Cell-Biomaterial Interactions for Ophthalmic Applications

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The evaluation of cell-biomaterial interactions has become increasingly important in the development of high-performance ophthalmic implants such as intraocular lenses, glaucoma filtration implants and keratoprostheses in recent years [1]. The optimisation of such interactions through surface bioengineering allows the generation of materials which elicit an appropriate host response for a particular application [2].

More recently, as research groups seek to use tissue engineering to reconstruct functional corneae, there has been increasing interest in the biological response of corneal epithelial cells, stromal keratocytes and corneal endothelial cells on biological scaffolds. This paper describes some studies which have been undertaken to better understand and control cell-biomaterial interactions for ophthalmic applications.

The interaction of mammalian cells with ophthalmic biomaterials is dependent on the initial adsorption of cell specific proteins onto material surfaces. These adsorbed proteins also modulate cell response, extracellular matrix production and the subsequent expression of cytokine/chemokines by the adherent cells. As protein conditioning is the primary event following implantation of materials into the eye, the ability to control this process offers a useful strategy to enhance tissue integration or minimise the inflammatory response.

The reduction of cellular adhesion to ophthalmic materials offers the opportunity to reduce clinical complications associated with the use of intraocular lens and glaucoma filtration implants. Previous studies have shown that the adhesion of inflammatory cells to intraocular lenses [3] and scleral fibroblasts to glaucoma filtration implants [4] can be achieved using a biomimetic, biocompatible phosphorylcholine-based polymers. Further studies have suggested that these materials minimise cell interaction by reducing the denaturation of surface associated proteins.

More recent studies have shown that by modifying the molecular composition of the phosphoylcholine-based polymer coatings it is possible to engineer surfaces to control cell-surface interactions providing the potential to minimise initial inflammatory cell interaction whilst allowing the longer term tissue integration [5]. Such materials have potential applications in the fabrication of keratoprosthetic skirt materials to assist with the integration of the device within the stroma.

The *in vitro* evaluation of cytokine release is also important in the selection of suitable materials for *in vivo* applications. Previous workers have reported differences in cytokine-derived macrophage behaviour on different materials [6]. Although initial studies demonstrated that modifying the molecular composition of the surface of a material it is possible to control the adhesion of keratocytes to keratoprosthetic skirt materials, further evaluation demonstrated that materials which support the adhesion of keratocytes give rise to differential expression of the proinflammatory cytokine IL-6 by the adherent cells [7].

These studies suggest that the biological response to ophthalmic biomaterials may be effectively modulated by controlling the molecular architecture of the material surface. As we increase our understanding of the effects of surface engineering on biological response it should therefore be possible to develop materials which could be used a bioactive constructs for the future fabrication of tissue engineered corneae.

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Enhanced Formation of Three-Dimensional Cellular Structures *Via* Biomolecular Engineering

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INTRODUCTION: The complex cell-cell and cell-matrix interactions within tissues are vital for their structural and functional integrity. Hence, to generate effective *in vitro* models and engineered tissues for medical applications, methods for inducing these interactions instead of relying on random cell contacts are essential. We have developed a technique that, *via* biotinylation of cells or matrix and subsequent treatment with avidin, accelerates cell-cell and cell-matrix adhesion, enabling the rapid formation of cell layers and multicellular 3-D aggregates [1]. This study demonstrates the application of this technique to the transfer of therapeutic keratinocytes to a wound bed model and the aggregation of embryonic stem (ES) cells into embryoid bodies.

METHODS: Surface engineering: Wound bed models, cell suspensions or cell monolayers were treated with sodium periodate and subsequently biotinylated using biotin hydrazide [1]. Where necessary, biotinylated cells were avidinated by incubating twice with 5 μg/ml avidin solution for 15 minutes each.

Cell layering: Cell suspensions were seeded on to wound bed models, allowed to adhere for different time periods and then washed. The number of adhered cells was then quantified microscopically or by the MTT-ESTA assay. Different combinations of untreated, biotinylated or avidinated cells and matrices were examined.

ES cell culture: Murine ES cells were suspended from adherent culture and natural formation of embryoid bodies (EBs) compared to biotinylated ES cells that had been cross-linked by avidin treatment. EBs at different stages of development were then dissociated, cultured in osteogenic conditions [2] and the formation of bone nodules from natural and engineered EBs compared after 21 days.

