/1-8.

2. J. Chen et al (2014) *Acta Mech. Sin.***30:**2-6

3. C.A. Clifford et al (2012) *Nanotechnology* **23:** 165704/1-7

# Nanomechanics of bioimplant infection related bacteria

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## INTRODUCTION:

Healthcare–associated infection is responsible for many biomedical implant failure. A recent study has shown that nanoprotrusions on silicon could kill various bacteria commonly presented at hospitals[1]. Such a nanostructure-bacteria interaction would result in the change of bacteria mechanics which can be assessed by nanoindentation[2]. In order to circumvent characterizing the localized mechanical properties of the cell, a relatively large penetration is preferred. However, in such case, the boundary conditions in the existing analytical model[3] violated due to limited dimension of the bacteria. The objective of this work is to develop a new model to tackle this.

## METHODS:

An axisymmetric FE model is adopted to investigate the nanomechanical responses of the bacteria indented by a conical tip (semi-included angle=60°) with a trapezoidal loading function as shown in Fig.1. The elastic modulus of the bacteria is assumed to be 1kPa. Its viscoelastic behaviour is described by Prony series model. A similar mathematical framework presented by Chen et al [4] is adopted to account for a large deformation relative to bacteria size.

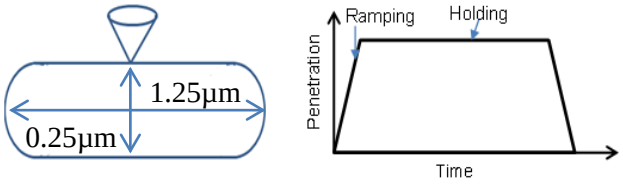


Fig. 1 Schematic of the indentation of rod bacteria and the trapezoidal loading function.

## RESULTS:

Fig. 2 displays the comparisons of nanoindentation responses during ramping and holding period, determined by the simulation, the existing model and the new predictive model.

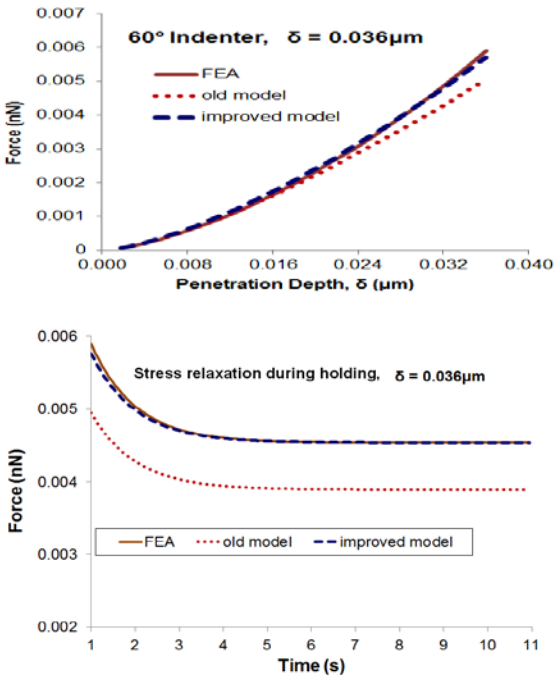


Fig2. Nanoindentation responses determined by the simulation and different models.

## DISCUSSION & CONCLUSIONS:

A new model has been developed to determine the viscoelastic properties of a rod-shaped bacteria for a large deformation relative to bacteria size. Such an approach is also applicable to others cells in tissue engineering. Understanding how nanomechanics of cells are regulated by scaffold materials could guide the surface design of scaffold materials.

## REFERENCES:

1. E.P. Ivanova et al (2013) *Nat Commun* **4**: 2838/1-7
2. L.Kailas et al (2009) *Ultramicroscopy* **109**: 775-780.
3. Y.T.,Cheng et al. (2005) *Appl. Phys. Lett.* **87**: 111914
4. J.Chen et al (2012) *J.Biomech.* **45**: 2810-2816

## ACKNOWLEDGMENTS:

EPSRC (EP/K039083/1) is acknowledged for providing financial support.

# Novel polymer topography to influence mesenchymal stem cell behaviour

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## INTRODUCTION:

A cells response to the physical environment can regulate gene expression and differentiation[1]. Tailored architecture and topography of implants can regulate cell activity to augment the use of autologous progenitor cell populations to rebuild a diseased tissue more successfully. Adaptation of implant surfaces with appropriate temporal and spatial cues, possibly in combination with biomolecules and/or biomolecular motifs has the potential to influence multipotent cell activity influencing cell orientation, organisation and potentially cell differentiation leading to control of tissue formation and structure.

The aims of this project are to explore the influence of polymer surfaces on mesenchymal stem cell (MSC) biology and exploit different polymer chemistries to functionalize surfaces with biomolecules. These studies will assist scaffold design and fabrication, to aid regenerative applications.

## METHODS:

Thin films of polymers were spread onto a flat substrate by spin coating at high speeds. Using a combination of immiscible polymers to generate a micro-scale landscape of opposing structures. Addition of water induces breath figure patterns, creating pores and crators to further accentuate the topographical pattern. Immiscible polystyrene (PS) and poly(methyl methacrylate) (PMMA) solutions were demixed at various ratio's (v/v) and spun at speeds > 8,000rpm under humid conditions[2] . Topographical surfaces were assessed using atomic force microscopy (AFM) and scanning electron microscopy. Primary adult human MSCs, isolated from bone marrow were characterized by flow cytometry and tri-lineage differentiation. Biological responses to surfaces were evaluated using immunofluorescece, histological staining and qPCR.

## RESULTS:

After evaluation of a range of demixed ratios/ concentrations/ solvents a selection of surfaces [PS:PMMA 40:60/50:50/60:40] 3%w/v in toluene] were further characterized. These generated opposing raised PMMA and low-lying PS islands. Breath figure patterns generated crater-like features ranging from 0.5-1µm in height and depth (Fig. 1A). The raised *caldera* attracted increased cell interactions, where cells concentrate focal adhesion plaques (Fig1.B) to the raised features; altering their morphology (Fig.1 C) and gene expression (Fig.1D). Adaptation of demixed polymer ratios altered the spatial distribution of *Caldera*.

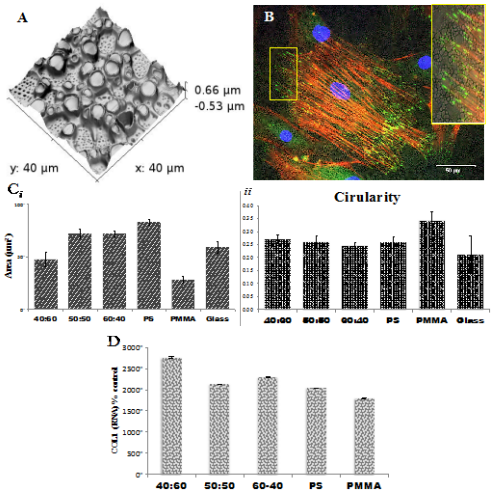


**Figure** 1. Demixed PS:PMMA [50:50] **A)** AFM 3D image **B)** Immunofluorescent staining of hMSC (P-2) 72hr. Focal adhesion plaques [*Vinculin*-FITC; green]; F-Actin cytoskeleton [*Rhodamine;* red]; Nucleus [Dapi; blue]. **C)** Quantification of cell morphology; ***i***) Cell area and ***ii***) Circularity. **D)** qPCR: COL1 expression (ΔΔCt) % control (glass) after 14days culturing on surfaces.

## DISCUSSION & CONCLUSIONS:

Demixed polymer blends provide tuneable topographies for the stimulation of various cell responses through regulation of adherence dynamics. Utilizing the immiscible polymer chemistries we can restrictively alter the dispersion pattern of humidity induced pores and *Caldera,* to induce a biological response. Tailoring these surfaces to influence specific cell types has the potential to manipulate the tissue formation and hence tissue function.

## REFERENCES:

1. C.M. Dekevsera *et al. polymer* 45, 2211-2219 (2004)
2. Park, M.S. and Kim, J.K. *Langmir* 20, 5347-5352 (2004)

## ACKNOWLEDGMENTS:

Authors would like to acknowledge support from Arthritis Research UK (Award 19429).

# A novel hdaci can improve osteogenic differentiation of human adipose derived stem cells

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## INTRODUCTION:

The ability to control stem cell differentiation into specific lineages is the key to functional tissue engineering. Histone deacetylase (HDAC) proteins play a key role in epigenetics and their inhibitor compounds (HDACis) are well researched cancer treatments. Novel HDAC3 selective HDACi, MI192, has potential in leukaemia, and rheumatoid arthritis treatments[1,2]. In recent years, researchers have investigated the effect of HDACis on stem cell epigenetics, with potential to control stem cell fate[3,4]. The aim of this study was to investigate the potential of using MI192 to control the osteogenesis of human adipose tissue derived stem cells (hADSCs), for bone tissue engineering.

## METHODS:

The novel inhibitor MI192 was synthesised by a palladium catalyzed allene gas reaction and characterised using NMR spectroscopy. hADSCs were pretreated with different concentrations of MI192 for two days, prior to culture in osteogenic inductive medium for five days. HDACi effect on cell proliferation was assessed with MTT assays. The effect of HDACi pretreatment on osteogenic differentiation was assessed with alkaline phosphatase (ALP) staining.

## RESULTS:

100 µM pretreatment of the HDACi MI192 caused ADSC death. Cell proliferation was halted at a lower concentration (30 µM). Pre-treatment of ADSCs with 30 µM HDACi MI192 for two days, followed by osteogenic media for five days, showed increased positive alkaline phosphatase staining, compared to the negative and positive controls (Figure 1).

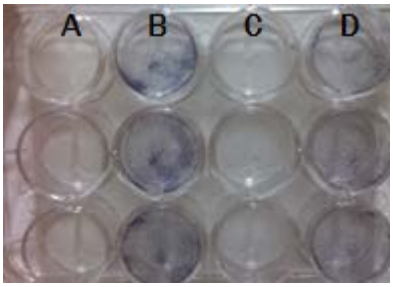


*Figure 1 - ALP staining of ADSCs. **A** - Basal media (no pretreatment), **B** & **C** - Cells pretreated with 30 µM (**B**) and 100 µM (**C**) MI192 for 2 days followed by 5 days in osteogenic media. **D** - Osteogenic media (no pretreatment).*

## DISCUSSION &CONCLUSIONS:

The results from this study indicate that the high concentration of MI192 is cytotoxic to cells. However, low concentrations only inhibit cell proliferation. 30 µM pretreatment of MI192 promoted ADSCs differentiation along the osteogenic linage. Novel HDACi MI192 is known to be HDAC3 selective, which suggests the potential of using the selectively inhibiting HDAC3 for bone tissue engineering applications.

## REFERENCES:

1. M. Boissinot et al (2012) *Leukemia Research* **36**: 1304-1310.
2. J. Gillespie et al (2012) *Arthritis and Rheumatism* **64**: 418-422.
3. J. Boer et al (2006) *Tissue Engineering* **12**: 2927–2937.
4. H. F. Duncan et al (2011) *Journal of dental research* **90**: 1377–1388.

## ACKNOWLEDGMENTS:

The authors would like to thank the EPSRC funded DTC TERM for financial support.

# Non-destructive *in vitro* tools for the comparison of degradable biomaterials

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## INTRODUCTION:

Degradable biomaterials are often required for regenerative medicine. These biomaterials are gradually resorbed and replaced by natural tissues. This rate of degradation often has an effect on cell proliferation and the production of extracellular matrix (ECM) proteins. Biomaterial degradation has been traditionally monitored using destructive techniques including weight loss and HPLC. This study, however looks at the production of fluorescent biomaterials whose degradation can be monitored online and compared to ECM production and cell proliferation to produce a turnover index which can be used as an online comparative tool for biomaterial selection.

## METHODS:

*Fluorescent biomaterials* – PLGA 50/50 (Sigma Aldrich) and Chitosan (Sigma Aldrich) were solvent cast in chloroform and acetic acid respectively. PLGA was plasma coated with ammonia followed by treatment with TRITC, whereas chitosan was directly treated with TRITC. Alexa fluor-488 fibrinogen (Life Technologies) was combined with fibrinogen (Merck) and thrombin (Sigma Aldrich) in the presence of calcium to produce a fibrin gel

*Cell Culture* – MLO-A5 were seeded on to the biomaterials ($5x10^4$cell/$cm^2$) and cultured in osteogenic media for 7 days. Chitosan was degraded by addition of 0.2 mg/mL lipase to the media. Proliferation rates were assessed using Alamar Blue™ (Life Technologies) and ECM production was monitored using an Osteopontin ELISA (R&D Systems). Turnover indices (TI) were calculated as ratios between degradation rates and proliferation or ECM production.

## RESULTS:

Fluorescently tagged biomaterials (*FIG 2*) were monitored with time and fluorescence was released into the supernatant at rates comparable to that observed using a weight loss technique.

MLO-A5 proliferation decreased on biomaterials with a higher degradation rate (*FIG 2(A)*), however the production and expression of osteopontin (*FIG 2(B)*) was increased.

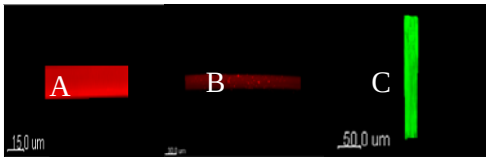
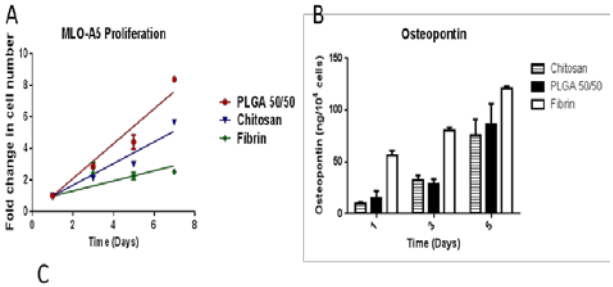



*Fig. 1 Fluorescent Biomaterials (A) chitosan; (B)PLGA 50/50 and (C) fibrin.*

A TI which can be used to compare the biomaterials can therefore be calculated. This showed that fibrin which degraded quickest had the lowest proliferation rate and the highest production of osteopontin (*FIG 2(C)*).

*Fig. 2 (A) MLO-A5 proliferation on fluorescent scaffold materials measured through Alamar Blue™ assays; (B) osteopontin production and (C) table of turnover indices.*




C

| | Cell Proliferation Turnover Index | Osteopontin Turnover Index |
|---|---|---|
| Chitosan | 0.028 | 15.54 |
| PLGA 50/50 | 0.092 | 19.60 |
| Fibrin | 0.028 | 83.47 |

## DISCUSSION & CONCLUSIONS:

The use of online monitoring of fluorescent degradation is advantageous as it is non-destructive and allows for the online monitoring of the production of engineered tissues. The use of a TI value allows for the comparison of degrading biomaterials. Our aim is to generate an *in vitro* model and tools which allow the correct biomaterial to be selected for translation to clinic.

## ACKNOWLEDGMENTS:

Authors would like to acknowledge Paul Roach for his help with plasma coating and Framework VII Programme BIODESIGN (Grant no: 262948) for providing financial support to this project.

# Porous chitosan constructs support bone marrow stem cells for osteochondral modelling

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**INTRODUCTION:** Regenerative therapeutic solutions are required to address the increasing prevalence of bone and cartilage diseases within the population. Limitations of existing treatments, such as bone graft reconstructions or biomaterial implants, suggest that osteochondral tissue constructs with the ability to support differentiation of mesenchymal stem cells (MSCs) into both osteoblast and chondrocyte lineages are desirable[1]. The current project aims to overcome issues arising from the divergent differentiation requirements for each lineage by providing scaffolds with spatially resolved environments, each supportive of one of these cell lineages. The work reported here describes the porogen and cross-linker optimisation for the production of bi-layered chitosan scaffolds containing two distinct pore sizes. Scaffolds were characterised before cell seeding and 3D culture in a perfusion bioreactor.

**METHODS:** Porous scaffolds were produced using a freeze-gelation method from a 4% chitosan solution mixed with polycaprolactone (PCL) porogen and cross-linked using either 0.5% glutaraldehyde or 0.3% genipin solutions. Porogen microspheres were produced by an emulsion method. Porogen particles and scaffold porosity were evaluated using scanning electron microscopy (SEM) and micro-computed tomography (MicroCT). Cytocompatibility was assessed after seeding of human mesenchymal stem cells (hMSC) onto fibronectin-treated scaffolds and culture in a perfusion bioreactor for up to 3 weeks.

**RESULTS:** Dual porosity scaffolds were produced and analysed by MicroCT, confirming different porosity and pore size distribution for bottom, middle and top sections of scaffolds made with PCL microspheres (Table 1).

| | Bottom | Middle | Top |
|---|---|---|---|
| *% Porosity* | 74 | 73 | 73 |
| *Pore size (μm) ± SD* | 287 ± 10.5 | 232 ± 17.5 | 196 ± 5.5 |

Large (300-425 μm) pores were designed to promote osteogenic differentiation of hMSCs and smaller, (180-300 μm) pores to support chondrogenesis. SEM analysis of the scaffolds illustrated the graded pore size distribution (Fig.1), and the absence of delamination between layers.

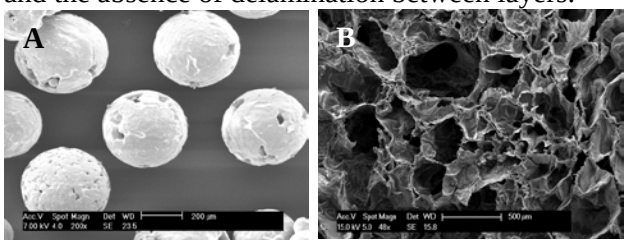


***Figure 1*** **SEM images of PCL porogen microspheres (A) and resulting bi-layered chitosan scaffolds (B).**

Scaffolds cross-linked with genipin displayed more uniform pore morphology than ones made with glutaraldehyde. Scaffolds were then seeded with hMSCs and cultured both on culture dishes and in a perfusion bioreactor for up to 21 days. Cell viability and differentiation were assessed at different time-points to measure osteochondral lineage maturation *in situ.*

**DISCUSSION AND CONCLUSIONS:**
A novel dual porosity chitosan scaffold was produced for osteochondral modelling. Results show that the PCL microspheres permitted good control over pore characteristics in the scaffolds. Subsequently, hMSC seeding methods were evaluated for optimal cell infiltration into the scaffold core to populate the construct. Scaffolds cross-linked with genipin allowed higher cell metabolic activity than glutaraldehyde, to a level comparable to tissue culture plastic. MSC differentiation analysed over 3weeks highlighted the influence of scaffold structure on cell compatibility in 3D.

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# BIOLOGICAL CHARACTERISATION OF THE PORCINE ACETABULAR LABRUM

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**INTRODUCTION:** Femoroacetabular impingement (FAI) can impinge on the labrum, causing damage and has been recognised as an initiator of hip osteoarthritis[1]. Labral damage compromises the functions of the tissue (enhancing hip stability and protecting the articular surface); therefore it is advantageous to repair the labrum, in order to restore the hip to normal or near-normal mechanical and physiological function[2]. The aim of this study was to characterise the acetabular labrum (in comparison to cartilage) both quantitatively and histologically.

**METHODS:** Quantitative analysis of porcine acetabular labrum, acetabular cartilage and femoral cartilage (mean age 5 months and removed within 24 hours of slaughter) was carried out on load-bearing and non-load-bearing regions. Glycosaminoglycan (GAG) and collagen concentrations were determined using quantitative assays[3]. Statistical analysis was carried out using a one-way ANOVA and student t-test (n=6). Histological analysis was used to qualitatively assess porcine acetabular labrum and cartilage. Samples were stained to identify the general architecture (haematoxylin and eosin [H&E]), collagen and elastin (Sirius Red and Miller's Elastin) and GAGs (Alcian blue) [3].

**RESULTS:** The acetabular and femoral cartilage had significantly higher concentrations of water and GAGs, and a significantly lower concentration of collagen compared to the acetabular labrum (Table 1). There were no significant differences between the load-bearing and non-load-bearing regions of femoral and acetabular cartilage. Two key tissue types were identified within the labrum using H&E (Figure 1A). Sirius red staining under polarised light (Figure 1B) showed characteristic collagen orientation for the cartilage. The cartilaginous labrum region showed up dark under polarised light suggesting a radial alignment with the more fibrous region appearing random. Both tissues stained positively throughout for Sirius red and neither showed positive staining for Miller's elastin (Figure 1C). The labrum showed strong Alcian blue staining in the middle zone while the articulating surface and outer regions of the acetabulum showed reduced Alcian blue staining, highlighted by the presence of pink periodic acid-Schiff counterstaining (Figure 1D).

*Table 1 Water content, GAG and collagen concentration of acetabular labrum and cartilage and femoral cartilage in load-bearing and non-load-bearing regions.*

| Sample | Water Content (%) | GAG Content ($\mu g.mg^{-1}$) | Collagen Content ($\mu g.mg^{-1}$) |
|---|---|---|---|
| ACL | 75 (+1.35; -1.38) | 186 ± 50 | 64 ± 11 |
| ACN | 75 (+2.01; -2.09) | 197 ± 50 | 63 ± 23 |
| FCL | 76 (+1.48; -1.66) | 176 ± 57 | 58 ± 15 |
| FCN | 75 (+2.37; -2.45) | 176 ± 76 | 71 ± 7 |
| ALL | 70 (+2.69; -2.76) | 67 ± 30 | 90 ± 21 |
| ALN | 67 (+3.23; -3.48) | 29 ± 6 | 99 ± 16 |

AC – Acetabular cartilage, FC – femoral cartilage, AL – acetabular labrum, L-load-bearing, N- non-load-bearing

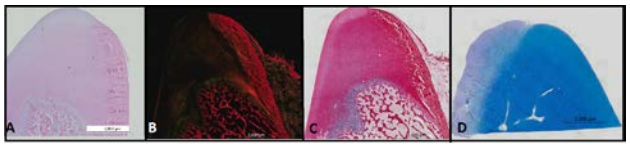


*Figure 1 Histological images of the acetabulum taken at x10 magnification. A – H&E, B – Sirius red polarised, C – Sirius red bright field, D – Alcian blue*

**DISCUSSION & CONCLUSIONS:**

This study has shown that the acetabular labrum demonstrates different material properties to the adjacent articular cartilage. The labrum has a higher collagen concentration to support its function of enhancing hip stability. The differences in fibre alignment may also support the tissues' functions; with articular cartilage requiring highly aligned surface fibres for articulation and the labrum requiring radial fibres for strength. GAGs enhance the tissues ability to support load and hence higher GAG concentrations were found in the load-bearing articular cartilage as opposed to the non-load bearing acetabular labrum.

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**ACKNOWLEDGMENTS:** This research was funded by the EPSRC DTC TERM.

# The effects of decellularisation on the mechanical properties of bone

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**INTRODUCTION:** Bone defects as a result of trauma and physiological and pathological bone resorption represent a major challenge and have become a worldwide health issue[1]. Regenerative medicine strategies involving decellularised extracellular matrix scaffolds are developing very fast[2]. This project aims to demonstrate and establish decellularisation protocols to make donor bone scaffold, which could be used to repair bone defects in recipient patients and to determine whether the decellularisation process alters the mechanical properties of the bone scaffold.

**METHODS:** Bovine femur samples were decellularised by six cycles of overnight incubation at 37°C using two protocols: A – 10mM Tris, 1mM EDTA, 0.1% v/v Triton X-100; and B – method A plus 0.5% w/v trypsin. Decellularisation was confirmed by the absence of DNA staining with DAPI. Mechanical testing (Bose ElectroForce 3200) and porosity (mercury porosimetry) measurements were carried out before and after the decellularisation process for bone samples using protocol A and protocol B.

**RESULTS:**

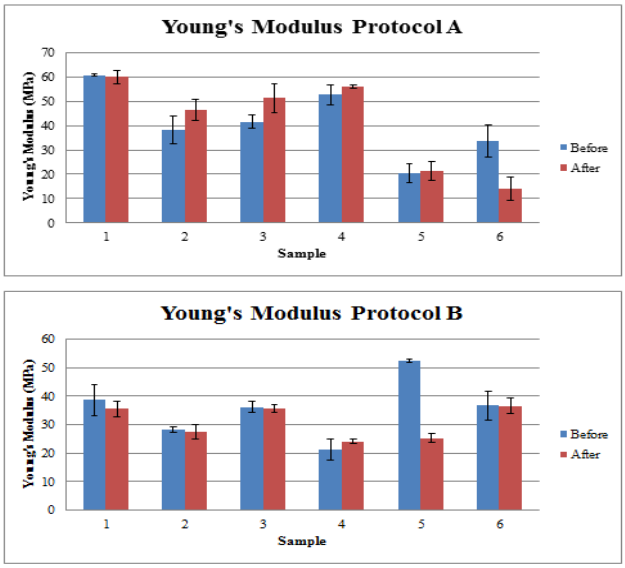


*Figure 1: The comparisons of Young's modulus (E) in bone samples before and after six cycles of incubations with protocol A or B. Results are the mean ± SEM of n = 3. p>0.05 compared with the control (ANOVA for repeated measures).*

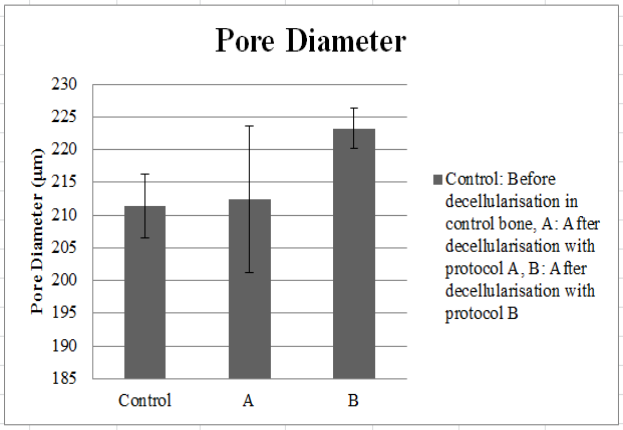


*Figure 2: The pore size diameter of bone samples before and after decellularisation with protocol A or B. Results are the mean ± SEM of n = 3. p>0.05 compared with the control (ANOVA for repeated measures).*

**DISCUSSION AND CONCLUSIONS:** The present study indicates that decellularisation process with protocols A or B did not affect the mechanical properties in terms of Young's Modulus (E, stiffness), and there was no significant difference in pore size diameters before and after decellularisation. The bone scaffold produced in all protocols had an average pore size of 211.349µm to 223.275µm. DAPI staining revealed that post-decellularisation by protocol B no measurable DNA was present on the bone samples. Protocol B using trypsin could therefore be used to make donor bone scaffold, which could be used to repair bone defects in recipient patients.

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# Hydrostatic force bioreactor-a novel tool for mechanical conditioning of cells, tissues and tissue constructs

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**INTRODUCTION:** Cell fate and tissue development are affected by mechanical stimulation and chemical cues. Bioreactors provide growth environments to engineer tissues and to investigate the effect of mechanical forces on cells and cell-scaffold constructs. Hence, to define outcome, evaluation of the bioreactor environment during culture is critical. A novel hydrostatic force bioreactor imposing low levels of cyclical hydrostatic force at 0.005 Hz to 2 Hz frequency and 0-280 kPa on standard tissue culture multi well plates was developed in collaboration between Instron Tissue Growth Technologies, Ltd and Keele University. This study aims to investigate the growth environment within the bioreactor to define outcome for regenerative medicine and clinical applications.

**METHODS:** Physiological variables such as dissolved oxygen and carbon dioxide concentration in the medium and pH have been investigated experimentally. Physical forces (shear stress and pressure) in the bioreactor were determined by mathematical modeling and numerical simulation. The effect of hydrostatic pressure on differentiation and maturation of stem cell types was assessed using human bone marrow derived mesenchymal stem cells and chick femur fetal skeletal cells in monolayer and 3D hydrogel as well as organotypically cultured ex vivo chick foetal femurs and human embryonic stem cells.

**RESULTS:** The concentration of dissolved $O_2$ and $CO_2$ in the medium increased after mechanical stimulation, whereas the pH of the medium decreased. These changes were dependent on the applied hydrostatic pressure and were reversible when samples were transferred to a cell culture incubator. Further investigation is necessary to investigate the effect of these changes on potential cell fate and tissue development. Mathematical modeling and numerical simulation showed that the distribution and magnitude of physical forces such as shear stress and pressure depends on the shape and position of the cell-seeded hydrogels in the tissue culture well plates. Finally, cyclic hydrostatic force worked synergistically with chemical cues resulting in an increase in mineralised densities of cell-seeded hydrogels and chick foetal femurs leading to enhanced osteogenesis. In addition, hydrostatic force enhances the bi-refringent properties of 3D collagen hydrogels which suggested increased alignment of collagen fibrils.

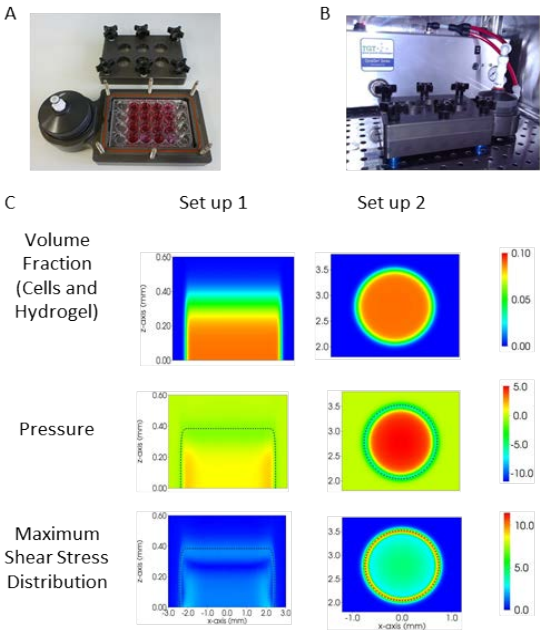


*Figure 1: Hydrostatic force bioreactor. Images show the bioreactor chamber (A) and the bioreactor set up in the incubator (B). Distribution of volume fraction, pressure and shear stress are shown for two theoretical experimental set ups (C).*

**DISCUSSION & CONCLUSIONS:** This novel bioreactor allows the application of hydrostatic force for gas-liquid interface culture at physiological relevant pressures and provides a growth environment for engineered tissues. The application of hydrostatic pressure resulted in changes of dissolved $O_2$, $CO_2$ and pH of the medium, which remained within the human physiological range. Moreover, this bioreactor is alignable to culture in standard culture environments and could be a suitable tool for pre-conditioning of cells and tissue for clinical tissue regeneration.

**REFERENCES:** Y. Reinwald et al. (2014), "Evaluation of the growth environment of a novel hydrostatic force bioreactor" (in press, Tissue Engineering Methods C); J.R. Henstock, et al. (2013) "Cyclic hydrostatic pressure stimulates enhanced bone development in the foetal chick femur in vitro". Bone 53, 468-477.

**ACKNOWLEDGMENTS:** The authors would like to thank the EPSRC Centre for Innovative Manufacturing in Regenerative Medicine (ECP007/1010) and Instron TGT, Ltd for funding.

# Promoting vascularization in bioactive microenvironments

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## INTRODUCTION:

Forming functional vascular network remains a great challenge in tissue engineering[1]. One of the strategies how to achieve this is a targeted delivery of pro-vasculogenic growth factors. Use of ECM proteins able to bind various growth factors and to present them in order to modulate cell behaviour looks promising[2]. In addition, spontaneous fibrillogenesis of fibronectin (FN) on specific polymers was recently discovered[3] what can enhance GF sequestration, and contribute to stimulation of cell differentiation.

## METHODS:

*Sample preparation and testing*: Polymers (poly (ethyl acrylate), PEA, and poly(methyl acrylate) PMA as a control) were spincoated on glass coverslips to obtain ~ 1 µm thin layer. Samples were incubated with FN to form a network, and coated with VEGF. Samples were tested for VEGF binding by ELISA and AFM.

*Cell culture*: HUVECs were used to test samples for stimulation of tubular network formation; cells were fixed and stained for actin and DNA.

## RESULTS:

Binding of VEGF to fibronectin fibrils on PEA was characterized by ELISA, and visualized by AFM. There, VEGF molecules bind to a central part of a FN arm (Fig. 1).

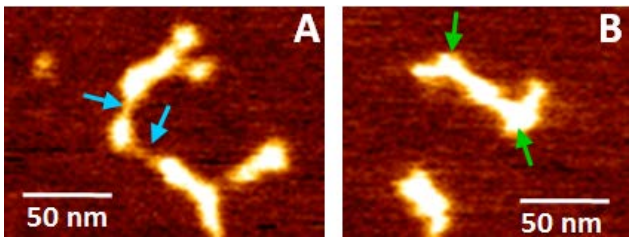


*Fig. 1 AFM phase images of FN on PEA without (A) and with (B) VEGF bound. Blue arrows show unoccupied FN binding sites for growth factors, green arrows show VEGF bound to FN.*

HUVECs cultured on samples for 6 days showed clear reorganization into network resembling structures (Fig. 2), while experiment with control polymer (PMA) did not show such cell behaviour.

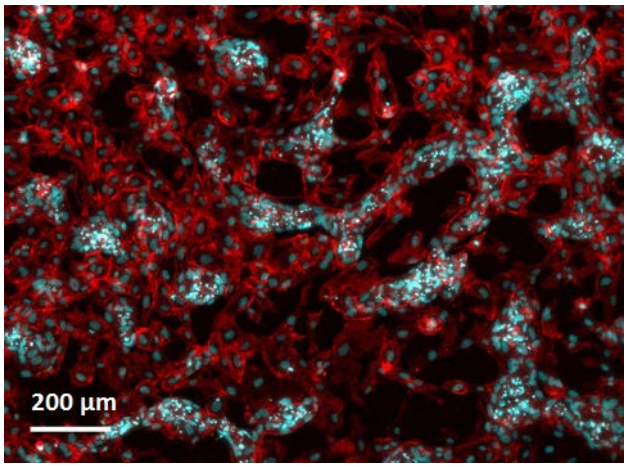


*Fig. 2 HUVECs forming a network on PEA with FN fibrils binding VEGF covered with fibrin matrix. Actin is in red, nuclei are in cyan.*

## DISCUSSION&CONCLUSIONS:

A specific combination of a FN network formed on PEA and VEGF was shown to be able to provide conditions for triggering HUVECs formation of network structures, and it will be used for further testing of primitive vascular plexus formation *in vitro*. It represents a promising microenvironment to support so much needed circulatory system development in various tissues to allow their regeneration.

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## ACKNOWLEDGMENTS:

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# Human airway smooth muscle maintain *in situ* cell orientation and phenotype when cultured on aligned electrospun scaffolds

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## INTRODUCTION:

Human airway smooth muscle (HASM) contraction plays a central role regulating airway resistance in both healthy and asthmatic bronchioles. *In vitro* studies that investigate the intricate mechanisms regulating this contractile process are predominantly conducted on tissue culture plastic (TCP), a rigid, 2D geometry, unlike the 3D microenvironment smooth muscle cells are exposed to *in situ*[1]. It is increasingly apparent that cellular characteristics and responses are altered between cells cultured on 2D or 3D topographies[2]. Electrospinning is an attractive method to produce 3D topographies for cell culturing as the fibres produced have dimensions within the nanometre range; similar to cells' natural environment.

## METHODS:

Polyethylene terephthalate (PET) was electrospun into uni-axially orientated nanofibres. The effect of this topography on HASM cell adhesion, alignment, morphology, and contractile protein characteristics was compared to both 2D-cultured and *in situ* smooth muscle.

## RESULTS:

Fibre orientation provides contact guidance to cells enabling the formation of fully aligned sheets of HASM cells similar to *in situ* smooth muscle. Moreover, HASM cells cultured on the scaffold present an elongated cell phenotype with altered contractile protein levels and distribution.

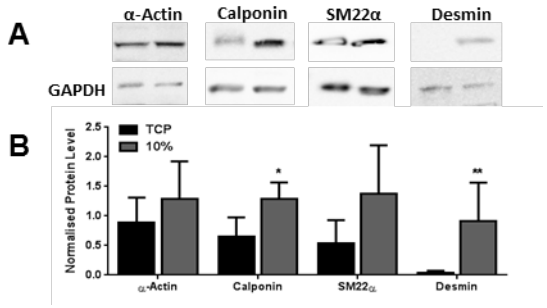


*Fig. 1 ASM protein levels when cultured on 2D or 3D aligned topographies*

## DISCUSSION & CONCLUSIONS:

The platform presented provides a novel *in vitro* model that promotes airway smooth muscle cell development towards a more *in vivo*-like phenotype whilst providing topological cues to ensure good cell alignment.

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## ACKNOWLEDGMENTS:

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# *In vitro* culture of colonic epithelial stem cells

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## INTRODUCTION:

Conditions such as inflammatory bowel disease which cause damaged intestinal epithelium are currently either treated with surgery to remove part of the intestine or with expensive medications which dampen the symptoms. Adult colonic stem cells could provide a new treatment option by forming tissue grafts that aid regeneration of damaged epithelium. Lgr5 is a marker of adult stem cells in various tissues. Lgr5+ 'organoids' have been cultured *in vitro* from mouse stem cells and transplanted into damaged intestine, where they have contributed to regeneration of epithelium[1,2]. Using this proof of concept, the first aim of this work is to culture stem cells from human colonic epithelium to form organoids containing Lgr5+ stem cells.

## METHODS:

Normal colon tissue was obtained with local ethical approval from resections at the Queens Medical Centre, Nottingham. The isolation of colonic crypts was adapted from previously published work[3]. The tissue was sterilised in 0.04% sodium hypochlorite for 15 minutes. The tissue was then incubated in 3 mmol/L EDTA + 0.05 mmol/L dithiothreitol (DTT) for 90 minutes at room temperature. The EDTA/DTT solution was then removed, 15 mL PBS was added, and the tube was shaken manually for 20 seconds liberating the crypts from the submucosa. The PBS containing the crypts was transferred to another tube, centrifuged gently and counted. Crypts were cultured as described previously[4]. 100 crypts per 25 µL Matrigel per well were plated in a 48-well plate, overlaid with 250 µL culture medium containing nicotinamide, Wnt-3a, EGF, Noggin and R-Spondin.

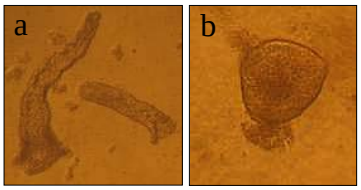
## RESULTS:

Individual crypts were isolated from normal colon, as shown in figure 1a. Within the first 24 hours in culture, the crypts became more rounded, forming cystic organoids. Proliferating cells have been found in budding organoids[4]. These budding structures were observed after 2 days in culture, as shown in figure 1b.

**Figure 1:** Crypts immediately after isolation (a) and a budding organoid 48 hours after plating (b)

## DISCUSSION & CONCLUSIONS:

Organoids have been cultured from crypts isolated from normal human colon, as has been described previously[5]. Further work to confirm the expression of stem cell markers such as Lgr5 will be done. The more long term aim of this research is to grow organoids on tissue engineering scaffolds such as electrospun fibres to investigate the effect on differentiation. The scaffolds may also provide an efficient and convenient method of cell delivery.

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## ACKNOWLEDGMENTS:

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# Effect of cultured rat primary hepatocytes on the mechanical properties of collagen gel matrices

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## INTRODUCTION:

Collagen gels are widely used as matrices for in vitro cell culture and also for a variety of tissue engineering applications. The mechanical properties of matrices have been shown to be important parameters which greatly influence cell behaviour[1]. For successful support of long term cell growth and function, it is important for matrices to retain their mechanical integrity during the period of use. The presence of cells has been shown to alter matrix properties[2]. The aim of this study was to investigate the effect of seeding primary hepatocytes on the mechanical properties of collagen hydrogel matrices.

## METHODS:

Adult rat primary hepatocytes were seeded at different densities on reconstituted type I collagen hydrogels (0.3% w/v) and maintained in culture for up to 7 days. Stiffness (aggregate modulus) and hydraulic permeability of the gels were evaluated at 48 h and 7 d using biphasic theory following confined compression testing[3].

## RESULTS:

Stiffness and hydraulic permeability of the gels were altered when primary hepatocytes were maintained on them (Fig. 1). Presence of cells generally led to lowering of stiffness and an increase in hydraulic permeability.

## DISCUSSION:

Primary hepatocytes influence the mechanical properties of collagen gels. The decrease in stiffness of collagen gels with presence of hepatocytes is most likely due to the cells degrading the matrix through the chemical action of degradation enzymes, and the physical exertion of mechanical forces on the gels by the cells. The increase in hydraulic permeability may also be due to the degradation of matrix which occurred with cell seeding.

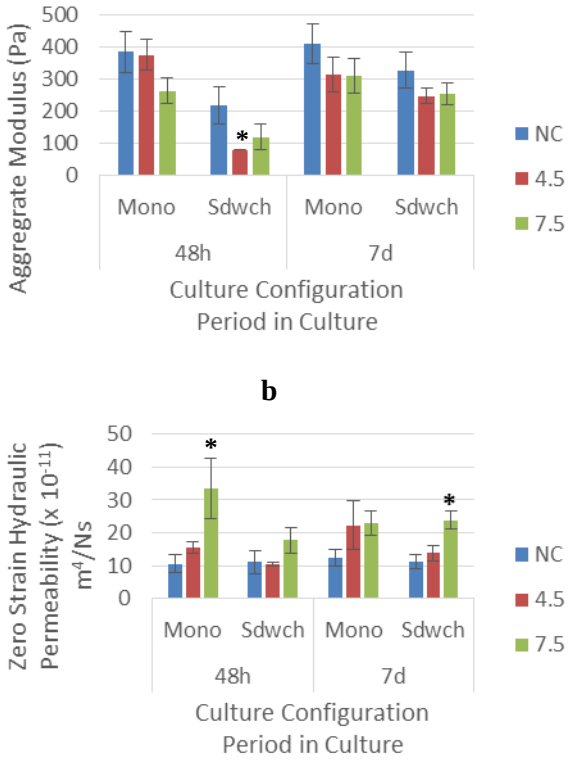


*Fig. 1: Effect of hepatocytes on stiffness (a) and hydraulic permeability (b) of collagen gels in monolayer (Mn) and sandwich (Sdwch) culture configurations. NC, no cells; 4.5, 4.5 x $10^5$ cells/well; 7.5, 7.5 x $10^5$ cells/well. Data are means ± SEM, n=3 independent experiments in triplicate, * represents significant difference from NC ($p < 0.05$) by ANOVA followed by Dunnett's multiple comparison test.*

## CONCLUSION:

The change in mechanical properties observed in this study has implications for use of collagen gels as matrices for long term use.

## REFERENCES:

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[3]Mow et al. (1980) J Biomech Eng. 102:73–84

# Preliminary evaluation of novel bioceramics for bone restoration

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## INTRODUCTION:

Since the development of the first Bioglass® in 1971, more than 40 different ceramic formulations have been used for bone reconstruction[1,2]. Nevertheless, regeneration of bone defects still remains a significant clinical challenge. The aim of this research work is the development of novel bioceramic materials with good bioactivity, biocompatibility and competent mechanical properties for bone tissue engineering applications.

## METHODS:

Two novel silicate-based glasses, containing different oxide combinations including $P_2O_5$, $B_2O_3$, $Na_2O$, CaO, $K_2O$, MgO, ZnO, SrO, $Ag_2O$, $V_2O_5$, $Cr_2O_3$ have been prepared by GTS Ltd, Sheffield-UK, along with apatite-wollastonite (AW), a material previously adopted in FDA approved medical devices which has been used as control. The synthesised materials, labelled AW, NCL2 (45 mol% $SiO_2$) and NCL7 (50 mol% $SiO_2$), were ground and sieved to have particle sizes under 53 µm. Glass-ceramic pellets were then prepared with an automatic hydraulic press and sintered at temperatures determined from hot stage microscopy. The bioactivity was investigated by soaking the pellets in simulated body fluid. Subsequently in vitro tests were carried out on the sintered pellets and the modulus in compression was evaluated using a universal mechanical testing machine.

## RESULTS:

Cell culture results showed a positive response in presence of both NCL2 and NCL7 sintered pellets (fig.1). According to the bioactivity definition[3], the novel materials possess a low level of in vitro bioactivity, although the amount of phosphorus increased from 0 to 4 weeks in immersion. The modulus in compression, measured on dense glass-ceramic pellets, (Table 1) was similar to that of AW.

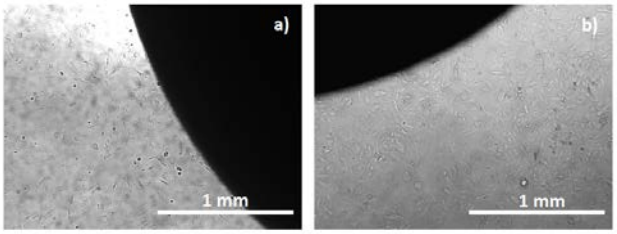


*Fig. 1 Rat OB cells cultured in presence of a) NCL2 and b) NCL7 composition b) after 1 week*

*Table 1 Dry Compression Modulus*

| SAMPLE | YOUNG'S MODULUS (GPa) | STANDARD ERROR |
|---|---|---|
| NCL2 | 1.43 | ±0.03 |
| NCL7 | 1.32 | ±0.01 |
| AW | 1.29 | ±0.03 |

## DISCUSSION & CONCLUSIONS:

Two different novel $SiO_2$-based glasses with complex compositions have been obtained by a melting process. Morphological observations, image analysis, mechanical and in vitro tests showed that the new silicate glasses are good candidates for bone tissue engineering applications.

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## ACKNOWLEDGMENTS:

The authors would like to acknowledge the support of Arthritis Research UK (Award 19429) and the FP7 RESTORATION project (Award CP-TP 280575-2).

# Oxygen difference in live melanoma muticellular tumour spheroids using novel platinum compound and time-resolved imaging microscopy

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## Introduction

More sensitive methods are continually in demand for the non-invasive detection of cancer cells, including melanoma. Quenching of phosphorescent compounds by oxygen is a novel approach for detecting hypoxia, which relies on the emission lifetime of compounds being sensitive to the oxygen concentration. Solid tumours display varied oxygen levels [1-3] so that there is a need to develop methodologies to determine these changes. Here we used novel non toxic phosphorescent platinum(II) complexes with long emission lifetimes in combination with Time Resolved Emission Imaging Microscopy (TREM) for hypoxic detection [4,5].

## Materials and Methods

A human melanoma cell line (C8161) was used to create multicellular tumour spheroids (approximately 200-300µm diameter). Samples were incubated with a Pt(II) compound based on (Pt(dipyridobenzene)Cl) with an appended primary amine unit. Variation in emission lifetime was investigated using 2-photon excitation and time resolved emission microscopy, as well as in parallel studies using 'Hydroxyprobe' and histology of tumour spheroids for comparison.

## Results

Spheroids were immersed in Pt(II), 100 µM solution for 12 hours, then washed with PBS, before imaging. Pt (II) lifetime distribution across spheroid ranges: 0.8-11.5 µs (Figure 1A). Time resolving lifetime clearly shows three distinct spheroid regions, outer proliferative rim (red, 0-3.0 µs), necrotic rim (green, 3.0-7.5 µs) and inner necrotic core (blue, 7.5-12.0 µs) (Figure 1B).

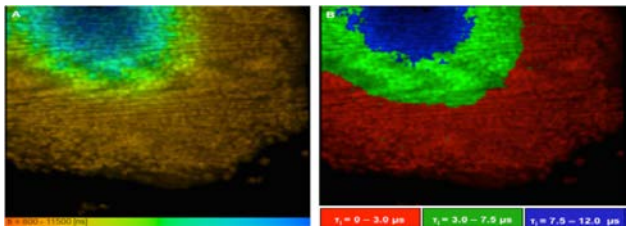

**Figure 1**. Lifetime distribution of Pt(II) in spheroid. (A) Continuous rainbow scale, range: 0.8-11.5 µs, (B) Discrete colours showing three distinct spheroid regions

## Discussion and Conclusions

We propose the measurement of intra-tissue $O_2$ as a potential parameter for assessing cellular activity associated with tumour development. Depletion of $O_2$ is known to increase the emission lifetime of the Pt(II) labels described herein, and thus permits real-time information on the concentration of molecular oxygen in a tissue sample. This method therefore has the potential to detect changes in partial $O_2$ pressure, and related responses (e.g. necrosis) providing a novel parameter for real time non-invasive detection of tumorigenic processes e.g. melanoma.

## References

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## Acknowledgments

We thank the BBSRC for funding AR with a PhD studentship and STFC for TREM expertise.