RESULTS: When applied to skin wound bed models, either employing collagen-coated plastic or de-epidermized dermis [3], the adhesion of biotinylated keratinocytes to avidinated matrix was significantly greater than that observed in controls. When this technique was employed with murine ES cells in suspension culture, the cross-linking of biotinylated ES cells by avidin treatment resulted in accelerated EB formation. What is more, the osteogenic differentiation of cells derived from engineered EBs was significantly greater than that of those from naturally-formed controls (figure 1).

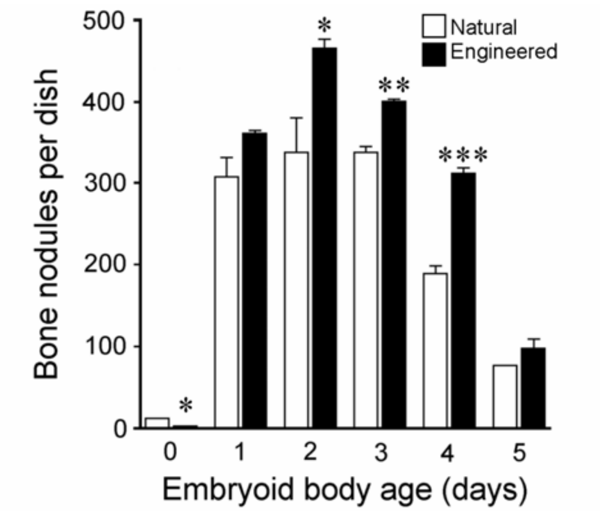

*Fig. 1: Effect of engineered aggregation on the osteogenic differentiation of ES cells from natural and engineered EBs. * Indicates P < 0.05, ** indicates P < 0.005 and *** indicates P < 0.001 as determined by un-paired t-tests.*

DISCUSSION & CONCLUSIONS: These results demonstrate that this technique has the potential to enhance the formation of three-dimensional cellular structures. By accelerating the establishment of cell-cell and cell-matrix interactions it may be possible to apply this method to the development of 3-D tissues, therapeutic cell delivery and specific differentiation of ES cells.

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Collagen Conduits for Neural Repair

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INTRODUCTION:

In peripheral nerve damage, the nerve distal to injury quickly degenerates. While it is easy to suture together two ends of nerve where a short gap exists *in vivo*, for longer gaps conduits or autologous nerve grafts are used to plug the gap. We have developed a form of nerve regeneration conduit based on high density, plastic-compressed collagen/fibronectin, as the basis of next generation biomimetic constructs.

METHODS:

Acellular, type I collagen gels were made, as previously described[1], and routinely compacted by a combination of compression and blotting. These compressed sheets were rolled to form spiral constructs approximately 10mm long by 4mm diameter. Four types of conduit were made. (i)Tight spiral collagen rod, (ii) collagen rod with central hollow core of 350 microns, (iii) collagen rod with 20% strain applied parallel to its long axis (iv) composite of collagen and orientated fibronectin, spirally wound together to give a collagen sheath and fibronectin core. Each conduit was implanted into a standardised 0.5cm nerve gap formed by sectioning the rat sciatic nerve. Constructs were recovered after 4 weeks and regeneration monitored histologically along the construct length.

RESULTS:

Conduits from each group integrated well with proximal and distal ends of the nerve injury. The plain collagen conduits seemed to integrate better on a gross level compared to the composite conduits. Schwann cell migration was seen throughout the construct. From the proximal end, this was present as part of a growth cone of new tissue from the proximal stump. The appearance of a schwann cell growth cone only at the proximal end seems to indicate the importance of neurite outgrowth for this formation.

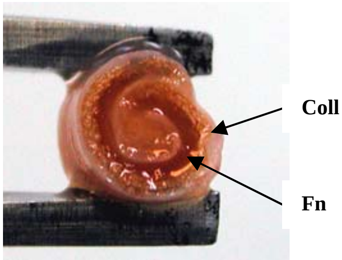

Figure 1. Two component spiral assembly construct of co-compressed collagen and fibronectin material sheets. Figure shows the gross, spiral structure (transverse, between forceps) formed by off-setting the two sheets prior to spiral assembly, to give a fibronectin (**Fn**) core and collagen (**Coll**) sheath.

DISCUSSION & CONCLUSIONS:

Nerve regeneration constructs fabricated by Plastic compression of collagen and fibronectin were made rapidly, were robust and simple to suture in to place, easy to prepare with meso-structure built in. These included heterogenous layers, zones and channels. The integration *in vivo* and in-growth of neural cell regenerate across the constructs indicates that they will be effective guides for repair. Their great strength lies in their ease of fabrication and ability to introduce biomimetic zones and structural features, at the cell scale (i.e. meso-level). We conclude that this approach to native protein conduits is a good basis for fabricating multi-functional implants.