# Analysis of positional and rotational variables for cell seeding on hollow fibres

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## INTRODUCTION:

In recent years mesenchymal stem cells (MSCs) have shown increasing potential for use as a cell therapy for treatment of damaged heart tissue caused by myocardial infarction. MSCs can be extracted in limited quantities from adipose tissue surrounding the heart for use as an autograph to regenerate new cardiomyocites, however expanding the MSC population to a clinically applicable number remains elusive. To this end a dynamic method of seeding MG63 human osteosarcoma cells onto poly(lactic-co-glycolic) acid (PLGA) hollow fibres has been investigated.

## METHODS:

PLGA hollow fibre membranes were fabricated using wet spinning phase inversion. A solution of 20% w/w PLGA in N-methyl-2-pyrrolidone (NMP) solvent was passed through a spinneret into deionised water, the fibres are left in the water, replaced twice a day, for three days to allow complete solvent removal[1].

Fibres of 1 mm external diameter were cut to lengths of 35 mm and placed within a cylindrical bioreactor housing of 0.1 ml. The fibre and internals of the bioreactor were sterilised with 70% ethanol for 12 hours at 6°C, soaked for 1 hour in Dulbecco's Modified Eagle Medium (DMEM), washed thoroughly with 1 ml DMEM and inoculated with 110,000 MG63 cells at a density of 100,000 cells/cm$^2$.

Bioreactors were attached to a Stuwart STR4 rotator drive at one of three different angles and spun at one of three rotational velocities for 6 hours at 37°C and 5% $CO_2$. Afterwards the residual media was collected, the bioreactor washed with PBS, and the hollow fibre trypsinised for 20 min and agitated to remove attached cells. Manual cell counts were also performed on the media and PBS wash.

## RESULTS:

Cell attachment on bioreactors angled horizontally (17%), diagonally (20%) and vertically (16%) showed no statistically significant difference in their rates of cell attachment compared to each other (n=12) or static controls (23%) (n=59). This was also true of horizontally orientated bioreactors spun at 2 RPM (17%), 6 RPM (17%) and 8 RPM (11%) (n=12). Intermittent spinning at 6 RPM for 25 seconds and resting for 220 seconds also yielded no improved rates of cell attachment (12%) (n=8).

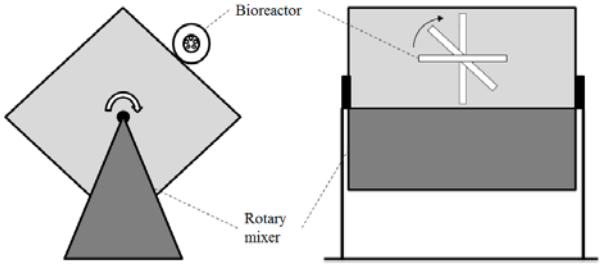


*Fig. 1: A side and front facing view of a hollow fibre bioreactor attached to a Stuwart STR4 rotator drive.*

## DISCUSSION & CONCLUSIONS:

All forms of dynamic cell seeding showed no significant improvement over the same system seeded in static conditions. Large variations in each data set lead to a corresponding average error of ±33%. This indicates a high degree of variability in cell attachment on hollow fibres which needs to be mitigated if hollow fibre bioreactors are to comply with good manufacturing practices.

## REFERENCES:

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## ACKNOWLEDGMENTS:

Funded by the University of Bath URS award.

# 3d micro-printing of nerve guides for peripheral nerve repair

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## INTRODUCTION

Nerve Guidance Conduits (NGCs) presently have a limited regenerative capacity, mainly due to the absence of physical guidance cues and materials, which do not ideally support the regeneration of injured neuronal and Schwann cell growth [1]. A need exists to fabricate more accurate NGC structures from more biocompatible materials to improve nerve regeneration following injury. The broad aims of this work are therefore to develop NGCs with improved bulk properties, physical design and surface chemistries to better support neuronal and Schwann cell growth, for surgical applications of nerve injury repair.

## METHODS

Caprolactone and polyethylene glycol pre-polymers were microwave synthesised, methacrylate functionalised and characterised by THF-GPC, MALDI-TOF, MS and NMR. Pre-polymers were UV cured into 2D sheets and 3D structures via stereolithography [2]. The microSL set-up consisted of a 405 nm CW laser with a maximum output of 100 mW. Laser output was directed onto a digital micromirror device (DMD) used as a dynamic mask in the projection microSL set-up. NGCs were assessed *in vitro* via cell viability testing and immuno-fluorescence labelling of neuronal cells, rat-derived primary Schwann cells and dorsal root ganglia (DRG). Common fibular nerve regeneration was assessed *in vivo* via implantation in to a mouse Yellow Fluorescent Protein (YFP) short gap injury model.

## RESULTS

NGCs of 5 mm length and 1.25 mm inner diameter were produced via microSL (Fig 1) with a minimum resolution of 50 µm from polyethylene glycol and caprolactone. *In vitro* testing using neuronal, Schwann and dorsal root ganglion culturing, cell viability testing and immuno-fluorescence labelling of ß-tubulin-III (for neuronal cells) and S100ß (for Schwann cells) demonstrating cellular adhesion and neurite outgrowth on these materials. *In vivo* implantation of NGCs in to a mouse YFP 3.0mm common fibular nerve injury model revealed axon regeneration equivalent to autograft control.

## DISCUSSION & CONCLUSIONS

In summary, photocurable biodegradable polymers manufactured with accurate 3D structures by additive manufacturing techniques have considerable potential for a new generation of medical devices, including implantable NGCs. Devices have improved physical, biochemical and biological properties which are demonstrating to be as effective as 'gold standard' autograft methods for short gap repair.

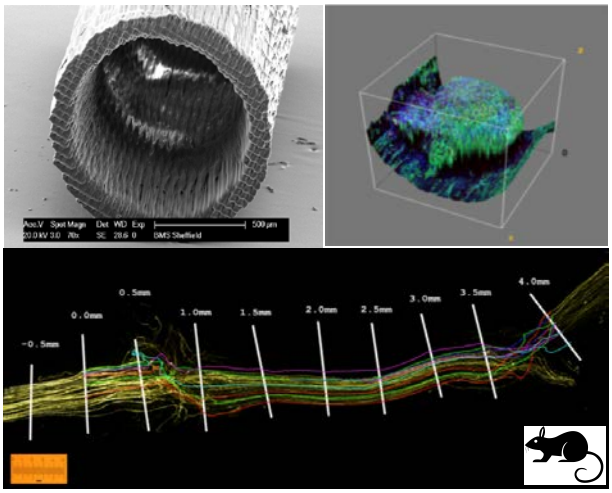


*Figure 1: A nerve guidance conduit fabricated by microSL: (top left). 3D 2-photon microscopy of a DRG growing in vitro in an NGC channel showing neurite and Schwann cell outgrowth (top right). In vivo axon regeneration through a microSL fabricated NGC in a mouse YFP common fibular short gap injury model (bottom).*

## REFERENCES

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3. Kaewkhaw R, Scutt AM, Haycock JW (2012). **Nature Protocols** 7: 1996-2004.

## ACKNOWLEDGEMENTS

EPSRC and NIHR-i4i for funding.

# Assessing the barrier performance of an *in vitro* co-culture model of the intestine

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## INTRODUCTION:

In vitro models of human tissues are fundamental to academic and industrial research. Considering the caveats of *in vivo* animal studies (e.g. high cost, low throughput and poor human correlation) and the 3R's principle of refining, replacing or reducing animal use in research, it is imperative to develop more sophisticated *in vitro* models that better simulate the *in vivo* situation. This can be achieved by developing physiologically relevant scaffold technologies and culturing multiple cell types together to simulate the cellular microenvironment and cross talk experienced *in vivo*.

## METHODS:

Nanofibre scaffolds were produced by electrospinning 10% poly (ethylene terephthalate) in a solution of dichloromethane and trifluoroacetic acid. Caco-2 cells (Epithelial) and CCD-18co (Myofibroblasts) were cultured on opposite sides of the Nanofibre scaffolds for 15-21 days. Throughout the culture period Trans-epithelial electrical resistance (TEER) measurements were used to assess barrier integrity. The apparent permeability (Papp) of two markers, Lucifer Yellow and Atenolol were used to assess the paracellular transport.

## RESULTS:

*Table 1. Apparent permeability values of Lucifer yellow and Atenolol*

| | Papp x10$^{-6}$ (±SD) | | | |
|---|---|---|---|---|
| | Transwell | | Nanofibre | |
| Substance | Caco-2 | Caco-2/18co | Caco-2 | Caco-2/18co |
| Lucifer Yellow | 2.59 (±1.51) | 2.69 (±1.75) | 64.33 (±18.21) | 56.63 (±14.21) |
| Atenolol | 0.53 (±0.13) | 1.63 (±0.11) | 1.06 (± 0.27) | 1.43 (± 0.94) |

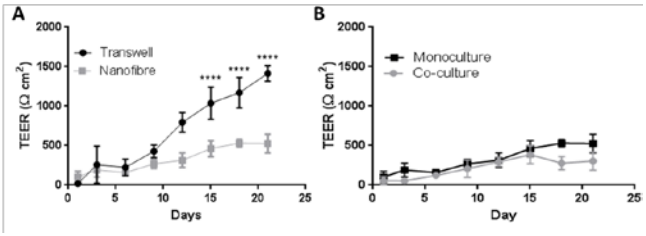


*Figure1. TEER measurments for Caco-2 monocultures and Caco-2/18co co-cultures on Nanofibre and Transwell scaffolds.*
***** p>0.0001*

Caco-2 cells cultured on nanofibre PET scaffolds show reduced barrier integrity with respect to TEER when compared to those cultured on Transwell™ supports (Figure 1). When Caco-2 cells were co-cultured with the 18co myofibroblast cells a reduction in TEER was observed which was significant for the Transwell™ system. Paracellular transport was assessed by Lucifer yellow and Atenolol transport (Table 1). Lucifer yellow transport increased around 20-fold for Caco-2 monocultures cultured on nanofibre scaffolds compared to the Transwell™ equivalent, this significant increase however was not observed for Atenolol.

## DISCUSSION & CONCLUSIONS:

Initial results suggest that culturing Caco-2 cells on nanofibre scaffolds can produce a phenotype more akin to the small intestinal epithelium, the functionality of the model, however, is still to be fully assessed.

## ACKNOWLEDGMENTS:

The authors would like to thank the EPSRC (EP/I01375X/1) and AstraZeneca for funding this work.

# The chorioallantoic membrane (cam) as a capillary bed for human bone angiogenic analysis: the potential of laponite clay gel for growth factor delivery ex vivo

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## INTRODUCTION:

Bone disorders, such as osteoporosis, often lead to fractures which require bone substitute materials to promote bone healing. Lack of vascular invasion is often the cause of failure and is the focus of many current bone tissue engineering approaches1. Here we propose the use of a clay gel (laponite) as a vehicle for growth factor delivery into human bone, and the use the chorioallantoic membrane (CAM) as an ex vivo bioreactor to test our hypothesis.

## METHODS:

To investigate this, 4mm diameter bone cores were obtained from fresh human femoral heads. Prior to incubation, bone fragments were perfused with (i) clay gel (laponite) and (ii) laponite with vascular endothelial growth factor ($rhVEGF_{165}$) or (iii) vehicle control. After 7 days' incubation either on the CAM or in organotypic culture, bone fragments were harvested and analyzed histologically (immunostaining for an endothelial marker, von Willerbrand factor (vWF) and standard bone tissue stainings).

## RESULTS:

Results showed that the highest embryo survival rate was for the bone grafts containing both laponite and VEGF (90%), followed by the laponite group (75%). Histochemical analysis showed marked CAM cell migration and invasion in the laponite groups. Tight and close interaction between the human and chick tissue was observed. In the VEGF treated group vWF immunostaining showed increased blood vessel formation around the graft. Bone cores containing articular cartilage inhibited vascular CAM invasion.

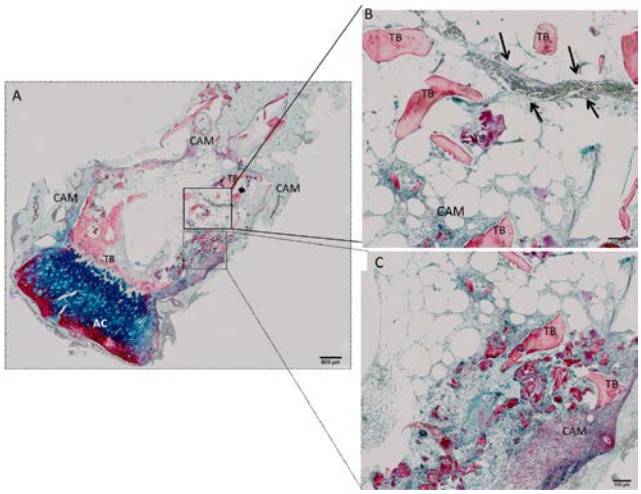


*Figure 1*: Histological section of bone graft with laponite and VEGF integrated and vascularized by the CAM within 7 days. (A-C) Alcian Blue (proteoglycans) and Sirius Red (collagen) staining. (A) Overview of bone graft surrounded by CAM. (B) Blood vessel (BV) invading marrow space. (C) CAM embedding human bone. Black arrows point to CAM blood vessels. TB (trabecular bone) AC (articular cartilage) CAM (chorioallantoic membrane).

## DISCUSSION & CONCLUSIONS:

This data shows that CAM membrane was able to integrate human bone cores within 7 days and that its vasoproliferative response and invasion was increased in the presence of VEFG. Additional drugs in combination with biomaterials could be screened using the CAM model as a surrogate blood supply.

## REFERENCES:

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## ACKNOWLEDGMENTS:

The authors would like to thank the NC3Rs for providing financial support to this project.

# Intracellular transduction of proteins in three dimensional gradients

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## INTRODUCTION:

Methodologies to deliver cargo proteins directly into cells are useful tools to elicit changes in cell behaviour and direct stem cell differentiation and self-renewal. Cellular processes such as proliferation, angiogenesis and differentiation are guided and regulated by gradients of different physical and chemical cues[1]. The ability to control the 3D gradient of these signalling molecules within matrices is therefore important for inducing desirable differentiation of stem cells. We have developed a highly efficient system we term Glycosaminoglycan (GAG)-binding enhanced transduction or GET[2] and wanted to explore if GET protein delivery could be controlled spatiotemporally and ultimately allow us to direct cell responses.

## METHODS:

Hydrogels (5x5x15 mm) with or without NIH3t3 mouse fibroblasts ($2x10^6$ cells/mL) were cast within a modified diffusion chamber[3] to create a 3 compartmental diffusion assembly of source-gel-sink. We used a GET- monomeric red fluorescent protein (mRFP) and compared this to non-transducing mRFP. Gradient profiles at different time points were defined as function of distance inside scaffold at 20 µm resolution by serially slicing the scaffolds perpendicular to the direction of protein diffusion using Leica CM1100 cryostat at -20 ºC. Protein content per slice was quantified fluorometrically using TECAN Infinite 200 PRO multimode reader supported with I-control ™ software.

## RESULTS:

Our system demonstrated that gradient profiles at different time points reflected the transduction capabilities of the GET protein, with non-transducing mRFP diffusing to equilibrium throughout the hydrogel volume. Compared to the mRFP, cellular uptake of GET mRFP completely depleted the hydrogel of free diffusible protein and demonstrated that the cells themselves acted as sink that retained the GET-mRFP.

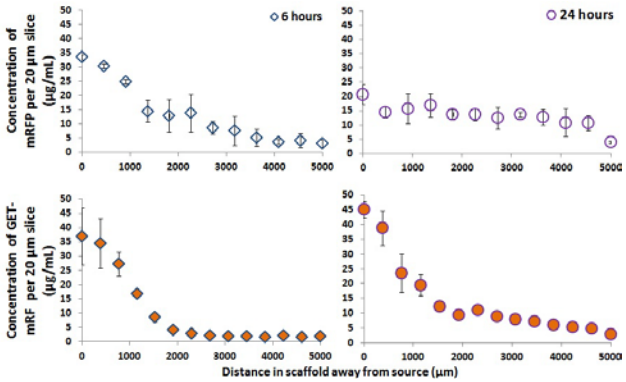


*Fig. 1: Diffusion gradient profiles of mRFP vs GET-mRFP across cellular hydrogel scaffolds at 6 and 24 hours.*

## DISCUSSION & CONCLUSIONS:

With the ability to control the intracellular delivery of functional proteins spatiotemporally, we believe that GET technology will allow us to create gradients in hydrogels to direct cellular behaviour and ultimately control the differentiation of stem cells in 3D for regenerative medicine and tissue engineering applications.

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## ACKNOWLEDGMENTS:

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# Tissue engineering skeletal muscle to investigate glucose uptake

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## INTRODUCTION:

*In vivo*, skeletal muscle contains, multi-nucleated aligned myotubes surrounded by an extracellular matrix [1], and functionally, skeletal muscle is responsible for a large proportion of post-prandial glucose uptake [2]. The development of skeletal muscle tissue engineered constructs which recapitulate both morphological and physiological characteristics *in vitro* provides the potential to investigate metabolic diseases, such as skeletal muscle insulin resistance.

## METHODS:

C2C12 skeletal muscle myoblast cells were seeded at $1x10^5$ cells in fibrin based constructs previously described [1]. Constructs were grown in growth medium supplemented with 250µg aminocaproic acid (AA) (DMEM, 20% FBS, 1% P/S). Upon confluence, media was changed to differentiation media (DMEM, 2% HS, 1% P/S, AA), for two days. Constructs remained in maintenance media for the duration of the experiment (DMEM, 7% FBS, 1% P/S, AA). Constructs were then analysed by qPCR for mRNA of GLUT1, GLUT4. Protein was obtained and separated by western blot to measure concentrations of protein kinase B (Akt) and GLUT4. Glucose uptake was quantified through scintillation counting using [$H^3$]-2-Deoxy-D-Glucose.

## RESULTS:

Time duration in differentiation and maintenance media resulted in contraction of fibrin based constructs ($P < 0.0001$, ANOVA). At 14 days total in culture constructs had developed multi nucleated myotubes (*figure 1*) and express total Akt protein, a key mediator within the insulin signalling cascade (*figure 2*).

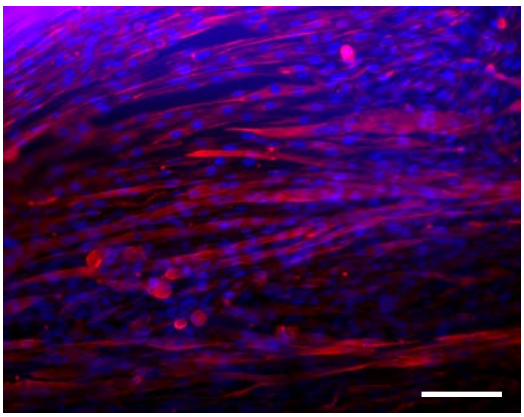

*Figure 1: Immunofluorescence of C2C12 cells in fibrin gels at day 14 in culture. Myodesmin (Red) counterstained with DAPI (Blue). Scale Bar = 100µM (N = 1 construct).*

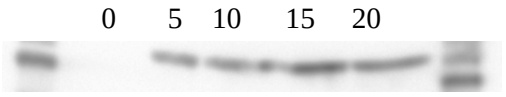


*Figure 2: Immunoblot for total Akt with dose dependent protein concentrations (µg) from 14 day old fibrin gels.*

## DISCUSSION & CONCLUSIONS:

These findings present the characteristics of a skeletal muscle tissue engineered constructs which express fundamental genes and proteins required for insulin stimulated glucose uptake.

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DeFronzo RA, Gunnarsson R, Bjorkman O, Olsson M, Wahren J. (1985). *J Clin Invest*; 76(1):149-155..

# Tissue engineered bone through the use of novel alginate/bone ecm hydrogels

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## Introduction

There is a growing socioeconomic need for new approaches to treating bone damage, especially within an increasingly aged population. Tissue engineering offers a new avenue to generate novel bone both *ex vivo* and *in vivo*. Here the authors have investigated the use of novel hydrogel constructs incorporating both inductive signaling, and an inducible cell source for the purpose of bone formation and regeneration.

## Materials and Methods

Stro-1 enriched human BMSCs and growth factor loaded polymer microparticles [1] were combined with alginate/bone derived ECM hydrogel (60-40%) [2]. VEGF (fast release, 50 ηg/mL), TGF-β3 (fast, 15 ηg/mL), BMP-2 (slow, 100 ηg/mL), PTHrP (fast, 100 ηg/mL), and VitD3 (slow, 25 ηM) were each selected for temporal release of angiogenic, chondrogenic, and osteogenic signals. Controls included HSA loaded microparticles (carrier protein) and blank hydrogels. 5mm segments were subcutaneously implanted for 28 days within MF1 nude mice. Bone formation was assessed by both micro-CT and histological analysis. ANOVA and appropriate t-tests were used to assess statistical significance.

## Results

Hydrogel constructs exhibited tissue invasion, mineralisation, and vascularisation independent of cell incorporation and loaded growth factor (Fig 1B). Dense bone tissue assessed by micro-CT (Fig 1A) correlated with mineralised bone assessed histologically (Fig 1C). Alginate/ECM hydrogel appeared highly osteoinductive.

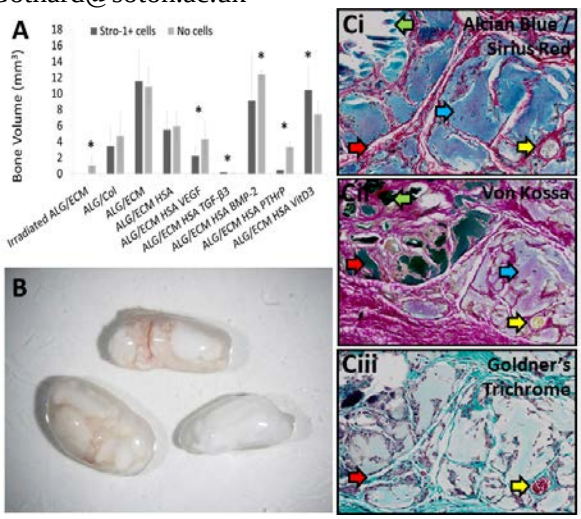


Fig 1: Subcutaneously implanted hydrogels (**B**) were assessed by micro-CT (**A**) and histological analysis (**C**). Green, red, blue, and yellow arrows depict mineralisation, tissue invasion, matrix deposition, and vascularisation respectively.

## Discussion and Conclusions

Novel alginate/ECM hydrogel constructs offer an effective method for tissue engineering new ectopic bone *in vivo*. Future study would assess the capacity for bone defect repair *in vivo*.

**References** [1] L. J. White *et al*, Mater Sci Eng C 33 (5) 2578-2583 2013. [2] M. J. Sawkins *et al*, Acta Biomater 9 (8) 7865-7873 2013.

**Acknowledgments**

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**Disclosures**

No conflicts of interest exist.

# Ameliorating the effects of tissue engineered skeletal muscle atrophy with mechanical overload: relevance to osteoarthritis?

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## INTRODUCTION:

Osteoarthritis (OA) affects >80% of the population aged 55yrs and older1. Both OA and age lead to a significant loss in muscle force2, mass (atrophy) and a decrease in the regenerative capacity of muscle following injury3. Therefore, it is of vital clinical relevance to study ways to ameliorate muscle atrophy in order to manage OA. A 3D in vitro model of replicative senescence has been established4 to further understand the characteristics of atrophic muscle. This atrophic model can be manipulated in vitro to witness the effects of mechanical overload on its structure and genetic response.

## METHODS:

MPD and CON C2C12 skeletal myoblasts were compared[6]. 4 x $10^6$ cells/ml in 3ml type-1 rat tail collagen were plated in glass chambers between floatation bars and were cultured for 14 days [4 d in growth medium (20% FBS), 10 d in differentiation medium (2% HS)]. The constructs were placed on the t-CFM to undergo an acute intermittent stretch protocol or to remain un-stretched to serve as a control. The
RNA was extracted from each gel in preparation for PCR analysis of the following genes: MGF, IGF-I, IGF-IEa, Myogenin, MMP9, MMP2, Myostatin, MyoD, MuRF1, MAF-bx, IGFBP5, IGFBP2, IGF-II, IGF-IIR and IGF-IR.

## RESULTS:

As previously witnessed[4], MPD C2C12 myoblasts have a reduced ability to fuse to form myotubes in monolayer, adhere and remodel the collagen matrix (Fig.1). Force data for the intermittent stretch protocol, and post-stretch PCR data will be presented at the conference.

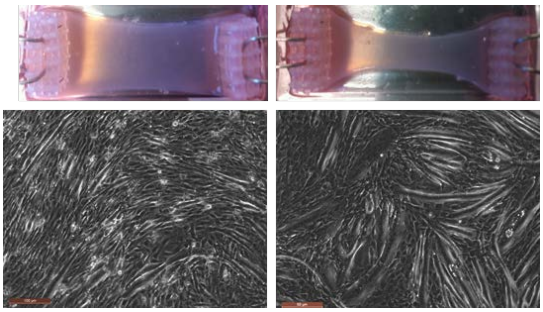

*Fig.1 1. Macroscopic images of 3D collagen constructs (a) MPD (b) CON 2. Monolayer light micrographs of myotubes (a) MPD and (b) CON*

## DISCUSSION & CONCLUSIONS:

These early results show some differences between MPD and CON cells/myotubes in 2D and 3D. It is expected that there may be differences in the up regulation of a number of genes after mechanical overload between MPD and CON. This would show similarities between the exercise response in an atrophic phenotype *in vivo*, providing a controlled model to investigate these phenomena. This could then be translated to the context of osteoarthritis in an ageing population.

## REFERENCES:

1. P.Bajaj et al (2001), *Pain* **93**: 107-14 2. W.Frontera et al (2008), *J Appl Physiol (1985)* **105**: 637-42 3. T.Pietrangelo et al (2009), *Exp Gerontol* **44**: 523-31 4. A.Sharples et al (2012), *Aging cell* 11: 986-95

## ACKNOWLEDGMENTS:

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# Cell micro-patterning on polyhema using 1,1'-carbonyldiimidazole as a surface modification intermediate

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**INTRODUCTION:** Tissue function during development relies on correct cell organization (at micro and macro scales) within defined architectures. In this study we introduce a novel cell micro-patterning technique that utilizes aerosol deposition of CDI through a micro-stencil and subsequent protein immobilization to induce region selectivity, creating cell adhesive properties on a poor cell responsive surface. This methodology aims to produce a rapid and accurate cell micro-patterned area that can remain fixed, without loss or spreading of cells, over a macroscopic area for periods previously demonstrated[1].

**METHODS:** Micro-stencils[1] acted as a mask to selectively deposit CDI aerosols to polyHEMA coated surfaces. Fibronectin (Fn) or FBS was subsequently immobilised to the activated surface. Genetically labelled (mRFP) NIH3T3 cells[1] were seeded onto micro-patterned surfaces in defined media conditions (FBS containing or FBS free, calcium free DMEM). Cell proliferation was assessed every 24 hours for 5 days using MTS assay.

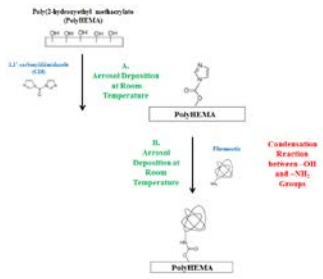


***Fig 1.*** *Schematic diagram outlining reaction mechanism of CDI aerosol deposition and subsequent protein immobilisation.*

**RESULTS:** NIH-3T3 cells attached and proliferated to CDI modified regions and cell patterns remained faithful for several days ***(Fig 2 & 3).***

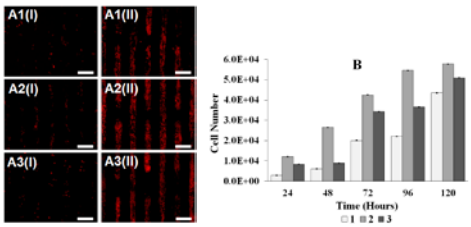


*Fig 2. Micro-patterning of cell lines. A. Fluorescent images and B. proliferation assay (MTS) of NIH-3T3 cultured with 1. 10% FBS immobilisation in FBS containing media, 2. Fn immobilisation in FBS containing media, and 3. Fn immobilisation in FBS free media. Images taken (I) 24 and (II) 96 hours post cell seeding. Scale bar = 20 µM.*

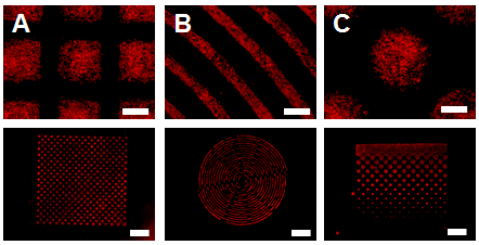


*Fig 3. Micro-patterning of more complex designs. Fluorescent images of NIH-3T3 cells cultured with Fn immobilisation in FBS containing media, showing A. Square, B. Spiral and C. Dot patterned structures. Images taken 96 hours post cell seeding. Scale bar = Top 20 µM and Bottom 2 mm.*

**DISCUSSION & CONCLUSIONS:** Changes to the surface chemistry of polyHEMA to a more adhesive cell substrate were successfully employed using aerosol deposition of CDI. Cells remained proliferative and responsive with limited spreading over a 120 hour period demonstrating high level precision and length scale micro-patterning for future tissue engineering applications and stem cell niche creation. This fabrication method represents a robust system that has micron resolution over a macro scale.

**REFERENCES:** [1]Paik, et al. (2012). Biotech Bioeng 109:2630-41.

**ACKNOWLEDGMENTS:** This work is funded by the EPSRC DTC for Regenerative Medicine.

# Heterogeneity of dental pulp progenitor cells

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## INTRODUCTION:

Mesenchymal Progenitor Cells (MPCs) are the subject of intense scientific interest in biomedicine. We have derived and characterised populations of clonal human dental pulp progenitor cells (hDPPCs). Understanding how these cells behave as part of a community is fundamental to understanding processes of tissue repair and their potential for therapeutic application. This project proposes to study the heterogeneic behaviour of hDPPCs by labelling and tracking cells with fluorescent nanoparticles (Qtracker705®). These nanoparticles have a conserved signal therefore loss of signal is observed following cell division.[1]

## METHODS:

Two clonal (A32 & B11) and one mixed hDPPC (M-hDPPC) population were cultured in α-MEM (10% Foetal bovine serum (FBS) and 100µM L-ascorbate). Cells were labelled with 4nM Qtracker® 705. After 24 hours, cells were sorted using FACS into two groups, high Qtracker®705 signal (Peak) and low signal (Lower). Cells were re-seeded at a density of $2x10^3$cells/$cm^2$ cultured for 72 hours and re-sorted using the same principle. RNA was extracted from the sorted cells. Biomarker analysis was carried out utilising RT-PCR for MPC markers, pluripotency markers, nerural crest and early neural markers.

## RESULTS:

Clonal and mixed hDPPCs demonstrate differences in their initial Qtracker®705 loading and different signal loss over time. B11 loading and signal loss is similar to M-hDPPC. A32 cells maintain signal over 72 hours but lose signal after 144 hours. Differences exist in the expression of MPC, pluripotency and neural markers between the sorted cell populations based on their Qtracker®705 signal.

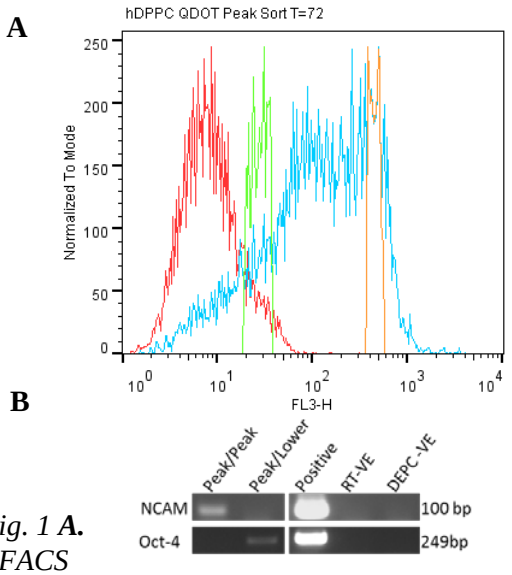


*Fig. 1* ***A.*** *FACS analysis of QTracker705® labelled peak M – hDPPC 72 hours post-sort with re-sorted regions in orange and green.* ***B.*** *RT-PCR of NCAM and Oct-4*

## DISCUSSION & CONCLUSIONS:

Major differences exist in cell behaviour between clonally isolated cell populations of the dental pulp and M-hDPPCs. Sorting cells in this way allows us to identify and characterise sub-populations of cells within a heterogeneous population based on simple labelling techniques. This is a novel technology utilising a biophotonic signal to identify different stem cell phenotypes from a heterogeneous population - a systems approach.

## REFERENCES:

1. H. D. Summers et al, (2013) *ACS Nano. 7:6129-6137*

## ACKNOWLEDGMENTS:

The authors would like to thank the EPSRC (Grant no: EP/F040954/1) for providing financial support to this project

Peak/
Peak

# Modification of peptide self-assembling hydrogels for cell culture applications

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## INTRODUCTION:

Self-assembling peptide hydrogels have been shown suitable for the culture of a range of cell types [1,2]. In our group, we have recently developed a family of β sheet forming peptides that have been successfully used for 3D cell culture applications [3,4]. Modifications to peptide sequence, in particular charge, has been discussed as a method to control the interaction of cells with peptide hydrogels. For this reason, a series of peptide hydrogels were produces which varied in charge density.

## METHODS:

Cells were extracted from bovine cartilage [5] cultured in 5 % $CO_2$ atmosphere at 37 °C and maintained in DMEM supplemented with 10 % FBS, 1 % antibiotic and 50µg/mL ascorbic acid. Peptide solutions were prepared at 30 mg $mL^{-1}$ and adjusted to pH 6.5 with 0.5M NaOH. Cells were suspended in media at $5x10^6$ cells per 300µL and cultured in 3D. Rheometry was performed on a TA AR-G2 with fixed strain and frequency of 1% and 1HZ. Gels were dissolved and cell counts performed using a haemocytometer.

## RESULTS & DISCUSSION:

Hydrogels were produced by doping FEFKFEFK with charge. Subsequent hydrogels were rheologically tested (figure 1) and showed similar mechanical properties. This allows for any variation in cell number to be due to charge alone. Cells were cultured in 3D for 21 days (figure 2). It was seen that all gels were suitable for maintaining bovine chondrocytes over the 21 days, with an increase in cell numbers. In particular, an increase in cell numbers was seen on those hydrogels with 25-50% charge.

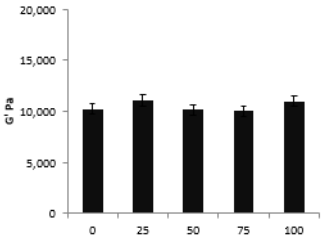


*Fig. 1 Rheological data of peptide hydrogels produced using FEFKFEFK (0), peptide carrying 25% charge (25), 50% charge (50), 75% charge (75) and FEFKFEFKK (100).*

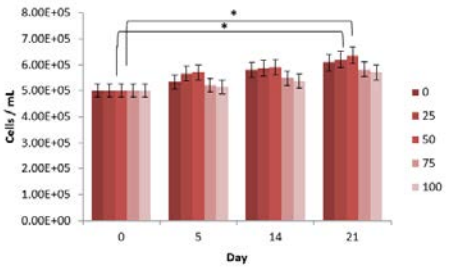


*Fig. 2 Cells/mL of peptides FEFKFEFK (0), peptide carrying 25% charge (25), 50% charge (50), 75% charge (75) and FEFKFEFKK (100).*

## CONCLUSIONS:

We have shown that a range of stiffness equivalent hydrogels can be produced. The variation of charge has been shown to influence cell numbers, when cells are cultured in 3D.

**REFERENCES:**[1] Holmes, TC et al (2000) *proceedings Nat acad sci* 97:6728-6733. [2] Kisiday, J (2002) *Proceedings Nat acad sci* 15: 9996-10001 [3] Mujeeb, A et al (2013) *Acta Biomater* 9(1):4609-17 [4] Guilbaud, JB et al (2012) MRS Proceedings 1417 [5] Barry, JJA (2004) *Biomat* 25:3559-3568

## ACKNOWLEDGMENTS:

Peptisyntha (a member of the Solvay Group), Belgium **(**http://www.peptisyntha.com**)**

# *In situ* synthesis of Biomimetic collagen and hydroxyapatite composite

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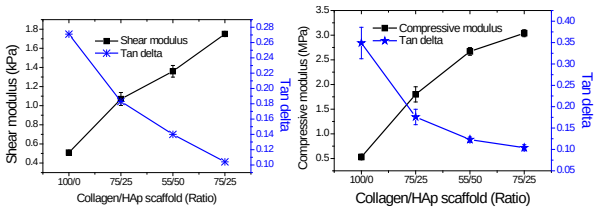
**INTRODUCTION:** Both Collagen and hydroxyapatite (HA) have merits as scaffold materials for bone regeneration [1]. Researches have shown that a combination of collagen and HA would provide an appropriate scaffold materials for bone tissue culture [2]. The *in vitro* performance of collagen-hydrodyapatite composite has well be documented, however, its dynamic mechanical properties at simulated environment has not yet sufficiently established. This work is to study the dynamic properties of the collagen-hydroxyapatite composite at physiological environment in order to determine the physical environment that the scaffold could provide in vivo.

**METHODS:** The collagen-hydroxyapatite composite scaffolds were prepared by an established in-site hydroxyapatite precipiration integrated freeze-drying method as reported elsewhere [3]. The microstructure and composition scaffolds were examined using SEM and XPS, respectively. Dynamic mechanical properties of the scaffolds were analyzed by using a dynamic mechanical analyzer (DMA8000, PerkinElmer). The dynamic stress scan and frequency scan methods in shear mode were performed on scaffolds in both dry and wet condition at 30$^{o}$C. The changes in modules and tan delta as a function of shear deformation were monitored. Compressive tan delta

**RESULTS:** SEM and TEM examinations revealed that nano-HAp precipitate onto and guided by the collagen fibers to form a composite with porous structure. Dynamic mechanical spectra for various collagen-hydroxyapatite composite scaffolds tested in wet condition at 37$^{o}$C under share mode are shown in Figure 1 a & b, respectively. It was observed that addition of hydroxyapatite into collagen matrix resulted in increase of the storage modulus of the scaffolds The higher the HAp content (within the range of study), the higher the modulus is. It was also observed that with the increase of modulus, the tan delta value of the scaffold decreased with the addition of HAp for both shear and compressive tests. The 1% collagen scaffold has a shear tan delta of 0.27, this decreased to 0.1 for the scaffold contains 75% (mass%) HAp. Correspondingly, 1% collagen scaffold has a tan delta of 0.35, this decreased to 0.104 for specimen contains 75% HAp.




(a) (b)

*Fig. 1: Variation of modulus of collagen-HAp scaffold with addition of HAp. (a) Shear modulus and (b) compressive modulus.*

**DISCUSSION & CONCLUSIONS:** The structure of collagen scaffold consisting of a complex mass of randomly knitted chains or large arrays of fibers. Under stress, the chains are stretched. The strain is a combination of inelastic and elastic strains. The viscoelastic behaviour of collagen is a combination of the elastic components and viscous component. With the addition of HAp, it increased the elastic component of the scaffold as evidenced by the increase of modulus and decrease of tan delta value. The influence of HAp on the dynamic property was dependent on the ration of collagen/HAp ratio.

**REFERENCES:** [1] M. Achilli, D. Mantovani (2010) Polymer 2: 664-680. [2] E. Andronescu, G. Voicu et al (2011) J Electron Microscopy 60: 253-259. 3 C Liu, Z. Xia et al. (2008) JBMR: 85B:519-528.

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# Towards the prevascularisation of tissue-engineered skin

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**INTRODUCTION:** Failure to achieve vascularisation is one of the major roadblocks in getting tissue-engineered (TE) substitutes translated from the laboratory to the clinic [1]. It is the aim of this project to create a model that induces neovascularisation within TE skin and in doing so allows the investigation of the effects of biochemical and mechanical factors on initial vascular ingrowth. We propose doing this through the use of a re-endothelialised biological vascular network pefused with a bioreactor to which TE skit will be added to explore formation of new vasculature into the TE skin. The current work focuses on the decellularisation of rat jejunum to establish the bioscaffolds with preserved vascular architecture and early stage recellularisation and angiogenic assay results.

**METHODS:** To produce the natural vascular nets jejunum was harvested from fresh rat cadavers. The main vessel of the mesentery was cannulated and flushed with heparin solution before treatment with 1% Triton-X 100 and 0.1% ammonium hydroxide. Distilled water was then circulated to remove residual detergent and the resultant matrix was sterilised using 0.1% peracetic acid. Residual peracetic acid was removed with sterile PBS. Blue dye was injected into the matrix to assess vascular patency. The presence of DNA, GAG, elastin and collagen before and after decellularisation were identified through histochemical staining and then quantified. The inherent angiogenic properties of the matrix were analysed using the chick chorioallantoic membrane (CAM) assay cultured for 8 days, with a poly-l-lactic acid (PLLA) negative control. To recellularise, a co-culture of dermal fibroblasts and human dermal microvascular endothelial cells (HDMECs) were injected through the mesenteric artery of the decellularised jejunum and subsequently perfused continuously with cell culture medium for 5 days at 37˚C. Samples were taken at days 1 and 5 for histological analysis and fluorescence microscopy.

**RESULTS:** A well-defined vascular tree with multiple branching was visible from blue dye injection. Quantitative analyses showed a 93% reduction in DNA following decellularisation. ECM components such as collagen were found to be retained whilst GAGs were diminished by around 40% and almost all elastin was removed by the process. The CAM assay showed good integration of the matrix with the host whilst indicating increased angiogenic properties of the decellularised matrix when compared to the PLLA control. Upon recellularisation, histochemical analysis showed that the cells attached to the matrix and occupied the blood vessels. Confocal microscopy showed a re-organisation of the HDMECs after 5 days in culture (Fig. 1), with the cells lining the vessel architecture.

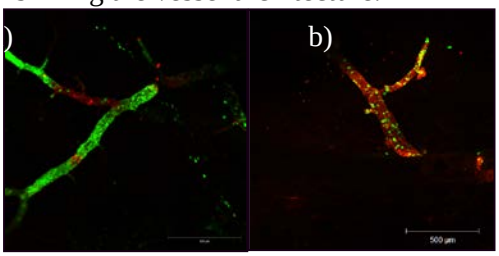


*Fig. 1: Confocal microscopy showing the recellularisation of the decelluarised jejunum with dermal fibroblasts (green) and HDMECs (red) after a) 1 day and b) 5 days in culture*

**DISCUSSION & CONCLUSIONS:** The decellularisation procedure was effective in preserving the architecture of the matrices whilst removing most of the cellular matter. The prp-angiogenic results from the CAM assay infer the preservation of growth factors whilst the recellularisation results illustrate that this matrix supports good cell attachment and reorganisation. We conclude that the re-endothelialised jejunum has the potential to act as a vascular bed from which neovascularization into TE skin can now be studied.

**REFERENCES:** [1] Novosel, E.C., C. Kleinhans, and P.J. Kluger, *Vascularization is the key challenge in tissue engineering.* Advanced Drug Delivery Reviews, 2011. **63**(4-5).

**ACKNOWLEDGEMENTS:** This work is funded by the EPSRC.

# Development of 3d skin models for *in vitro* testing

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## Introduction

For human 3D skin models to be physiologically relevant they must have a barrier function and appropriate epidermal architecture, with the correct proportion of proliferative through to differentiated cells. Importantly, fibroblasts help maintain proliferative epidermal cells and the formation of a dermal epidermal junction (DEJ), and also play a role in paracrine signaling. Critically, the inclusion of fibroblasts affects the response to potential irritants and the propagation of an acute inflammatory response. This is lacking in current 3D models. The aim of the present study was therefore to compare scaffold designs for supporting keratinocyte and fibroblast co-culture and organization in order to produce 3D skin models which can be used to study skin irritants.

## Materials and Methods

Scaffolds: (i) electrospun poly-L-lactic acid (PLLA); (ii) PLLA / poly (3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) / PLLA trilayer[1] and (iii) Alvetex® (Reinnervate Ltd.) a highly porous polystyrene scaffold were investigated. Scaffolds were populated with $1x10^6$ human dermal fibroblasts (HDF) for 7-10 days. Collagen type-1 was coated over a subset of samples. Then $5x10^5$ of normal human keratinocytes (NHK) or HaCaT human immortalized keratinocytes were added. Cultures were submerged for 3 days, prior to air-liquid (A/L) interface culture for 10, 14, 17 or 21 days. Skin irritation using sodium dodecyl sulphate (SDS) was assessed by the '42 bis' protocol[2] by MTT viability and ELISAs for IL-6 and IL-8.

## Results

The presence of fibroblasts with either HaCaT or NHK keratinocytes in Alvetex® produced a more developed morphology after 21 days at an A/L, than constructs without fibroblasts. NHKs developed a denser stratum corneum than HaCaT keratinocytes. HaCaTs cultured on electrospun scaffolds had a distinct close packed squamous appearance (Figure 1). When challenged with SDS All models responded to SDS at $10^{-4}$M without any signs of toxicity. Using this non-toxic SDS concentration, it was found that SDS upregulated IL-6 and IL-8, as detected by ELISA, in the medium of all samples. NHKs generated greater levels than HaCats and challenge with SDS revealed NHKs to be more sensitive to irritation than HaCaT keratinocytes. HaCaTs developed a dense epidermal layer on electrospun PLLA, but had more organisation on the PHVB/PLLA trilayer scaffold

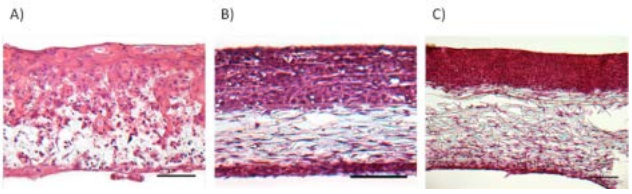


**Figure 1**. HDF and HaCaT keratinocytes cocultured for 21 days at an A/L on: A) Alvetex®, B) PHBV / PLLA electrospun trilayer and C) electrospun PLLA. Bar=100µm.

## Discussion and Conclusions

NHKs produced a better-stratified epithelial formation than HaCaT keratinocytes and responded more to SDS challenge than the cell line. Work is on-going to evaluate which scaffold model is the most appropriate for production of 3D skin models for *in vitro* testing and to investigate the role of the fibroblast in the response of skin to irritants as determined by production of IL-1α, IL-6 and IL-8.

## References

**1**. Bye FJ et al. Biomaterials Science. 1, 942, 2013.
**2**. Statement on the validity of in vitro tests for skin irritation. ESAC, ECVAM 2008.

## Acknowledgments

EPSRC for funding and Reinnervate Ltd for samples.

**Disclosures** - The authors have no competing financial interests except Przyborski as a consultant for Reinnervate, who produce Alvetex technology.

# Porous biodegradable microspheres that form highly porous scaffolds after injection

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## INTRODUCTION:

The use of injectable scaffolds for cell delivery can improve cell engraftment and survival [1]. Polymer microspheres can be used as such a scaffold for tissue repair [2]. For this application it is essential that scaffolds have the mechanical properties, porosity and pore diameter to support new tissue formation. This study demonstrates a method for fabricating porous PLGA microspheres that form scaffolds at body temperature, creating an injectable system capable of supporting cell growth in vitro.

## METHODS:

Porous PLGA microspheres were produced by double emulsion using PBS as a porogen and treated with ethanolic sodium hydroxide. Scaffolds were fabricated by mixing microspheres with carrier solution and incubating at 37°C. Mechanical properties were assessed by TA.HD+ texture analyser**.** Porosity and pore diameter were analysed by micro-computed tomography. Viability of NIH-3T3 fibroblasts cultured on scaffolds was determined using PrestoBlue assay (Invitrogen).

## RESULTS:

Porous PLGA microspheres were fabricated with an average size of 83µm. Treatment with ethanolic sodium hydroxide for 2 minutes increased surface porosity (Fig.1A) and enabled microspheres to fuse at 37°C into scaffolds (Fig1B). Average compressive strength of scaffolds after 24 hours at 37°C was 0.9 MPa. Scaffold porosity was 81.5% on average, with mean pore diameter of 54µm and maximum pore diameter of 306µm. NIH-3T3s attached and proliferated on the scaffolds *in vitro* (Fig1C).

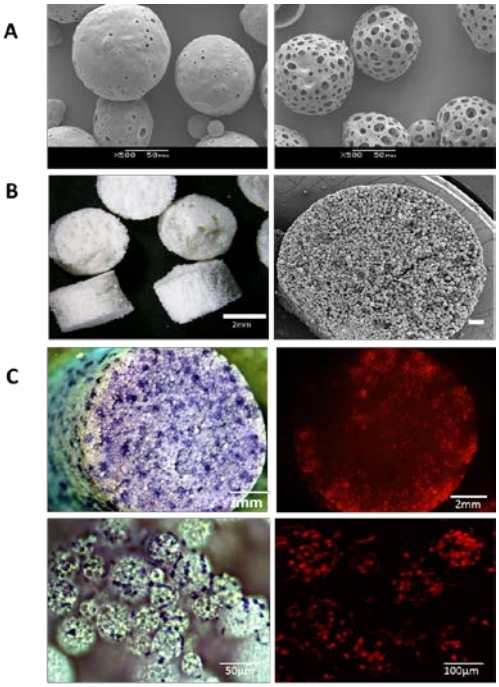


Fig. 1 (A) SEM of porous microspheres, non-treated (left) and EtOH-NaOH treated (right). (B) Scaffolds formed with treated microspheres. (C) Cells on scaffolds stained with toluidine blue (left), red fluorescent cells (right).