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Sterilisation of PLGA flat sheet and hollow fibre tissue engineering scaffolds

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INTRODUCTION: The design and production of scaffolds for tissue engineering is becoming increasingly sophisticated. Scaffold properties such as mechanical strength, architecture and degradation rate are known to have a significant influence on the ultimate success of the engineered tissue. Post-production scaffolds require sterilisation prior to use in cell culture. Typical sterilisation techniques, such as autoclaving, can have a significant impact on the properties of the scaffold. In this study the effects of four sterilisation methods (ethanol, UV, peracetic acid and antibiotics) are investigated on two different scaffold architectures (flat sheets and hollow fibres); success of sterilisation and effect on the scaffold architecture and pore size are investigated.

METHODS: Polymer scaffolds were fabricated by solvent exchange from a 20%w/w solution of 50:50 poly(D,L-lactic-co-glycolic) acid (PLGA) (Medisorb, Alkermes) in 1-methyl-2-pyrrolidinone (NMP) (Arcos Organics). Flat sheets were cast on glass sheets and immersed in a water bath; hollow fibres were extruded through a spinneret nozzle, solvent exchange occurred via water in the lumen and an external water bath.

Flat sheet scaffolds were prepared by placing the polymer in 13mm diameter cells (Minucells and Minutissue), hollow fibres were cut into 20mm lengths. Samples were sterilised with either: 70% ethanol (Fisher Scientific) [1], UV irradiation [2], Peracetic acid solution: (0.1% peracetic acid (Sigma-Aldrich), 4% ethanol [3] or Antibiotic antimycotic solution (Sigma-Aldrich) for varying durations.

Sterilisation effectiveness was determined following incubation in cell culture medium (10%FCS, 1% NEAA, 1%SP, 88%DMEM, Sigma Aldrich) for up to 48h. Effects on the structure were determined though SEM. Changes in pore size were measured through gas permeation experiments.

RESULTS: All sterilisation methods tested showed no signs of infection during culture when sterilised for typical durations as quoted in literature [1-3]. It was also found that all sterilisation techniques resulted in some degree of damage to the polymer structure; increased treatment duration lead to increased damage (Fig. 1). Polymer structures were seen to exhibit larger pores, tears associated with increased fragility of structure and folding and wrinkling of the surface.

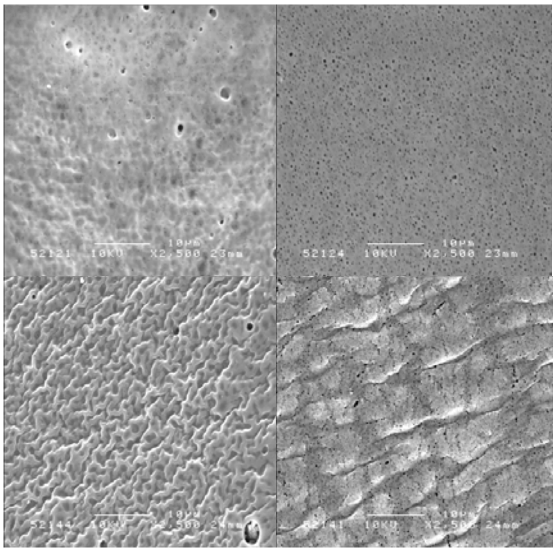

Fig. 1: Effect of antibiotic sterilisation on flat sheet polymer morphology: control bottom (top left) control top (top right) sterilised bottom (bottom left) sterilised top (bottom right).

DISCUSSION & CONCLUSIONS: All sterilisation techniques were associated with changes in the scaffold topography in terms of pore size and surface roughness. These changes may be beneficial; increasing the ability of cells to penetrate and adhere to the scaffold. However, they may also have detrimental effects on the mechanical integrity and degradation rates of the scaffold *in vivo*. The use of peracetic acid and antibiotic treatments for typical durations were found to have the most significant changes on the scaffold structure. The required ethanol treatment of 30mins led to a small increase in pore diameter, which may potentially enhance nutrient transfer through the scaffold.

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Nanomechanics & Nanomanipulation of Soft Biological Materials for Tissue Engineering Applications

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INTRODUCTION: Nanomechanics and nanomanipulation of soft biological materials, such as molecular, cell and tissue, are essential for the advancement of the tissue engineering. For example, applying nanoscale force and displacement as mechanical stimuli to engineered tissue construction, and for sorting and manipulating cells or molecules to nanofabricate *de novo* biomimetic systems, are in great need of both the better understanding of nanomechanics and the new techniques of nanomanipulations.