## DISCUSSION & CONCLUSIONS:

This study demonstrates a method for fabricating porous PLGA microspheres that form solid porous scaffolds at body temperature, creating an injectable system capable of supporting cell attachment and proliferation.

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2 Bible, E., *et al* 2012. Biomaterials, 33(30): p. 7435-46.

## ACKNOWLEDGMENTS:

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# Holographic optical tweezers as a tool for the study of stem cell microenvironments

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## INTRODUCTION:

The initial cellular organisation within early embryonic stem cell structures profoundly influences differentiation. Such fundamental cellular interactions also play pivotal roles within adult tissues. We have used holographic optical tweezers to precisely control and manipulate cellular position in three dimensions, providing a high precision tool for the study of stem cell microenvironments.

## METHODS:

Holographic optical tweezers were constructed similar to a previous system[1] and various cell types were trapped and manipulated into 3D structures. Cultures were fixed into position using PEG[2] and agarose hydrogels or an avidin-biotin crosslinking method[3].

## RESULTS:

Mouse embryonic stem cells (mES), mesenchymal stem cells (MSCs) and calvaria cells were positioned into precise 3D structures (Fig 1). MSCs were also seeded onto ECM fragments to replicate adult stem cell niche architectures (Fig 2 a/b). In addition mES were co-positioned with electrospun fibres (Fig 2c) and spatiotemporal control of chemical factors was demonstrated with positioned polymer microparticles releasing calcein-AM (Fig 3).

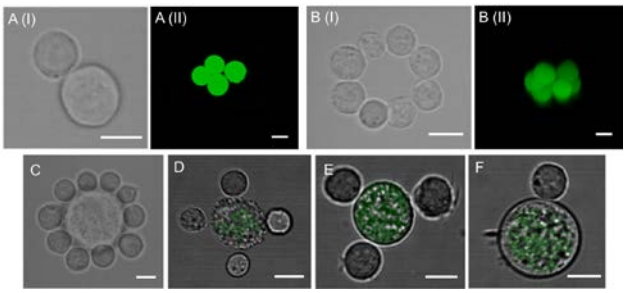


Fig. 1. mES (A-B), mES/calvaria (C-D), mES/MSCs (E-F) positioned into 3D structures. Scale = 14 µm.

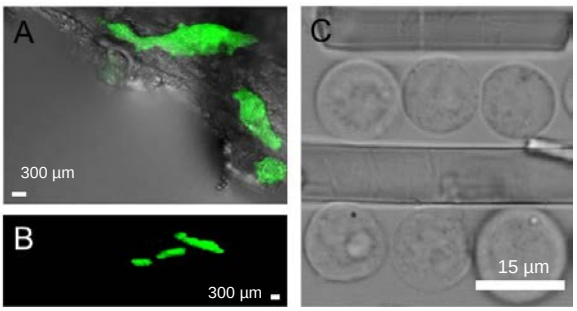


Fig 2. A/B - Single MSCs patterned onto ECM and cultured for 72hrs, C - mES and PLGA fibers.

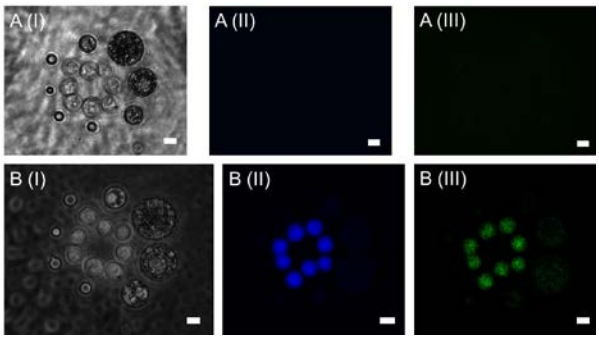


Fig 3. Patterned mES and microparticles releasing calcein-AM (blue and green) were cultured from 1 (A) to 10 days (B) in an agarose gel. Scale = 14 µm.

## DISCUSSION & CONCLUSIONS:

We have developed a novel method to precisely position cells and polymer fibres forming stable constructs with varying configurations. Spatiotemporal control of chemical factors was also achieved with positioned polymer particles. This method has huge potential for the study of cellular microenvironments.

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## ACKNOWLEDGMENTS:

ERC under the Seventh Framework Programme (FP7/2007-2013)/ERC grant agreement 227845.". Martin Ehrbar and Yannick Devaud.

# Cyclic hydrostatic pressure enhances bone growth and repair in an *ex vivo* non-union defect model

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## INTRODUCTION

Mechanical loading of bone *in vivo* results in the generation of dynamic hydrostatic forces as bone compression is transduced to fluid pressure in the osteocyte canalicular network. It has been previously shown that physiological hydrostatic loading regimes result in increased bone growth in *ex vivo* embryonic chick femurs[1]. The aim of this study was to investigate the effect of physiological mechanical loading on bone repair using a non-union defect in the embryonic chick femur model.

## METHODS:

$10^5$ hMSCs were seeded into 0.5mg/ml collagen hydrogels. Defects were created along the mid-section of the (e11) chick foetal femur and the hydrogels inserted. A hydrostatic pressure regime of 0-280kPa at 1Hz, was applied to the femurs for one hour per day in a custom designed bioreactor for 14 days. The constructs were supported using a glass needle. Bone formation was assessed after 14 days by X-ray microtomography and quantified by histology.

## RESULTS:

Total femur volume was significantly higher in hydrostatically stimulated samples ($p$=0.01). Hydrostatic stimulation significantly increased the bone volume in the implant ($p$=0.04) which was complemented by improved trabecular architecture and increased mineralized tissue bridging the defect site (fig. 1 b&d).

*Table 1.µCT of femurs after 14 days culture*

| | Total Femur Volume ($mm^3$) | % Bone Volume (Defect) |
|---|---|---|
| Unstimulated | 9.68 | 2.17 |
| Stimulated | 15.56 | 10.00 |

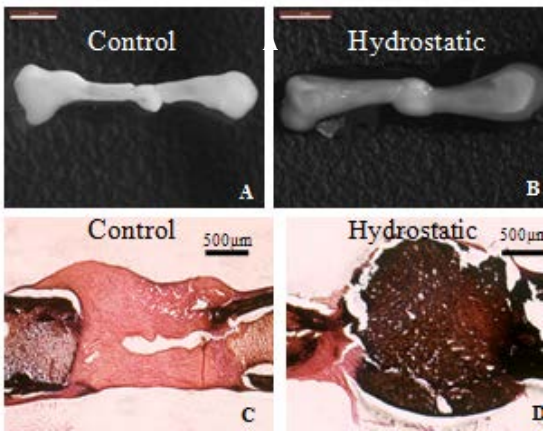


*Fig. 2. (A&B) Whole mount imaging demonstrating enhanced cortical growth and improved integration of the construct in stimulated femurs. (C&D) Von Kossa staining for mineralisation shows osteogenesis in the implant in stimulated femurs.*

## DISCUSSION & CONCLUSIONS:

Hydrostatic stimulation enhanced femur growth and increased bone volume in collagen - hMSC implants in *ex vivo* chick femurs. Hydrostatic stimulation significantly increased mineralisation in hMSC collagen implants. The study also demonstrates a suitable model for future study of non-union repair *in vitro*.

## REFERENCES:

[1] Henstock, JR, Rotherham, M, Rose, JB, & El Haj, AJ. (2013). Cyclic hydrostatic pressure stimulates enhanced bone development in the foetal chick femur in vitro. *Bone*, *53*, 468–477.

## ACKNOWLEDGMENTS:

The authors would like to thank TGT/Instron and the EPSRC DTC in regenerative medicine.

# *In vitro* detection of ppix fluorescence in human colon cancer cells- towards better pre-surgical photodiagnosis and staging of bowel cancers

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## INTRODUCTION:

Most bowel or colorectal cancer diagnosis and staging occur after the surgical removal of the suspicious tissue. The use of a real time fluorescent marker for bowl cancer tissue detection, in combination with laparoscopic or endoscopic techniques would take us a step closer towards better pre-surgical detection and staging (stratification) of these diseases in human patients. Our aim is to study the feasibility of utilizing protoporphyrin IX (PpIX) fluorescence in colorectal cancer cell lines, to distinguish them from non-tumour cells in *in vitro* model systems for diseases such as peritoneal carcinomatosis.

## METHODS:

Monolayer cultures of human colon cancer cell lines (E.g. T84 & HT29) were investigated in comparison to non-tumour cells (E.g. HEK293 & 3T3). Incremental concentrations [0-1mM] of the pro-drug 5-aminolevulinic acid (5-ALA), and incubation times (0-6h) were investigated for levels of PpIX fluorescence detectable with a spectrofluorometer (Ex 405nm and Em 625nm) of 4 central points per well of 96 well black plates with transparent base (4 replica wells per plate). The % change above basal (no 5-ALA treated cells) was calculated using the formula below, and then plotted (Fig 1A). **% Change = [(Treated-Basal)/Basal] x 100**

## RESULTS:

PpIX fluorescence increased with increasing concentrations of 5-ALA and incubation times. HT29 tumour cells displayed the greatest detectable PpIX fluorescence in comparison to other cells tested in these experiments. 1 mM 5-ALA treated HT29 cells were approximately 1300 % brighter than non-treated cells, while T84 tumour cells are approximately 300 % brighter than non-treated cells after 6h incubation (Fig 1A). HEK293 cells under investigation showed lower levels of PpIX fluorescence than HT29 tumour cells (Fig 1B).

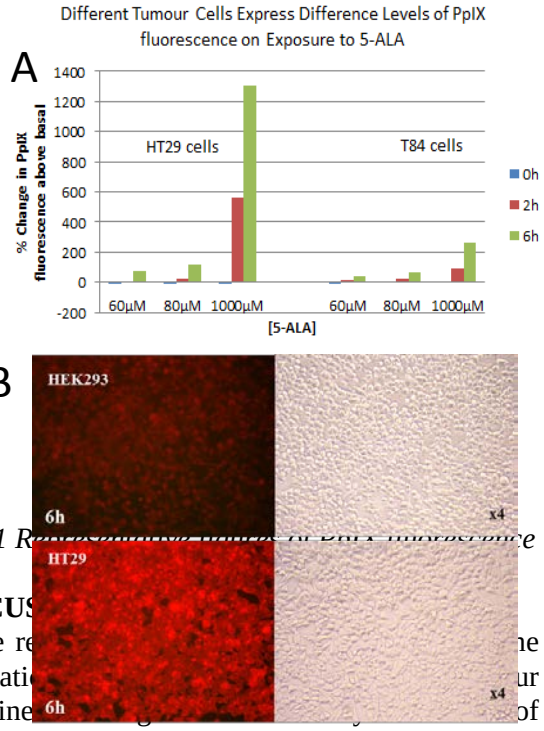


*Fig. 1 R[illegible]*

## DISCUS[illegible]

These re[illegible]ne generati[illegible]ur cell line[illegible]of PpIX fluorescence detected in bowel cancer cells/tissues may be a useful aid in classifying bowel cancer patients for treatment prior to surgery and/or chemotherapy.

## ACKNOWLEDGMENTS:

The authors would like to thank NIHR and the CRUK Leeds Centre-Development Fund (Grant no: C37059/A16369) for providing financial support to this project.

# Production of growth factors for use in regenerative medicine application

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## INTRODUCTION:

Bone Morphogenetic Protein 2 (BMP2) is an osteoinductive growth factor which plays an important role in bone regeneration and repair. BMPs can be isolated directly from bones but the purification protocol is complicated and the yield is low (~1µg/kg) [1]. Another method to produce BMP2 involved constructing transgenic tobacco plants for eukaryotic expression system for BMP2. However, this approach required a long period of time for a growth and a very complicated purification protocol [2]. Commercially available BMP2 is produced in the prokaryotic hosts *Escherichia coli* (*E.Coli).* The BMP2 is produced as inclusion bodies but the solubilising and refolding procedures are complicated and costly [3].The high cost of BMP2 is prohibitive for tissue engineering research.

**METHODS:** This work aims to develop a low cost method to simply the production of BMP2 for tissue engineering, by producing it in its soluble form using a special tag dihydrolipoyl-dehydrogenase (Lip) which enhances its expression and solubilisation. A two-step purification was implemented to purify Lip-BMP2 based on the affinity and ion-exchange chromatography.

**RESULTS:** The expressed protein was in its soluble form in the supernatant, no further solubilising and refolding steps were needed The BMP2 was functionally active as demonstrated by the induction of alkaline phosphatase activity in C2C12 cells.

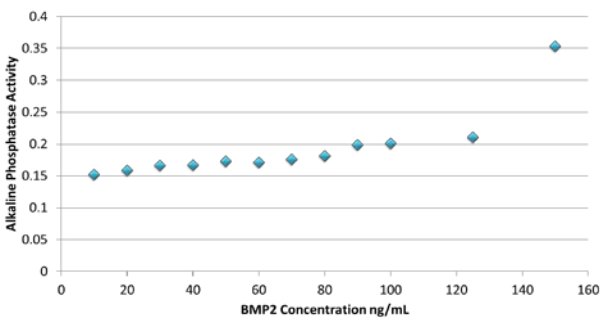


*Fig. 1 : Biological activity of purified dimer BMP2 in C2C12.*

## DISCUSSION & CONCLUSIONS:

This work was carried out in order to develop an effective procedure to simply the production of active BMP2 without any refolding and solubilisation steps. The purified dimer BMP2 showed biological activity as demonstrated by the induction of alkaline phosphatase activity in C2C12. This procedure shown to be highly reproducible, it was able to produce active dimer BMP2 in short period of time with low cost and with no solubilisation and refolding steps.

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# Serum-free medium development for the expansion of bone marrow derived-mesenchymal stem cells

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[2]School of Mechanical and Systems Engineering, Newcastle University, UK
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**INTRODUCTION:**
Adult human stem cells offer significant promise in regards to the repair and regeneration of damaged and degrading tissues and organs but due to poorly characterised and expensive expansion processes are rarely developed into mature commercial products. In an effort to help reduce the variability inherent to many adult stem cell-related culture methodologies and characterisation procedures, we have begun to develop a completely defined serum-free medium for use in the expansion of human bone marrow-derived mesenchymal stem cells (hBM-MSCs).

**METHODS:**
BM-MSCs were initially isolated from the trabecular bone of human femoral heads before being cultured using conventional serum-containing medium over a period of approximately five weeks. Utilising a series of fractional factorial experiments, the effects of a range of different medium supplements on total cell number and morphology were assessed.

**RESULTS:**
The results of this investigation appear to suggest that a number of the tested supplements may aid in the expansion of human bone marrow-derived mesenchymal stem cells during serum-free culture; including Fibroblast Growth Factor-2 (FGF-2) and Insulin-Selenium-Transferrin-Ethanolamine (ITSX).

**DISCUSSION & CONCLUSIONS:**
Whilst it is important to note that the total cell numbers generated under serum-free conditions were significantly lower than those seen when utilising serum supplemented medium, these results do support the idea that an optimal collection of growth factors could help sustain hBM-MSC growth within a defined environment. Future work will focus on encouraging serum-free cell adhesion and working towards hBM-MSC isolation using a completely defined medium formulation.

**ACKNOWLEDGMENTS:**
The authors would like to thank the Engineering and Physical Sciences Research Council (EPSRC) and Arthritis Research UK (ARUK) for providing financial support to this project.

# Correlating mesenchymal stem cell delivery with pain in an oa rat model.

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[2] Arthritis Research UK Pain Centre, Nottingham University, Nottingham, UK and
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## INTRODUCTION:

Osteoarthritis (OA) is characterised by pain, inflammation and ultimately destruction of the synovial joint. Existing pharmacologic treatments are aimed at alleviating symptoms (pain and inflammation). These approaches do not target the disease process itself thus allowing the disease to progress in severity. Mesenchymal stem cells (MSCs) have been identified as suitable candidates in treating OA. They can be utilised as a therapy to address structural destruction and inflammatory aspects of the disease. To date, the relationship between MSCs delivery and pain has not been investigated. In this study, we have implemented a rat model of OA to investigate the effects of administering MSCs as a therapy on pain behaviour. Further to this, novel approaches to image and track implanted cell population's *in vivo* using superparamagnetic iron oxide nanoparticles (SPION) and MRI technologies were applied.

## METHODS:

Murine Mesenchymal stem cells (mMSCs) were isolated, expanded and CM-DIL labelled prior to implantation. Meniscal transection model (MNX) of OA was surgically induced in adult Sprague Dawley rats. Upon OA induction, $1.5x10^6$ mMSCs (SPION-labelled or unlabeled) or serum free media were intra-articularly implanted. Pain behaviour (changes in weight-distribution and hindpaw mechanical withdrawal thresholds) was assessed up to 42 days post-surgery. MRI (Bruker 2.35T MRI scanner) of the knee joint was performed at the final time point and joint histopathogy was evaluated.

## RESULTS:

Administration of MSCs significantly reduced weight bearing asymmetry, but not hindpaw withdrawal thresholds, indicating potential anti-nociceptive properties of MSCs. Good contrast was generated with SPION-labelled cell population located within the synovial cavity after 29 days by MRI and confirmed histologically.

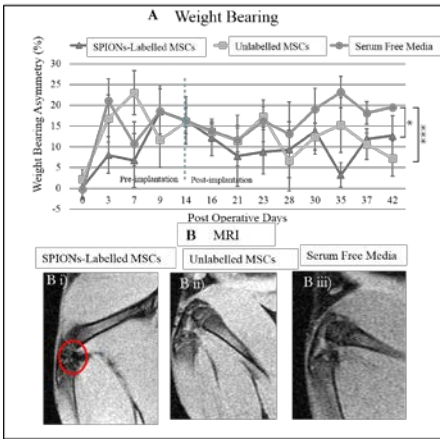


*Figure 1. (A) Pain assessment; by weight-bearing asymmetry in response to the intra-articular implantation of SPION-labelled, unlabelled mMSCs and serum free media over 42 days. B) MRI tracking of implanted cell populations following intra-articular implantation of SPION-labelled, unlabelled mMSCs and serum free media. Red circle indicates location of SPION-labelled cell populations.*

## DISCUSSION & CONCLUSIONS:

This data highlights the potential anti-nociceptive properties of MSCs. The differential effects of the MSCs on weightbearing asymmetry versus hindpaw withdrawal thresholds suggest that this intervention may alter established peripherally-driven OA pain, but not established centrally-mediated OA pain. Whether reduced pain is linked to better repair or the anti-inflammatory actions of the MSCs will be further investigated. MRI tracking of cell populations proved to be a practical means of monitoring cell fate *in vivo*.

## ACKNOWLEDGMENTS:

Acknowledge the support of the EPSRC and ARUK for funding sources

**Declaration:** The authors declare no competing interests

# The role of insulin-like growth factor (igf) binding proteins in osteogenic differentiation of human dental pulp stromal cells derived from healthy and carious teeth.

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## INTRODUCTION:

The IGF axis plays an important role in osteogenic differentiation [1]. Although some of the molecular details associated with IGF ligand and receptor roles in osteogenesis are becoming apparent, much less information is available regarding the potential functions of IGF binding proteins (IGFBPs) in this process. Our research aims to investigate the role of the IGF axis in the osteogenic differentiation of human dental pulp stromal cells (HDPSCs) derived from healthy and carious teeth. Our translational aim is to investigate the potential use of IGF axis members for future therapeutic application in bone/dentine regeneration.

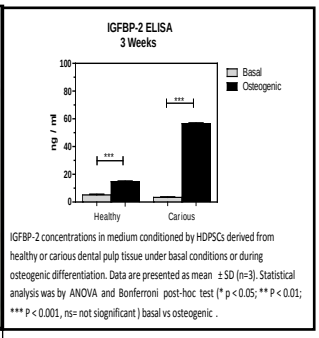
## METHODS:

Freshly extracted third molars (3 healthy and 3 carious) were collected from adult age matched patients (20-40 years old). Pulp cells were isolated, and expanded until passage 4 then cultured in either basal medium (α-MEM, plus 20% FBS, 1% Penicillin Streptomycin and 1% L-Glutamine) or osteogenic conditions (basal medium + 10 nM dexamethasone and 50 μg/ml L-ascorbic acid) for 1 and 3 weeks. Osteoblastic differentiation was investigated in both cultures using alkaline phosphatase (ALP) and Alizarin red staining. qRT-PCR was used to confirm the expression of bone markers (*ALP*, OC, *RUNX2*) and to investigate the changes in gene expression of the IGF axis (10 genes). Concentrations of IGF binding proteins (IGFBPs) in conditioned media were determined by ELISA. All data were statistically analysed using ANOVA and Benferroni post-hoc test to report significant differences. Results were considered statistically significant at $p<0.05$.

## RESULTS:

Healthy and carious HDPSCs showed positive staining for ALP indicating osteoblastic differentiation. However, carious HDPSCs showed higher proliferation rates and more intense ALP staining. Osteogenic differentiation was confirmed by osteogenic markers expression in HDPSCs isolated from both healthy and carious teeth; again, these markers showed higher expression in HDPSCs from carious teeth. The gene expression of specific IGF axis members was altered under osteogenic conditions in both healthy and carious HDPSCs, compared with basal cultures. There were reproducible changes in IGFBP-2 and -3 protein expression in all healthy and carious teeth.


ALP staining at 3Weeks




## DISCUSSION &CONCLUSIONS:

We have clearly demonstrated reproducible and reciprocal changes in IGFBP-2 and -3 expression in HDPSCs during differentiation to an osteogenic phenotype. HDPSCs derived from carious teeth had greater potential to differentiate toward the osteogenic lineage compared to those derived from healthy teeth. These findings can have a future impact on clinical approaches for bone and dentin regeneration.

## REFERENCES:

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## ACKNOWLEDGMENTS:

“The authors would like to thank King AbdulAziz University –Jeddah (KAAU) and the Royal Embassy of Saudi Arabia – Cultural Bureau (UK) for providing financial support to this project ”.

“The authors also would like to acknowledge the WELMEC, a centre of excellence in biomedical engineering ( funded by Wellcome trust and EPSRC) for funding Reem El-Gendy.

# Comparison of 3d micro-printed polycaprolactone conduits and fibrin conduits for peripheral nerve repair

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*[1]Department of Integrative Medical Biology and [2]Department of Surgical and Perioperative Sciences, Umeå University, Umeå, Sweden, [3]Department of Material Science and Engineering, University of Sheffield, UK*

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**INTRODUCTION:** Peripheral nerv
affect 3% of trauma patients[1]. In ca
injuries, an autologous nerve graft i
standard technique used to repair
Artificial nerve conduits constructed
natural or synthetic materials have bee
but their ability to promote regeneratio
to nerve grafts[2]. Advanced fabrication
might enable the production of mor
designed conduits with suitable chemical properties to better support axon growth and Schwann cell biocompatibility. In this study we have compared a new 3D-structured polycaprolactone (PCL) conduit with simple tubular constructs made from fibrin glue.

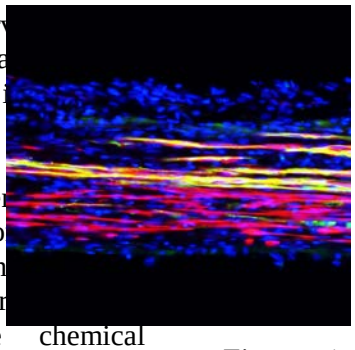

*Figure 1. Beta-III-tubulin immunostaining for regenerating axons (green) and S100 staining for Schwann cells (red) on the wall of the PCL conduit. Nuclei are shown by DAPI staining (blue).*

**METHODS:** Caprolactone pre-polymer was microwave synthesised, methcrylate functionalised and then UV cured into 3D structures via stereolithography (PCL conduit)[3]. Tubular fibrin conduits were moulded from two-compound fibrin glue (Tisseel™ Duo Quick; Baxter)[4]. The constructs were used to repair a 10mm rat sciatic nerve gap. Regeneration was compared with autologous reverse nerve grafts at 3 weeks.

**RESULTS:** *In vitro* testing demonstrated cellular adhesions and neurite outgrowth on the caprolactone material. *In vivo*, the 3D structured PCL conduits supported a 6-fold higher level of axon regeneration into the distal stump, compared with the fibrin conduit. Numerous regenerating axons were adherent to the wall of the PCL conduit, often in close association to the infiltrating Schwann cells (Figure 1). The Schwann cell ingrowth was more extensive in the PCL conduit compared with the fibrin conduit. Both conduits supported early vascularisation and RECA-1 positive endothelial cells also attached to the walls of the PCL conduit.

**DISCUSSION & CONCLUSIONS:** In summary, the 3D micro-printed PCL conduit demonstrated the usefulness of photocurable, degradable polymers as a tool to manufacture a new generation of conduits in peripheral nerve repair.

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**ACKNOWLEDGMENTS:** This study was supported by the Swedish Research Council, European Union, Umeå University, the County of Vasterbotten, the NIHR i4i and the EPSRC, UK

# *In vitro* degradation of novel resorbable polymers for maxillofacial applications

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## INTRODUCTION:

Mandibular fractures are usually aligned using internal fixation plates which support the bones whilst they heal. Each year there are significant numbers of revisions required as a consequence of post-operative complications associated with current plate designs. The development of new biodegradeable fixation plates will reduce the need for these revisions, consequently reducing the economic burden on the health system, and improving quality of life for the patient.

We report use of an *in vitro* model to assess the initial degradation profile of a synthesised biodegradable polymeric material for use in maxillofacial bone fracture fixation.

## METHODS:

Hydroxyethyl methacrylate terminated poly-lactic-*co*-glycolic acid (HEMA-terminated-PLGA) was synthesised *via* ring opening polymerisation of lactide and glycolide, (85 : 15 Mol %, 120 kDa). Commercially available PLGA (85 : 15, Sigma Aldrich) of molecular weights 50-74 kDa and 190-240 kDa were used as controls. Polymeric plates were prepared using compression moulding.

The plates (n = 5) were placed into vials containing phosphate buffered saline (PBS) (8 mL) in an incubator (37 °C) for 0, 1, 2, and 6 weeks, the PBS was changed weekly. On removal the plates were assessed for their mechanical properties (Instron 3-point bend test, 1mm/min).

## RESULTS:

Table 1: Young's modulus of polymer plates (standard deviations can be seen in parenthesis).

| | HEMA-term PLGA (120 kDa) | PLGA (50-75 kDa) | PLGA (190--240 kDa) |
|---|---|---|---|
| Time / weeks | Young's modulus / GPa | Young's modulus / GPa | Young's modulus / GPa |
| 0 | 6.7 (2.0) | 4.1 (0.3) | 8.4 (2.8) |
| 1 | 4.5 (0.3) | 4.1 (0.8) | 4.8 (0.4) |
| 2 | 6.3 (0.5) | 2.5 (1.1) | 7.2 (0.3) |
| 6 | 5.0 (0.2) | 0.4 (1.5) | 5.4 (0.1) |

Analysis of the data reveals a difference between the Young's modulus of PLGA (50-75 kDa) and HEMA-terminated-PLGA after 2 and 6 weeks, indicating that HEMA-term PLGA is able to maintain its stiffness for longer. HEMA-term PLGA yields similar stiffness properties to PLGA (190-240 kDa).

## DISCUSSION & CONCLUSIONS:

The results show that HEMA-terminated-PLGA maintains stiffness for 6 weeks in PBS, behaviour which is comparable with high molecular weight commercial PLGA, and which suggests that the HEMA-terminated-PLGA could be used within a biodegradable fixation plate.

## ACKNOWLEDGMENTS:

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# Controlled production of poly(3-hydroxybutyrate- co-3-hydroxyhexanoate) (PHBHHx) nanoparticles for use in tissue engineering

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**INTRODUCTION:** Control of size and quality of nanoparticles during production is critical for their success in tissue engineering. This would allow reliable and reproducible manufacture of smaller nanoparticles for applications such as nanoparticle-mediated gene transfection. This holds much promise in regenerative medicine as non-viral gene vectors present a significantly reduced safety hazard compared to viral based gene vectors.

**METHODS:** A Design of experiment (DOE) method was utilised to determine the sensitivity of process variables and the repeatability of producing PHBHHx nanoparticles of a desired size. The DOE approach uses parallel multivariate designed experiments to investigate the action and interaction of variables to improve understanding, and therefore control of complex processes. We then encapsulated PDGF-ββ for controlled and sustained release to induce human mesenchymal stem cell expression of smooth muscle actin (SMA).

**RESULTS:** A size range of nanoparticles was produced by varying the number of sonication cycles whilst maintaining the same PHBHHx concentration at high and low sonication power. This process was first completed with blank nanoparticles to obtain a base curve for nanoparticle size production over two batches (to account for the associated common cause variability). This process produced a nanoparticle range of 90 - 215 nm with zeta potential (-35.2 ± 7.79) and PDI (0.168 ± 0.029) within accepted limits. This identified two curves of sonication power at 100 W and 500 W that could be used to produce nanoparticles of a controlled size. A controlled and sustained release of encapsulated PDGF-ββ over 80 hours which induced SMA transcription in hMSCs was observed.

**DISCUSSION & CONCLUSIONS:** In this study we created nanoparticles as small as 90 nm with a PDI of well below 0.3, which is far smaller than the theoretical minimum of 122.4 nm using PLGA-BSA.

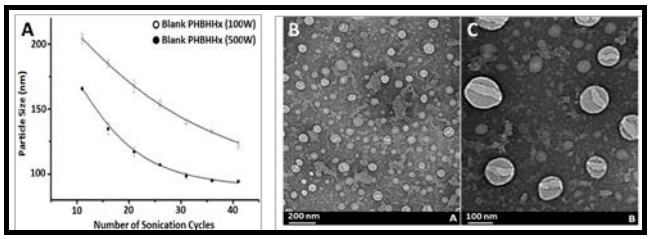


*Fig. 1(A) Nanoparticle prediction Curve, (B&C) SEM of PHBHHx nanoparticles*

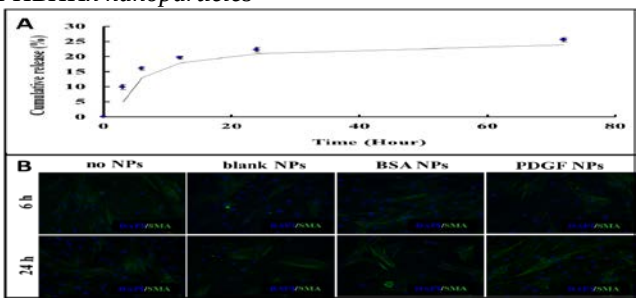


*Fig. 2 Cumulative release curve for PDGF-ββ (A) and NP encapsulated PDGF-ββ induced differentiation on hMSC*

This shows that the use of PHBHHx as a drug encapsulating polymer has greater potential compared to PLGA, producing smaller nanoparticles as required for intravenous injection and transport across the blood-brain barrier as well as opening up new avenues in the use of nanoparticles in gene transfection. The encapsulation and subsequent release of PDGF-ββ successfully induced SMA transcription in hMSCs. Our findings have added to the body of evidence that PHBHHx can be used not only in drug delivery, but also in sustained release of growth factors for controlled stem cell differentiation.

**ACKNOWLEDGMENTS:** Hyanji Scaffold Project: European Commission Framework 7 Program: SP3 People (PIRSES-GA-2008-230791)

# Cross-flow membrane emulsification for tunable-sized Monodisperse microparticles

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## INTRODUCTION:

In the field of tissue engineering (TE) and regenerative medicine, polymeric microparticles play distinct role, according to size[1]. Microparticles of 50µm and above sizes have been used as building blocks to construct injectable scaffold *in vitro*[2] or as cell carrier systems to form scaffold *in situ* [3]. To enhance efficiency of the scaffold in terms of repeatable initial cell numbers per scaffold and eventually to have better control over the interpretation of experimental data, monodispersity in the microparticle size distribution is crucial. Cross-flow membrane (CFM) emulsification is a robust technique that has been used to fabricate monodisperse microparticles but mostly of mean size 36µm or below[4]. This study therefore aimed at preparing monodisperse microparticles of larger sizes that can be used as scaffold building blocks in TE applications using CFM emulsification technique.

## METHODS:

Polycaprolactone (PCL) solution in dichlomethane was passed through the membrane under $N_2$ pressure into PVA solutions flowing across the membrane and emulsion droplets were generated into the PVA solution (Fig.1). Subsequent solvent evaporation yielded solid PCL microparticles.

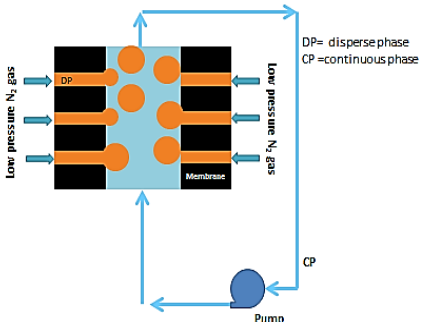


**Fig.1:**
**Schematic illustrates CFM emulsification process.**

## RESULTS:

PCL microparticles of uniform shape and narrower size distribution were obtained by the technique in comparison to that of microparticles prepared by conventional high speed homogenization method (Fig.2).

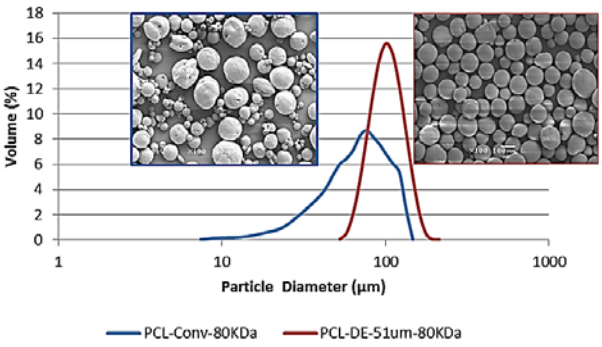


**Fig.2: Particle size distribution of PCL microparticles prepared by conventional (blue c**urve and blue-bordered SEM image) and cross-flow membrane emulsification **(red curve and red-bordered SEM image).**

## DISCUSSION & CONCLUSIONS:

In this study, we demonstrate the potential of cross-flow membrane emulsification, to prepare larger sizes monodisperse microparticles. The sizes were mainly controlled by selecting appropriate membrane pore size, working pressure and PVA concentration.

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## ACKNOWLEDGMENTS:

The authors would like to thank Islamic Development Bank for funding to this project.

# Human osteoblasts within soft peptide hydrogels promote mineralisation *in vitro*

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## INTRODUCTION:

Biomaterials with cell culture and tissue engineering applications are a promising alternative to treat degenerative diseases such as periodontitis[1]. The octa-peptide FEFEFKFK, where F is phenylalanine, E glutamic acid and K lysine, spontaneously self-assemble into β-sheet rich fibres that self associate to form a rigid hydrogel when above a critical concentration[2,3]. Here the mechanical properties and the ability of the gel to support the 3D culture of human osteoblasts (HOBs), the production of bone proteins and bone mineralisation are explored.

## METHODS:

3% wt FEFEFKFK hydrogels were used to culture HOBs. The elastic (*G'*) and viscous (*G''*) moduli of gels were recorded as function strain (0.01-100%) at 1 Hz and oscillatory frequency (0.1-100 Hz) at 1% strain using an ARG2 rheometer. HOBs were grown under standard cell culture conditions. Live/dead and Picogreen assays were carried out following manufacture's instructions. Detection of collagen I (Col I), was carried out using ICC staining. Quantification of bone proteins as well as the presence of calcium deposits were determined using colorimetric and Alizarin red assays respectively.

## RESULTS:

HOBs within gels, presented good viability (Figure 1A), and proliferation (Figure 1B), increases and remain steady over 14.

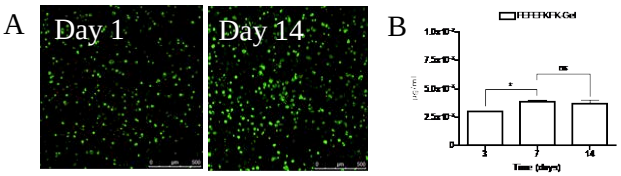


*Fig. 1A. HOBs viability. 1B. HOBs DNA content.*

FEFEFKFK G' increased in the presence of cells probably due to deposition of extracellular matrix (Figure 2).

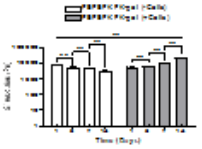

Fig. 2. *FEFEFKFK mechanical properties with and without cells as control.*

HOBs produced Col I, osteocalcin (OCN) and alkaline phosphatase (ALP) inside gels (Figure 3), over 14 days of culture (Figure 4 A, B & C).

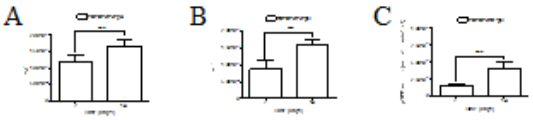


Fig. 3 A, B and C. production of Col I, OCN and ALP within gels respectively.

Calcium deposits were detected inside gels up to the 14 days explored here (Figure 5).

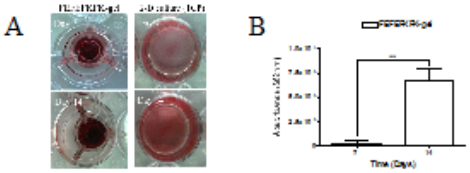


Fig. 4 A and B. Presence and absorbance values obtained from calcium ions deposited within gels and TCP respectively.

## DISCUSSION & CONCLUSIONS:

The FEFEFKFK gel supports the viability and proliferation of HOBs for 14 days. During this time HOBs express osteogenic proteins, and the gel mechanical properties increases. Thus, the FEFEFKFK system has potential to be used as 3D scaffold to culture HOBs with regenerative applications.

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## ACKNOWLEDGMENTS:

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# Mechanical and Viscoelastic Properties of Collagen-Elastin Hydrogels for Lung Tissue Engineering

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**INTRODUCTION:** The aim of this study was to produce a material capable of mimicking the characteristics of the smallest functional component of the lung, the alveoli. The main constituents of lung ECM are collagen and elastin, which play a key role in the biomechanical behavior of healthy lung tissue[1-2]. For this reason they are of particular interest in lung tissue engineering. The average Young's modulus of a single alveolar wall was calculated to be ~5kPa[3]. In this study collagen-elastin hydrogel constructs were examined for their mechanical properties. The incorporation of human lung fibroblasts was also observed.

**METHODS:** Hydrogel constructs were made using type I rat-tail collagen (BD Bioscience), soluble bovine elastin (Sigma Aldrich) and primary human lung fibroblasts using a previously described protocol[4]. Collagen only constructs and hybrid constructs with varying collagen to elastin ratios (4:1, 2:1 & 1:1) were produced. All gels contained a constant collagen concentration of 3.5mg/ml. The hydrogels with collagen only and with a collagen to elastin ratio of 1:1 were seeded with a cell density of either $2.5x10^3$ or $2.5x10^5$ cells per construct prior to gelation. The constructs were incubated for 8 days at 37ºC, 5% $CO_2$.
The hydrogels were tested for their mechanical and viscoelastic properties using a non-destructive spherical indentation technique previously described[4]. The thickness of the gels was measured using optical coherence tomography (OCT) system[5].

**RESULTS:** The addition of elastin increased the stiffness of the hydrogel construct (Figure 1). A correlation was found to exist between the elastin concentration and the elastic modulus for the constructs (Figure 2). Live/Dead imaging showed the addition of elastin had no adverse effect on cell viability. Similar cell morphology was shown for all cellular constructs.

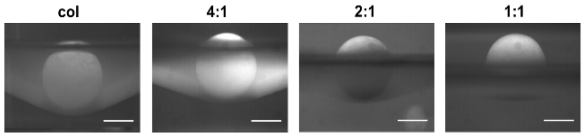


*Figure 1: Central deformation of collagen constructs with different concentrations of elastin. Scale bar=2mm.*

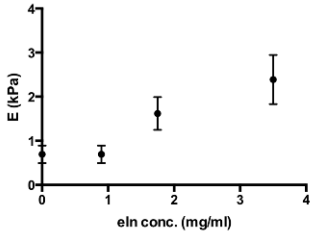


*Figure 2: Mean Young's modulus ± standard error at different elastin concentrations.*

**DISCUSSION & CONCLUSIONS:** The addition of elastin into a collagen hydrogel enhanced the mechanical properties of the construct. Increasing the elastin concentration resulted in a corresponding increase in Young's modulus. Cell-seeded constructs achieved a Young's modulus equivalent to the theoretical value for a single alveolar wall. Collagen-elastin hydrogels may have potential for use as a scaffold for lung tissue engineering applications.

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**ACKNOWLEDGMENTS**.
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# Tissue engineering a physiologically and spatially relevant 3D *in vitro* model of colorectal cancer

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**INTRODUCTION:** Collagen type I hydrogels are commonly used for cell culture as they provide a 3D environment in which to study cell behaviour. However, hydrogels do not accurately model tissue matrix density *in vivo*. Increasing collagen density alters scaffold properties such as matrix stiffness, a crucial parameter governing cell behaviour and can help recreate normal tissue barrier function *in vitro*. With this in mind, we have developed a spatially accurate 3D *in vitro* model of colorectal cancer known as a 'tumouroid' that recapitulates the dense architecture of tumours and the surrounding stroma.

**METHODS:** We created two ACMs (artificial cancer mass) of different matrix densities based on the removal of interstitial fluid in collagen type I hydrogels (plastic compression)[1]. These ACMs contained colorectal cancer cells (HT29 or HCT116), and were placed into a surrounding collagen hydrogel to construct tumouroids. Growth, morphology and invasion into the acellular compartment were assessed over 21 days. EGFR (epidermal growth factor receptor) expression was assessed by qRT-PCR and tumouroid response was investigated after treatment with the anti-EGFR monoclonal antibody cetuximab.

**RESULTS:** ACM matrix density was 2.63% ± 0.16% and 9.59% ± 0.64% for either partially compressed or fully compressed collagen hydrogels respectively (% w/v). Cells were cultured for 14 days and formed 3D cellular aggregates. EGFR expression levels revealed a 2-fold and 3-fold increase in 3D cultures for both HT29 and HCT116 cells in comparison to 2D monolayers respectively ($p<0.05$; $p<0.01$). Cetuximab efficacy was significantly lower in HT29 3D cultures in comparison to 2D monolayers whereas HCT116 cells in both 2D and 3D were non-responsive to treatment in agreement with their *KRAS* mutant status.

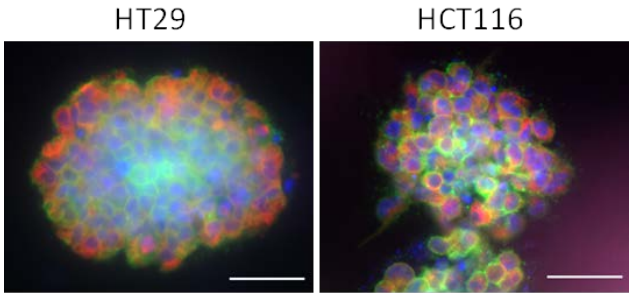


**Fig 1: Immunofluorescent analysis of cytoskeletal proteins on colorectal cancer cultures.** HT29 and HCT116 cells in 3D cultures were maintained for 14 days, fixed and stained for tubulin (red) and F-actin (green – phalloidin). Nuclei were stained with DAPI (blue). Scale bar – 50µm.

**DISCUSSION & CONCLUSIONS:** Cells behave similarly to the *in vivo* scenario, forming tumour-like cellular aggregates with visible cell-cell junctions *in vitro*. We established that cetuximab efficacy is significantly lower in 3D cultures in comparison to 2D monolayers, independent of EGFR expression levels. This signifies the increasingly important role of matrix density and cellular architecture on drug uptake and distribution.

## REFERENCES:

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## ACKNOWLEDGMENTS:

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# Investigating self-renewal of mesenchymal stem cells on nanotopography

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## INTRODUCTION

Adult mesenchymal stem cells (MSCs) can be induced to undergo differentiation[1] or maintain self-renewal[2] with culture on nanotopography. Exploiting the latter property and retaining expression of stem cell markers would allow for expansion of autologous stem cells for use in future clinical treatments. A highly ordered nanopit arrangement (SQ) has previously been shown to promote self-renewal, and this work seeks to determine optimal conditions for MSC growth and to greater understand what mechanisms drive MSCs to undergo self-renewal on this surface.

## EXPERIMENTAL METHODS

MSCs were isolated from human bone marrow aspirate using magnetic activated cell sorting and antibodies against surface markers (STRO-1/CD271). These were seeded onto polycarbonate SQ, an osteogenic near-square arrangement of nanopits (NSQ) and planar controls. Pit dimensions were 120nm diameter, 300nm centre-centre spacing, 100nm depth, with NSQ incorporating ±50nm disorder. MSC marker expression was assessed by fluorescence immunostaining and image analysis, with cell cycle protein levels detected using in-cell western methods. Coomassie blue staining, and scanning electron microscopy (SEM) was used to investigate growth patterns and cell morphology. Statistics: paired students t-tests with p values <0.05 taken as significant.

## RESULTS AND DISCUSSION

Cell seeding density was important for effective use of our SQ nanotopography. Optimisation of cell number revealed that a degree of clonal growth (fig.1A) may be important for self-renewal and that some changes in cell cycle regulation were apparent in MSCs cultured on the different surfaces. On SQ, contact guidance of filopodia was observed (fig.1B) in comparison to more random filopodial distribution on flat and NSQ.

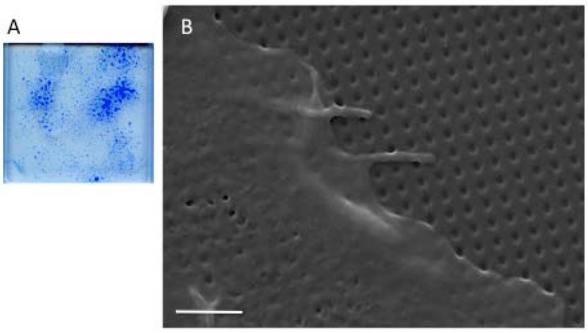


**Figure 1**. Optimisation of seeding density with subsequent morphology analyses (A) Coomassie stained MSCs on SQ at our optimal seeding density after 28 days culture showing colony growth (B) SEM imaging of MSCs on SQ showing contact guidance of filopodia. Scale bar: 1μm

Our observations suggest an association between adhesion-related events on SQ and intracellular biochemical changes.

## CONCLUSION

We have identified an optimal seeding density for nanotopographical retention of stem cell marker expression and propose that contact guidance constraints together with changes in cell cycle regulation play a part in directing MSC self-renewal.

## REFERENCES

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## ACKNOWLEDGMENTS

LCYL is funded by MVLS, University of Glasgow (UoG), MJD is funded by grants from BBSRC, EPSRC and MRC. We thank Julia Wells for STRO-1$^+$ MSCs and Peter Chung (UoG) for SEM imaging.

# The effects of polymer grade and sterilisation on electrospun fibre material properties and cell response

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## INTRODUCTION:

Electrospinning biopolymers, such as poly(ε-caprolactone) (PCL), is a popular method for producing scaffolds that mimic the extracellular matrix of many tissues[1]. Yet, research intended for overall translation to the clinic and human use requires various regulations to be adhered to, which includes material purity and recognised sterilisation processes. This study investigated the effects of polymer grade (chemical or medical) and sterilisation (ethanol or gamma) on the material properties and cell response of electrospun scaffolds.

## METHODS:

Grades of PCL investigated: medical (Purac) and chemical grade (Sigma). Three-dimensional (3D) fibrous scaffolds were prepared as described in Bosworth *et al.*,[1]. 3D scaffolds were sterilised in increasing concentrations of ethanol (50-100 %v/v) or gamma irradiated at 25 kGy (Synergy Health) prior to material characterisation, including tensile testing (Instron 1122, load cell 0.01 kN) and the morphology of seeded L929 fibroblasts (50,000 per $cm^2$) was assessed up to 48 hours by Scanning Electron Microscopy (SEM). Data was not Normally distributed. A Kruskal-Wallis test with Dunns post-tests was used for comparison of data sets for each PCL grade and Mann-Whitney test for comparison of as-spun (dry) scaffolds.

## RESULTS:

The results demonstrated a clear difference in tensile strength and stiffness depending on PCL grade, with statistical significance when comparing the as-spun (or dry) scaffolds (Fig.1). Fibroblasts attached to scaffolds irrespective of grade/sterilisation technique after 4 hours (Fig.2). By 48 hours, cells appeared flattened and spread-out on gamma irradiated scaffolds, whereas a rounder morphology was observed for cells seeded on ethanol sterilised fibres.

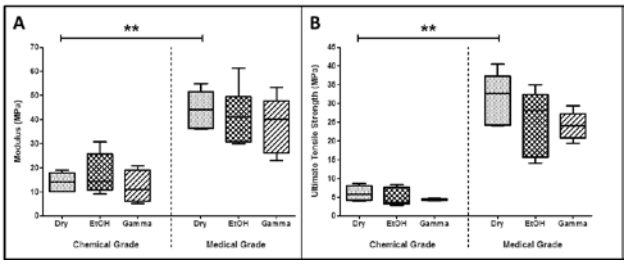


*Fig. 1 – Tensile data comparing PCL grades and method of sterilisation for (A) Young's Modulus (MPa) and (B) Ultimate Tensile Strength (MPa). (n=5; p<0.01).*

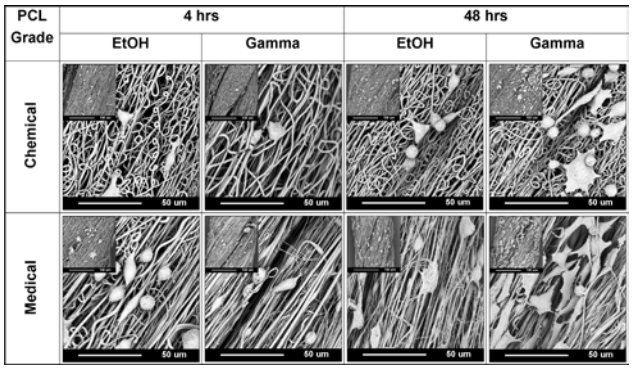


*Fig.2 – SEM images demonstrating cell morphologies on PCL scaffolds. Scale bar = 50 µm (inset 100 µm).*

## DISCUSSION & CONCLUSIONS:

The data highlighted a significant difference in tensile properties depending on the material grade. Similarly, cell response appeared to be more favourable for cells cultured on gamma irradiated scaffolds. This study demonstrates the importance of incorporating the right materials and sterilisation processes early in the project timeline to aid translation to the clinic.

## REFERENCES:

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## ACKNOWLEDGMENTS:

This study was supported by the MRC-DPFS (Grant no: G1000788-98812).

# Bioprinting of vascularised liver tissues

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**INTRODUCTION:** Metabolically active tissues must reside within 200 μm of a capillary lumen to gain sufficient oxygen and nutrients for survival. Incorporation of a perfusing blood supply in tissue engineering design is vital for the scalability, survival and integration of almost all tissues with the exception of a few poorly vascularised tissues such as skin epidermis and cartilage. Delivery of angiogenic growth factors, genes and regenerative cell types as well as combination therapies have been employed to promote vascularisation. However, the integration of these engineered tissues with the host remains a monumental challenge. To meet this challenge, the establishment of functional vasculature in engineered tissue constructs is likely to be required before transplantation. To this end, a recent research highlight has been the use of a sacrificial lattice of carbohydrate fibres to fabricate vascular-like tubule networks.1 Here, we demonstrate a novel method for engineering vascularised tissues using 3D printing. A construct with an embedded tubule network in a hydrogel matrix has been successfully printed at cell-compatible conditions. The tubule network was then perfused with endothelial cells to form vasculatures.

**METHODS**: A Fab@HomeTM printer was used to print the tubule network and the hydrogels. To form tubule network, a thermo-responsive material and alginate were printed into a pre-designed structure. The printed network was then dissolved away at physiological conditions to form tubes which were later perfused with endothelial cells. In addition, human umbilical vein endothelial cells (HUVECs) were mixed in Matrigel, and printed into a network within alginate. Cells were labelled by live/dead fluorescent stains or labelled by a red cell tracker, and imaged using a fluorescent microscope.

**RESULTS:** HUVECs after 8-days culture were labelled with live/dead fluorescent stains (Figure 1). A tubule network facilitating the perfusion of HUVECs was printed within alginate (Figure 2).

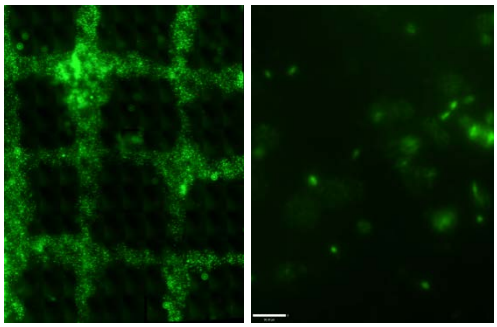
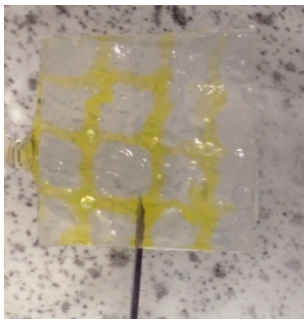


*Figure 1 A stitched image of a printed network with HUVECs embedded in Matrigel (Left). An image of encapsulated HUVECs showing spreading of some cells (Right).*



conditions, which is an advance compared to methods in which materials are printed at elevated temperatures unsuitable for cells and biomolecules. The cell-compatible printing environment permits the patterning of the tubule networks which mimic blood vessels, biomolecules and multiple cell types in a single printing process. Future work will look to improve, measure and validate the architecture and function of the engineered vasculature.

**CONCLUSIONS:** The method reported here allows the fabrication of a vascular network at physiological

**ACKNOWLEDGEMENTS**: The authors would like to thank the Nottingham Research Fellowshipscheme and the ERC Advanced Grant (227845) for supporting this work.

**REFERENCES:** [1] Miller, et al (2012) *Nat Mater* **11**:768-774.

# Topographical modification of nanocomposite polymer vascular graft surfaces to optimise endothelialisation

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## INTRODUCTION:

The success of a vascular graft is well-known to depend on its ability to develop an endothelial layer within the luminal surface. However, current graft materials in clinical use do not support this and patency rates of these grafts low[1]. Surface topographical features are well-known to have an influence on cellular behaviour[2]. The aim of this study is to examine both the optimal plasma treatment and topographical feature size to enable endothelial cell attachment on a nanocomposite polymer for use within a vascular bypass graft setting.

## METHODS:

Nanocomposite polymer, polyhedral oligomeric silsesquioxane and polycarbonate urea urethane (POSS-PCU) were embossed with both microgrooves (MG) of pitch 25µm and nanopits (NSQ), 120nm pits with centre-centre spacing of 300nm with ±50nm offset. These were then treated with different oxygen plasma treatments (40W, 60W and 80W for 60 seconds). The replication fidelity of the patterns was confirmed using AFM and SEM and water contact angle was used to measure the success of the plasma treatment. Human umbilical vein endothelial cells (HUVECs) were seeded onto POSS-PCU and Live/Dead staining were used to measure growth. Immunostaining was used to visualise the focal adhesions.

## RESULTS:

AFM and SEM both confirmed the high replication fidelity of both the micro and nanofeatures on POSS-PCU. Water contact angle showed that hydrophilicity of the polymer can be fine-tuned by increasing the power of the $O_2$ plasma treatments ($p < 0.05$). HUVEC proliferation was seen to be optimal when seeded on a plasma treated surface with an average of 68° being achieved. There is also further preference of NSQ over MG features and planar surfaces after plasma treatment. Endothelial cell morphology and function was seen to be retained, especially on the plasma treated NSQ surfaces. There were also increased focal adhesions seen on these surfaces.

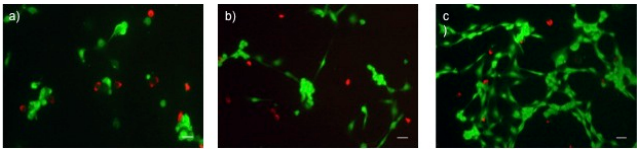


***Fig 1. Live/Dead staining of HUVECs cells on a) planar b) micropattern and c) NSQ surfaces respectively.*** *Green indicates live cells and red dead cells (scale bar - 20*µm*).*

## DISCUSSION & CONCLUSIONS:

This work shows the preference of HUVECs towards topographical features in the nanoscale over micro and planar surfaces, especially after plasma treatment. These are important observations as luminal surfaces of vascular grafts can be tailored-made using a combination of plasma treatment and topographical features to allow for self-endothelialisation potential.

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## ACKNOWLEDGMENTS:

The authors would like to acknowledge the Wellcome Trust, Royal Free Charity and the European Society for Vascular Surgery for research grants.

# A comparative genome wide transcriptional analysis of human bone marrow, synovial and periosteal mesenchymal stem cells

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**INTRODUCTION:**

Osteoarthritis (OA) is a progressive degenerative disorder affecting several compartments of the joints, including articular cartilage. Current available treatments are mainly symptomatic. Recently, cell therapy with chondrocytes or mesenchymal stem cells to repair focal lesions of the joint surface has been intensely pursued[1-6]. However, there are only a few clinical trials with cell therapy in OA[4-6]. Although these studies demonstrated promising outcome in terms of patient well-being and cartilage repair, patient to patient variability to heal damaged cartilage hinders consistent outcome. To enhance consistency, cell therapy requires a better understanding of MSCs but little knowledge exists at the genomic level, limiting the scope of molecular studies and expression analyses of genes of interest. To overcome this limitation in this study, we hope to identify molecular signatures associated with tissue-specific MSCs.

**METHODS:**

We performed microarray of culture-expanded human MSCs from three different joint tissues (bone marrow=BM, human periosteum=HP, Human synovial membrane=HSM). In some cases, HSM and HP samples were obtained from the same donors. Twenty two microarrays were performed from 17 donors, using Affymetrix GeneChip Human Exon 1.0 ST arrays, to compare gene expression profiles of culture-expanded human mesenchymal stem cells (MSCs) between donors and across three different tissue sources.

Preliminary data analysis was done after the data was processed and statistics (ANOVA, posthoc, MTC $p<0.05$) performed. Differentially expressed genes were validated by q-RT-PCR.

**RESULTS:**

Analysis of the microarray datasets revealed striking differences in gene expression profiles between MSCs from the three tissue sources. In particular, MSCs from BM show a gene expression profile that is distinct from HSM and HP-MSCs (Fig.1). A total of 347 genes and 47 pathways [which include TGF Beta (10 matches), MAPK Signaling (9 matches), Endochondral Ossification (8 matches), EGF-EGFR Signalling Pathway (8 matches), Notch Signalling Pathway (4 matches), etc.] were differentially regulated. The q-RT-PCR study confirmed in multiple donors top most differentially regulated genes.

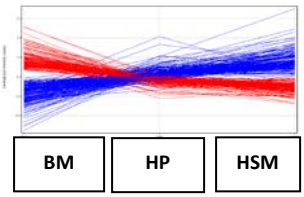


**Fig.1a**

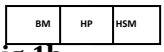


**Fig.1b**

*Figure* (1a) Profile plot (1b) Hierarchical clustering.

**DISCUSSION & CONCLUSIONS:**

We have demonstrated that HSM and BM-MSCs express distinct sets of genes. These could be a potential list of signature molecules for MSC identity in clinical applications and will provide opportunity to study them in joint diseases such as OA.

**REFERENCES:**

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**ACKNOWLEDGMENTS:** We are thankful to the ARUK Research Council for providing financial support to this project.

# Elucidating novel roles for dentine matrix components in directing stem cell biology

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**INTRODUCTION:** Attenuated bone repair is traditionally resolved using autologous bone grafts, although with varying degrees of success. Previous studies have identified demineralised dentine matrix (DDM) to be highly bioactive, stimulating augmented bone repair in compromised situations[1,2]. The potency of DDM is attributable to a potent cocktail of growth factors acting synergistically to elicit optimum biological responses. Growth factors can also bind to matrix proteins, notably decorin and biglycan, which may aid biological activity by sequestering to the matrix, protecting from proteolysis and possibly modulating cell signalling[3,4]. Our current study aims to assess the responses of bone marrow stem cells (BMSCs) to DDM and identify factors within the matrix eliciting a bioactive role.

**METHODS:** Commercial BMSCs, representing a heterogeneous population, were selected further for α5β1 integrin immature cells by preferential adherence to fibronectin (FNA). FNA BMSCs were characterised for population doublings (PDs) and stem cell markers using RT-PCR and compared with unselected cells. DDM was extracted from mineralised human dentine with 10% EDTA. FNA BMSCs were cultured in DDM-conditioned media for 28 days to quantify differentiation towards osteoblast lineage via Von Kossa staining. Effect of DDM on cell expansion was assessed by MTT assay, apoptotic activity by luciferase activation of active caspase-3/7 and chemotactic properties using Boyden chambers. Growth factors and matrix proteins decorin and biglycan in DDM were assessed by Western Blotting.

**RESULTS:** FNA BMSCs express mesenchymal stem cell markers, including CD105, CD90 and CD73 and exhibit a greater sustained proliferative capacity compared to unselected BMSCs. Cells cultured in media supplemented with 10μg/mL DDM exhibited positive staining for mineral deposition compared to untreated (fig 1). DDM indicated a dose-dependent response in reduction of cellular expansion over 4 days and apoptotic activity after 48 hours. Cell migration is enhanced by DDM albeit attenuated with increasing concentrations. Western blot analysis identified the definitive presence of TGF-β1 in DDM.

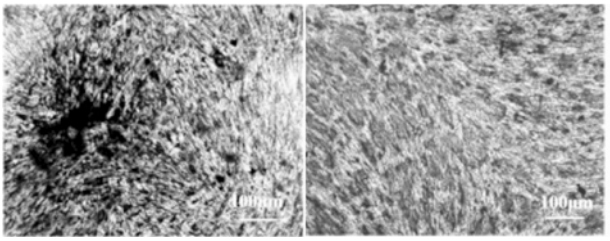


*Fig 1: von Kossa stain of cells cultured in 10μg/mL DDM (left) and control (right) medium for 28 days*

**DISCUSSION & CONCLUSIONS:** DDM stimulates BMSC differentiation towards an osteoblast phenotype and deposition of a mineralised matrix. In addition DDM appears to contain bioactive components that aid cell survival and migration. Although TGF-β1 has been identified within the DDM, it is hypothesized that a combination of components acting synergistically are responsible for the overall enhanced bioactivity of DDM.

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## ACKNOWLEDGMENTS:

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# Arthroscopic delivery of bio-active materials: a preliminary study

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**INTRODUCTION:** Current trends in tissue engineering for the restoration of chondral and ostheochondral lesions rely mostly on the in-vitro fabrication of scaffold structures, plugs and other constructs of various shapes and compositions which must be transplanted and fitted on the defect site with maximum accuracy and biocompatibility. While a number of studies have demonstrated the capacity of 3D Printing, or Additive Manufacturing (AM) for creating biocompatible tissue repair constructs[1-3],few have considered the potential for in-situ delivery of bioactive materials. This work describes the principles of a novel arthroscope–based material delivery system for the in-situ fabrication of bioactive implants adopting some of the principles of 3D printing.

**METHODS:** A proof of concept system was built consisting on: a peristaltic pump system with Flow rate 0.001 to 3400 mL/min (Cole Palmer), silicone tubing (3mm internal diameter), and a single LED UV-light source. Three specimens were produced with different layer thicknesses (0.5mm, 0.75, 0.9mm). An off the shelf acrylate-based photo curable resin (wavelength 420 -480) has been used to measure the system's capacity for delivering layered materials in a controlled manner.

**RESULTS:** The system consistently delivered material volumes uniformly distributed across the different layers. High repeatability was achieved on cylindrical shape constructs with a regular cross section, however the UV curing mechanism exhibited its dependence on parameters such as light exposure time, distance from source and layer thickness as some uncured residues were trapped between layers for the 0.9mm thickness part.

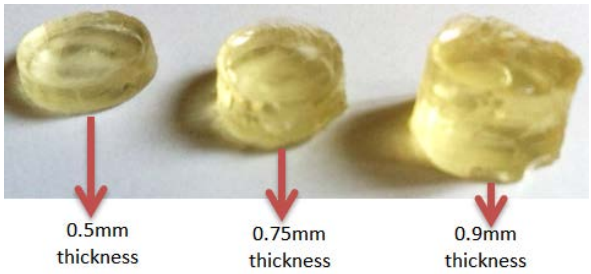


*Fig. 1 Resulting UV-cured plugs with different layer thicknesses*

**DISCUSSION & CONCLUSIONS:** The deposition mechanism showed its effectiveness delivering precise fluid material quantities over small timescales (10-12 mins) for the plugs to be made. The UV/light source cross linking activation must be tuned in order to reduce solidification time, thus accelerating the overall processing time-window. Future work includes making use of biopolymers designed specifically for the technique and further experimentation on thin extrusion profiles.

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## ACKNOWLEDGMENTS:

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http://mede-innovation.ac.uk/

# A mathematical framework for nerve regeneration in implantable conduits

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## INTRODUCTION:

Matching the performance of autografts with engineered scaffolds remains a challenge in peripheral nerve repair. A combination of 3D biomimetic architecture interspersed with 2D surfaces has been hypothesized to be an ideal environment for neurite regeneration[1]. However, the problem of how best to arrange material within a conduit is an open one[2]. Optimizing material parameters such as density, cross-sectional geometry and spatial distribution would require an extensive programme of experimental testing. By contrast, developing a modelling framework that is capable of testing key parameters may accelerate the design process, and reduce the dependency on animal testing.

## METHODS:

A model was devised to simulate neuronal growth inside a cylindrical conduit. The trajectories of neurites are generated according to a 3D random walk process with a spatially dependent probability distribution. To mimic preferred neuronal growth on particular surfaces, a topographical bias is included. Parameters can be adjusted to mimic realistic dimensions and growth rates, as well as the rate of sprout formation at the proximal stump.

## RESULTS:

The sensitivity of the hit ratio (proportion of neurites exceeding a prescribed longitudinal distance over a 2 week period) to changes in the porosity was investigated. Simulations were performed for a conduit featuring sheets of material. Fig. 1 shows the existence of an optimal porosity, representative of the competition between the total substrate available to aid growth and the obstruction of neurites at the entrance to the conduit.

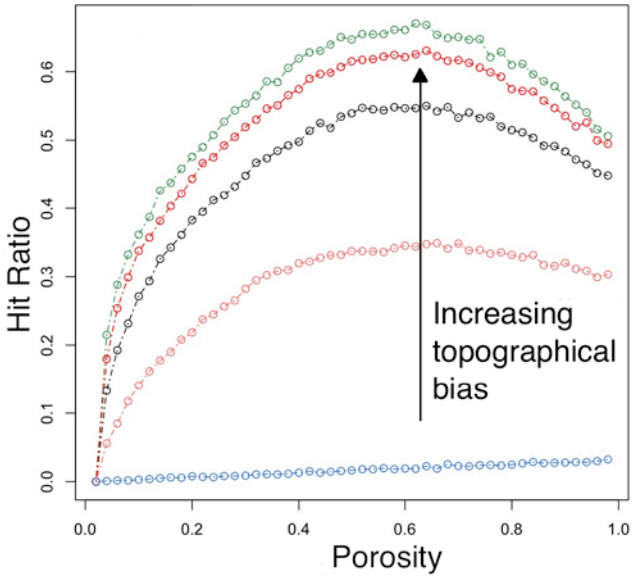


*Fig. 1 Simulated hit ratios for different porosity values. Different curves correspond to varying topographical biases.*

## DISCUSSION & CONCLUSIONS:

We demonstrate the utility of a stochastic model for peripheral nerve regeneration and its ability to mimic experiments. The model presented predicts the existence of an optimal porosity value for one spatial arrangement of material, which could be adapted to investigate more complex designs.

More importantly, perhaps, is the promise indicated by this dual-approach, whereby wet & dry approaches can be built and updated simultaneously - experiments can verify and refine a model, and a model can help motivate experimental specifications and investigate novel biological hypotheses.

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# Determination of drug permeability in caco-2 monolayers under dynamic conditions using a novel magnetic force bioreactor technology

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## INTRODUCTION:

The Caco-2 cell line is the gold standard for the prediction of drug absorption and permeability *in vitro* by mimicking the small intestine[1]. However, this model does not closely resemble the physiology of the intestine as it lacks dynamic motion similar to peristalsis *in vivo*. In our research, we have developed a dynamic Caco-2 cell model which aligns with the standard assay using the MICA technology[2].

We have selected test compounds which characterise the properties across the biopharmaceutical classification system (BCS) classification[3]. In this way, we can assess whether a dynamic *in vitro* assay more closely resembles the adsorption seen *in vivo*.

## METHODS:

The Caco-2 cells were labelled with bio-coated magnetic nanoparticles (MgNPs). With the use of our MFB technology (MICA BioSystems, UK), it is possible to interact with the MgNPs placed on the cell by applying an oscillating external magnetic field. The permeability experiments were performed with and without MFB (standard assay)[4]. The integrity of cell monolayer was assessed before and after the permeability studies by trans-epithelial resistance measurement (TEER), Lucifer Yellow passage in order to validate the experiments. Immunocytochemistry were used to assess the impact of MFB on the Caco-2 cell.

## RESULTS:

MgNPs were successfully bio-coated, in order to apply MFB technology. A summary of all the permeability data obtained from our standard permeability assay (as internal control), and our developed dynamic permeability assay is provided in Table 1. The Figure 2 shows the cell monolayers before and after the application of MFB.

*Table 1 Results from Caco 2 permeability assays.*

| Compound | BCS Class | % of $P_{app}$ increase | $P_{app}$ standard assay (nm/s) | $P_{app}$ Dynamic assay (nm/s) |
|---|---|---|---|---|
| Ketoprofen | I | 63% | 27 | 44 |
| Antipyrine | I | 88% | 34 | 64 |
| Piroxicam | II | 17% | 24 | 28 |
| Amoxicillin | III | 20% | 15 | 18 |

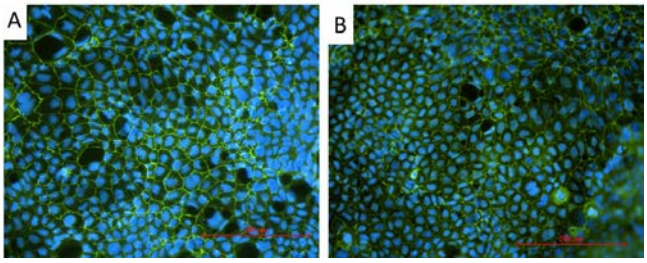


*Fig.2 Immunocytochemistry of Caco-2: the cells were stained with an antibody against a tight junction protein, before (A) and after (B) the MFB performance.*

## DISCUSSION & CONCLUSIONS:

The use of our novel dynamic platform increased the permeability in all the standard drugs selected for the experiments respect to the control gold standard Caco-2 assay. The monolayer integrity was unaffected by the MFB technology or addition of magnetic particles.
The developed dynamic screening established an *in vitro* dynamic condition which more closely mimics the *in vivo* absorption rates.

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## ACKNOWLEDGMENTS:

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# Development of a stem cell therapy for repair of the degenerate IVD: Influence of pH on nucleus pulposus and mesenchymal stem cells

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## INTRODUCTION:

Development of a mesenchymal stem cell (MSC) therapy for treatment of intervertebral disc (IVD) degeneration provides a challenge due to the unique microenvironment of the disc. Understanding how this hostile microenvironment may affect implanted MSCs is fundamental for the development of cell based tissue engineering/regeneration strategies for the treatment of IVD degeneration (a major cause of low back pain). To date, however, it is not known whether low pH affects expression of pain-related neuropeptides in MSCs or NP cells. The aim here was to assess the effect of a physiologically relevant low pH on cell viability, proliferation, intracellular pH and gene expression of pain-related and pH-regulating factors, in both human MSCs and IVD cells.

## METHODS:

3 x human bone marrow MSC and 3 x human nucleus pulposus (NP) disc cell samples were expanded and then cultured in MEM or DMEM medium respectively, at a range of pH (pH 7.4, 7.1, 6.8, 6.5 and 6.2) for 1 and 7 days. Live/dead staining, Annexin V/PI staining, Pico Green assay, SNARF-5 staining and qRT-PCR were used to assess cell viability, apoptosis, proliferation, intracellular pH and gene expression of pH-regulating carbonic anhydrases IX/XII and pain-related factors (CGRP, Substance P and MCP-1), respectively.

## RESULTS:

Intracellular pH decreased in line with external pH for both MSCs and NP cells. Both MSC and NP cell viability and proliferation were affected by changes in pH, with proliferation occurring at pH 7.4 and 7.1, a halt in proliferation at pH 6.8 and a reduction in viable cells (non-apoptosis related) at pH 6.5 and 6.2. The gene expression profile of CAIX&XII (data not shown), CGRP (Figure 1), Substance P and MCP-1 (data not shown) differed between MSCs and NP cells after 1 day of culture, with gene expression of pain-related proteins increased in MSCs but not NP cells; however MSCs altered their gene expression profile to mirror that of NP cells after 7 days of culture (Figure 1).

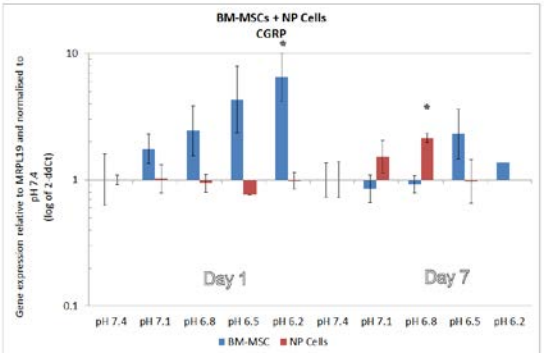


*Fig. 1 Gene expression of pain-related factor calcitonin gene-related peptide (CGRP) in MSCs and NP cells cultured at different pH*

## DISCUSSION & CONCLUSIONS:

Acidic pH (similar to that found in degenerate IVDs) has a detrimental effect on MSC and NP cell viability and proliferation. This data suggests that MSCs may require "conditioning" prior to implantation into a degenerate disc. Low pH initially causes MSCs to increase their expression of pain-related factors, with expression levels decreasing similarly to NP cells after 7 days. This suggests that pre-conditioning of MSCs in a low pH may be necessary prior to implantation into a degenerate IVD in order to minimise a pain response.

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## ACKNOWLEDGMENTS:

The authors would like to thank the Arthritis Research UK (Grant no: 19884) for providing financial support to this project.

# Developing an *in vitro* model to measure airway smooth muscle contraction

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## INTRODUCTION:

Airway smooth muscle (ASM) is the key effector cell in regulating airway contraction. Previous *in vitro* studies into ASM contraction have largely been limited to 2D single cell cultures and collagen gel contraction studies. Developments in tissue engineering have generated novel technologies for developing 3D models that better mimic the natural ECM. One method for producing such matrices is electrospinning, where non-woven mats of polymer fibres can be made by passing a polymer solution through a highly charged capillary. This is an attractive approach due to its simplicity, low cost and the wide range of both synthetic and natural polymers that are available to be electrospun.

The aim of this work was to culture contractile airway smooth muscle tissue using primary human ASM cells cultured on aligned electrospun scaffolds (fabricated from PET and gelatin) and in collagen gels. The contractile forces generated by the smooth muscle cells in response to contractile agonists can then be measured using a custom made culture force monitor (CFM).

## METHODS:

Solutions of PET and gelatin were electrospun onto a rotating mandrel (separately) to produce aligned fibrous scaffolds. Electrospun gelatin was cross-linked using 1-ethyl-3-(dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxyl succinimide (NHS) in an ethanol: water solution (9:1). Scaffolds were sterilised prior to seeding with smooth muscle cells. In addition, collagen gels were seeded with smooth muscle cells. Cells were cultured to confluency before cell-scaffold/gel constructs were placed on the CFM and stimulated chemically. The force of contraction was measured.

## RESULTS:

The electrospinning process produced highly aligned non-woven fibres (50% of fibres within 5° of the mean angle).The fibre diameter of the produced scaffolds ranged from 250nm to 3.5µm dependant on concentration of polymer used. The PET and gelatin scaffolds differed gratly in their elastic properties with the PET scaffolds being much stiffer than gelatin. ASM cells were successfully cultured to confluency on aligned electrospun scaffolds forming a uniaxial population. Contraction of smooth muscle cells in collagen gels was measured on the CFM in response to contractile agonists.

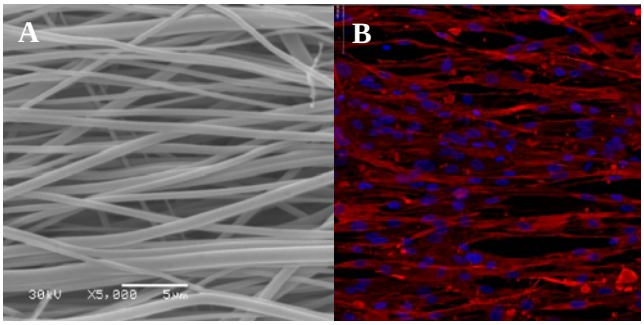


*Figure 1: (A) SEM image of aligned electrospun PET and (B) an immunohistochemical image of ASM cells cultured on aligned PET stained for smooth muscle marker SM22α and DAPI.*

## DISCUSSION & CONCLUSIONS:

Electrospun scaffold properties can be tailored for purpose by changing the electrospinning parameters such as polymer type, concentration and flow rate. Additionally, the electrospinning method can produce highly aligned fibres regardless of the other parameters. ASM cells adhere to and proliferate on the electrospun scaffolds forming a highly aligned cell population. The contractile force generated by smooth muscle cells in response to a chemical agonist can be successfully measured using the CFM. This method of measuring cell contraction will be repeated using the electrospun scaffolds in place of collagen gels.

## ACKNOWLEDGMENTS:

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# Novel hepatocyte-like cells and biomaterial for bioartificial liver design

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**INTRODUCTION:** Bioartificial liver (BAL) devices are a potential interim therapy to bridge patients with liver failure to transplantation and therefore reduce patient mortality from lack of donor livers by replicating liver function. The aim of this project is to develop a BAL device by using a novel hepatocyte-like cell (HLC) source (through the transdifferentiation (or conversion) of pancreatic cells to liver cells) and by developing a novel polymer for cell culture, PolymerX, to create a hollow fibre bioreactor (HFB). The rat pancreatic cell line AR42J-B13 readily displays normal liver cell functionality upon induction to HLCs [1, 2].

**METHODS:** AR42J-B13 cells were cultured on glass coverslips in 6 well-plates at a density of 30,000 cells per well. Cells were treated without or with 1 μM Dexamethasone (Dex) and 10 ng/ml Oncostatin-M (OSM) for 21 days and immunostained for pancreatic and liver markers to assess phenotypic changes. Characterisation of PolymerX was performed upon flat sheet membranes. Membranes were cast through phase inversion of 20% (w/w) PolymerX in NMP. Deionised water ($DIH_2O$) or 70% (v/v) industrial methylated spirits (IMS) was used as the nonsolvent. Membranes then underwent surface treatment for 1 min, 2 min or 5 min. Surface energy was analysed using the contact angle method by sessile drop. Statistical analysis of contact angle measurements was by a one-way ANOVA with Tukey's post-hoc tests using SPSS 21.

**RESULTS:** AR42J-B13 cells treated with Dex and OSM expressed glutamine synthetase (GS) and carbomoylphosphate synthetase I (CPS) (Fig. 1). Membranes undergoing surface treatment for 1 min showed significant reduction in the mean contact angle compared to the untreated control but thereafter showed no further significant change; this trend was seen for both nonsolvents (Fig. 2).

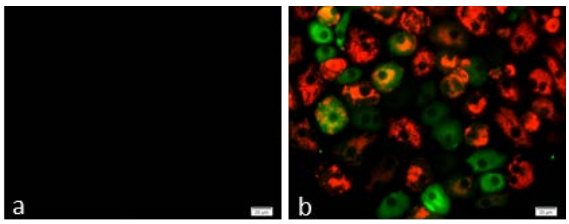


*Fig. 1: Immunofluorescence of AR42J-B13 cells cultured on glass coverslips. Cells were treated without (a) or with (b) 1μM Dex and 10ng/ml OSM for 21 days and stained for GS (green) and CPS (red).*

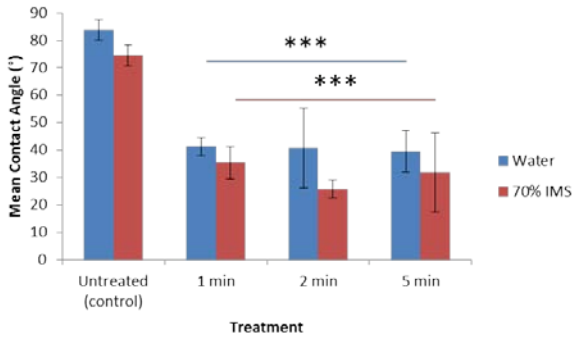


*Fig. 2: Surface treatment effect on PolymerX surface energy on 3 randomly selected membrane sections. Error bars = ± 1 SD. ***p<0.005.*

**DISCUSSION & CONCLUSIONS:** Surface treatment of PolymerX showed an increase in hydrophilicity compared to the control. The utility of AR42J-B13 cells to transdifferentiate to HLCs under Dex and OSM treatment has also been shown. Future work will assess the ability of the pancreatic cells to convert to HLCs on the novel polymer on both flat sheets and on the exterior of hollow fibre membranes.

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**ACKNOWLEDGMENTS:** We would like to thank the BBSRC and the University of Bath for funding.

# Materials processing for the manufacture of musculoskeletal Medical devices at the point of need

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## INTRODUCTION:

Osteochondral defects result in severe pain and disability for millions of people worldwide and massive healthcare costs. There is a recognized and urgent need for developing novel treatments based in bone tissue engineering and three dimensional scaffolds design. The use of Additive Manufacturing (AM) has been growing in recent years due to its ability to directly print 3D porous scaffolds with pre-designed shape and patient customized, solvent-free, controlled pore size and interconnected porosity [1]. This paper reports the results of an initial scoping study on the development of new processes to support the in-clinic manufacture and configuration of hybrid bioactive devices for large defects which are load bearing, functionally gradient, and biologically enhanced.

## METHODS:

As a starting point the aim was to develop a modular composite of a porous polylactic acid (PLA) block and wollastonite (A-W) cylinders. The overall aim is to have anatomical geometries derived from patient data. A Fused Filament Fabrication (FFF) 3D printer was selected to fabricate the PLA rectangular part with a 0º/90º laydown pattern and the AW cylinders were produced by a Z310 Plus 3D printer (Z-Corp, USA) using prepared AW and Maltodextrin powder. Afterwards the A-W green specimens were sintered at 1150ºC for 2 hours. Measurements were performed before and after sintering and all specimens were observed with a stereo microscope (Nikon SMZ1500).

## RESULTS:

The specimens were successfully manufactured as presented in Fig.1.A-C and the modular composite was assembled (Fig.1.D). The PLA block presents an interconnected porous structure characterized by approximately 300 µm pore size (Fig.1.C) which was defined by the CAD model geometry and the laydown pattern. Moreover the walls are also porous and interconnected with the surrounding porous structure (Fig1.B), which is crucial for achieving an interconnected porosity throughout the hybrid device. Shrinkage of 15% and 18% for both diameter and length was observed after sintering of the AW cylinders.

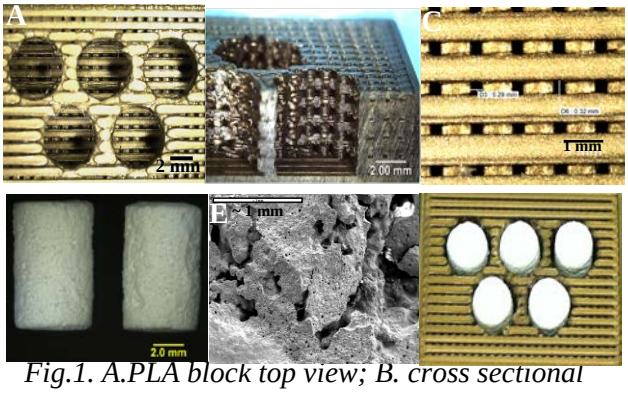


*Fig.1. A.PLA block top view; B. cross sectional view; C. porous structure; D. AW cylinders after sintering; E. SEM of porous AW structure; and F. hybrid composite.*

## DISCUSSION & CONCLUSIONS:

The composite was successfully assembled and it is characterized by an interconnecting porous structure which is crucial for a good osteointegration. Additionally the pore size and geometry can be controlled during the design step.

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## ACKNOWLEDGMENTS:

The authors would like to acknowledge the support of the EPSRC Centre for Innovative Manufacturing in Medical Devices MeDe Innovation; http://mede-innovation.ac.uk/).

# Remotely Controlled Mechanotransduction via Magnetic Nanoparticles: Applications for Injectable Cell Therapies.

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**INTRODUCTION:** Bone requires dynamic mechanical stimulation to form and maintain functional tissue, yet mechanical stimuli are often lacking in many therapeutic approaches for bone regeneration. Magnetic nanoparticles provide a method for delivering these stimuli by directly targeting cell-surface mechanosensors and transducing forces from an external magnetic field, resulting in remotely controllable mechano-transduction. In this investigation, functionalised magnetic nanoparticles were attached to the mechanically-gated TREK-1 K+ channels of human mesenchymal stem cells1. These cells were microinjected into an ex vivo chick foetal femur (e11) as a model for endochondral bone formation2,3. An oscillating 25mT magnetic field was used to induce mechanotransduction in the injected MSCs via the nanoparticles. Further analysis was performed in vitro in both monolayer and 3D hydrogel cultures4.

**METHODS**: Human MSCs and TREK-1 antibody-conjugated magnetic nanoparticles were introduced into the cartilaginous epiphyses of an organotypically cultured ex vivo chick foetal femur using a glass capillary needle. 20nl of material was injected containing 103 cells per injection, whilst injecting unlabelled (nanoparticle-free) hMSCs was used as a control. The femurs were cultured organotypically for 14 days. An oscillating magnetic field was applied for 1 hour per day to remotely activate TREK-1 signalling.

**RESULTS**: Control (unlabelled) hMSC-injected femurs were shown to mineralise predominantly in the bone collar (diaphysis), whilst secondary mineralisation sites were observed in the epiphyses of the femurs injected with TREK1-labelled hMSCs (fig. 1). Significant mineralisation occurred in the region of the epiphysis surrounding the injection site as revealed by µCT and alizarin red staining for calcium.

**DISCUSSION & CONCLUSIONS:** In these experiments we demonstrated the effectiveness of targeting the TREK1 ion channel to remotely activate mechanotransduction and promote osteogenic effects at injection sites in an organotypically cultured chick foetal femur. Further research has shown that this method has the potential to act synergistically with other tissue engineering approaches and amplifies the effects of BMP2 delivered from microspheres, possibly by amplifying intracellular SMAD signalling4.
Current work is ongoing to identify the differentiation and signalling mechanisms underlying the extensive mineralisation observed in the microinjection model.

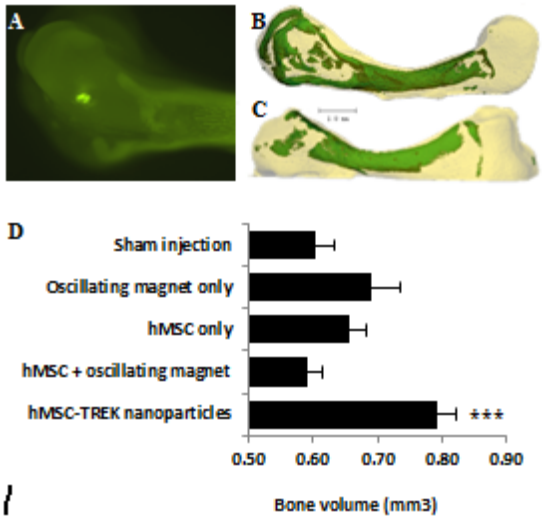


Fig. 1. The location of DiO-labelled hMSCs in the epiphyseal injection site within the chick foetal femur (a). After 14 days in vitro culture, the sites at which the TREK1-nanoparticle tagged cells were injected (b) were more mineralised than epiphyses injected with unlabelled hMSCs, (c) resulting in significant increases in bone formation in the TREK1-nanoparticle treated group compared to controls (d, n=9).

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2Smith EL, Kanczler JM, Roberts CA, Oreffo ROC (2012) Tissue Eng. Part C 18: 984-994.
3Henstock JR, Rotherham M, Rose JB, El Haj AJ (2013) Bone 53: 468-77.
4Henstock JR, Rotherham M, Rashidi H, Shakesheff KM, El Haj AJ. (2014) SCTM (under review)

# Scaffolds' characterisation for a multilayered construct simulating the tooth periodntium

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## INTRODUCTION:

Orthodontic forces are usually small forces used to move crowded or un-erupted teeth into place. The tooth periodntium is a complex tissue comprising a soft ligamentous tissue (periodontal ligament (PDL)) enclosed between 2 hard tissues (bone and cementum) [1, 2]. To understand the effect of orthodontic forces on capillaries and the balance between bone resorption and deposition ,in this study we have compared 4 different types of scaffolds as candidates to select: one scaffold representative of hard tissue components (bone and cementum)and one to represent the soft tissue component (PDL) of the periodontium to be used in a proposed multilayered construct simulating the tooth periodontium.

## METHODS:

For the hard tissue component: PLGA 50/50 Poly (D,L-lactide-co-glycolide),and Sol-Gel sacffolds were compared. For the soft tissue component we have compared commercially available Geistlich Bio-Gide® and Flexcell® membrane. Each scaffold was seeded with ($2.5x10^5$) human periodontal ligament cells (HPDLCs) . The comparisons included assessing:

A. Cells' viability, adhesion, proliferation, and matrix formation (Confocal and SEM imaging) after 2 weeks of culture in basal media B. Mechanical properties were evaluated by measuring average compressive strength before and after cell seeding.

**RESULTS:** All types of scaffolds have displayed typical viable fibroblast like appearance in confocal images. SEM images indicated the presence of cells in sheets or layers, spreading and stretching within the scaffolds confirming the biocompatibility of theses scaffolds to HPDLCs and indicating various amounts of matrix formation by the cells within the scaffolds.

The average compressive strength of Sol-Gel scaffolds increased from 0.014±0.001 MPa in scaffolds without cells to 0.835 ± 0.3 MPa in cell seeded scaffolds after 2 weeks of culture under the same conditions. For the soft tissue component, the Bio-Gide® showed a higher initial compressive strength 0.020 ± 0.03MPa compared to collagen coated Flexcell® 0.0069±0.011Mpa.

## DISCUSSION & CONCLUSIONS:

Since the four scaffolds tested in this study showed biocompatibility with the HPDLCs we considered the compressive strength as the deciding parameter for this study.

This study showed that matrix produced by HPDLSCs after 2 weeks has improved the Sol-Gel scaffold's mechanical properties. Hence, it was considered to be more suitable in representing hard tissue compared to PLGA.

Due to its higher compressive strength, Bio-Gide® was considered to be more suitable to represent the soft tissue component of our proposed multilayered construct.

**Acknowledgement:**
The authors would like to acknowledge the Egyptian cultural bureau for providing financial support to this project and the WELMEC, a centre of excellence in biomedical engineering (funded by Wellcome trust and EPSRC) for funding Reem El-Gendy.

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# 3d printing of high internal phase emulsions for cell culture applications

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## INTRODUCTION:

Stereo-lithographic production of 3D porous scaffolds using Poly-High Internal Phase Emulsions (Poly-HIPEs) have great potential for applications in tissue engineering as a support matrix. The tunability of the foam like morphology, and structural properties of a PolyHIPE scaffold are dependent on monomer choice, the addition of other components, and the processing conditions [1]. Typically the 3D printing capabilities of stereolithography has a trade-off between the resolution and subsequent fabrication time. We present a hybrid of structuring techniques to incorporate poly-HIPE's pre-processing control over micro porosity for macro structuring of highly porous materials [2]. Both the flexibility and porous nature of this fabrication approach makes it an excellent candidate for potential use for fabrication of scaffolds for *in vitro* cell culture

## METHODS:

A water in oil emulsion was created using various blends of the water-immiscible monomers 2-Ethylhexy acrylate (EHA) and Isobornyl acrylate (IBOA) with a triacrylate crosslinking agent, photoinitiator and the surfactant Hypermer B246 stabilize the emulsion during the addition of water and agitation at 350 rpm. The viscous white liquid was cured into woodpile structures by selectively exposing the top surface to Ultraviolet light (355 nm) isolated from a ND:YAG microchip laser.

## RESULTS:

SEM analysis of both poly-HIPE disks and 3D grid arrays produced via stereolithography were used to assess the porosity and surface features (Fig. 1). The tunability of the mechanical properties of PolyHIPE monomer blends were studied via nanoindentation and tensile testing. Cell culture studies have shown the porous discs to support proliferation of human fibroblast cells.

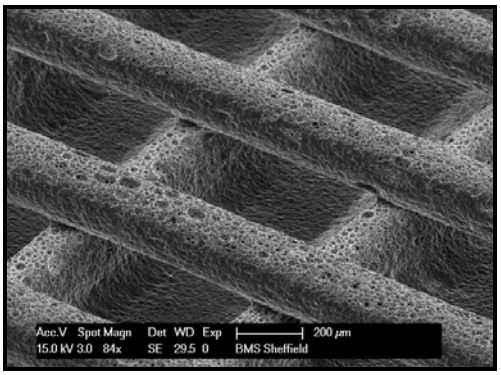


*Fig. 1 SEM image of a PolyHIPE woodpile structure, pores can be seen on the top surface*

## DISCUSSION & CONCLUSIONS:

The Poly-HIPE's internal porosity is preserved during the stereolithography fabrication. This hybrid technique addresses the resolution trade-off between macro structuring with micro resolution, while maintaining user control over porosity parameters throughout the process. We illustrate the potential of this fabrication approach and that the scaffold supports the proliferation of human dermal fibroblasts. We foresee this material having a promising future with a wide range of tissue engineering applications.

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## ACKNOWLEDGMENTS:

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# LOW DENSITY CULTURING MAINTAINS HMSCS IN THEIR HIGHLY PROLIFERATIVE STATE AND IMPROVES THEIR OSTEOGENIC DIFFERENTIATION POTENTIAL

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## INTRODUCTION:

Human Mesenchymal Stem Cells (hMSCs) enjoy widespread use in tissue engineering and regenerative medicine. Hundreds of clinical trials are performed and numerous articles are published each year linked to this very important adult stem cell type. However, reliable expansion of hMSCs, without compromising their stem cell capabilities, remains a challenging task. In this study the effect of expanding hMSCs using the traditional method (from 40% to 70% confluence – Protocol A) was compared to a low cell density technique (from 10% to 50% confluence – Protocol B) on proliferation, and osteogenic and adipogenic differentiation potential.

## METHODS:

Commercial hMSCs were expanded to passage 4 using either the Protocol A (40% to 70%) or Protocol B (10% to 50%) method and were used for experiments at passage 5. To assess the proliferation rate cells were seeded at 1575 and 3150 cell/cm$^2$. Cell numbers ($n$=6) were measured each day up to day 4 using the PicoGreen assay. To assess differentiation potential cells were seeded at 3150 cell/cm$^2$ and cultured for 14 days in growth, osteogenic and adipogenic medium. Samples at day 14 were assayed for cell numbers (PicoGreen); gene expression of osteogenic (alkaline phosphatase, collagen type I, osterix, osteopontin, osteocalcin) and adipogenic markers (adiponectin, leptin) (qRT-PCR); alkaline phosphatase activity; and lipid formation.

## RESULTS

Protocol B cells produced greater cell numbers and showed a significantly higher (+10-15%) proliferation rate. At day 14 Protocol B samples

contained 23% more cells in growth, 12% more in cells in osteogenic and 78% more cells in adipogenic medium compared to Protocol A. Differentiation marker gene expression was comparable between the two protocols. Alkaline phosphatase activity was three times higher, while lipid levels were significantly lower when normalised to cell numbers, with Protocol B.

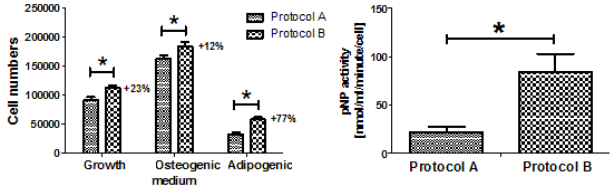


*Fig. 1 – Cell numbers in growth, osteogenic and adipogenic medium (lelft) and alkaline phosphatase activity in osteogenic medium (right) at day 14*

## DISCUSSION & CONCLUSIONS:

Our results show that low density culturing improves the proliferation rate of hMSCs, not just during expansion but in differentiation experiments as well. This can potentially have a great impact on both research and clinical applications. The two culturing methods were comparable at the gene expression level, however at the secretional level Protocol B cells showed better osteogenic, while Protocol A cells showed better adipogenic differentiation. This study highlights the importance of identifying the optimal culture conditions for stem cells.

## ACKNOWLEDGMENTS:

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