METHODS: In-situ measurement of important parameters such as elastic modulus, flexural rigidity and visco-elastic behaviour etc. for a single biological or artificial cell have been made possible only recently by using a range of newly devised "indentation" methods by our research group, such as improved nanoindentation apparatus and long-focal side-view imaging system (Fig. 1) [1].

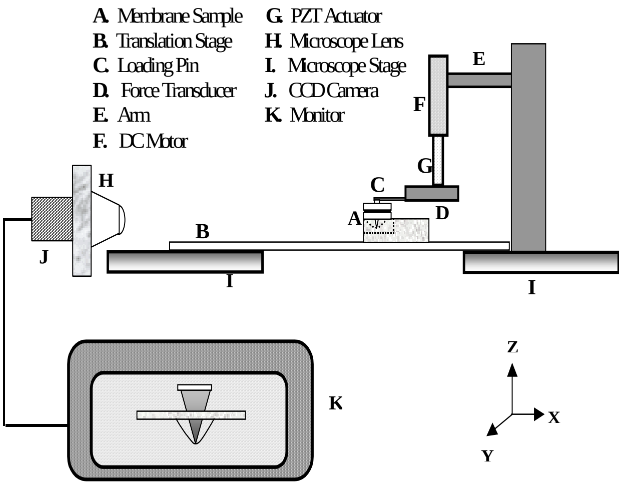

Fig. 1: The schematic view of improved nanoindentation system

Recently, optical tweezers has emerged as a novel tool for manipulating cells and macromolecular structures and for performing sophisticated biophysical characterization. We have used this instrument to interrogate the shape transformation of a moving unilamellar vesicle, and Computation Fluid Dynamics technique has been developed for calculating the flow-induced deformation [2].

We have lately developed a theoretical model to portray adhesion between biological vesicles and a flat substrate in the presence of osmosis. The theory leads to new prediction in cell-substrate adhesion and has an impact in tissue engineering and drug delivery.

RESULTS: Nanomechanical characterisation of the thin soft tissue membrane, e.g. cornea and cell-embedded hydrogel, has been performed using our novel indentation method. Such characterisation is not accessible by conventional methods. The optical tweezers study provides new insights into the mechanics of suspending cell under flow and interpretation of lateral drag force of deformable vesicle [3] and some results are shown in Fig. 2.

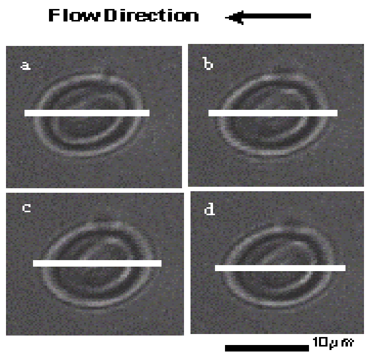

Fig. 2: Deformations of laser-trapped liposome vesicle at room temperature (23.0 ^{o}C) under different flow velocities: (a) static, (b) 16.0 µm/s, (c) 31.0 µm/s, (d) 46.0 µm/s.

The new vesicle-substrate adhesion theories were validated by experimentally using high-resolution reflection interference contrast microscope, which is capable of resolving the cell-substrate contact area in a very high accuracy.

DISCUSSION & CONCLUSIONS: These new instruments and theories provide powerful tools in the study of nanomechanics of soft biological materials for tissue engineering applications.

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Effect of Fluorescent Micropatterned Self Assembled Monolayers on Cell Behaviour

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INTRODUCTION: Microstructured chemical surfaces have gained increasing attention in cell biology. Patterning of solid substrates with biomolecules can be used not only to control cell adhesion and growth but also to regulate cell function. It is vital to understand how cells react with surfaces and how geometrical constraints affect cell function.[1-3] This communication describes the technique of patterning self assembled monolayers (SAMs), with different functional groups (-CH_3, -COOH) using microcontact printing (µCP) and the effect these patterned surfaces have on fibroblast cells (h-Tert).

METHOD: In this project, elastomeric [poly(dimethylsiloxane) (PDMS)] stamps having micrometric dimensions were used. These stamps were inked with a thiol solution and used to pattern the substrates with alternating functional groups of thiols. Initially the project involved the study of self-assembled monolayers on gold substrates and using fluorescent markers, to aid the visualisation of the patterned surfaces, however it led to an interesting study, wherein these fluorescent markers behaved as adhesive platforms for cell attachment. The substrates were stamped (patterned) with a monolayer of carboxy group thiol (-COOH) and ultimately immersed into a solution of PEG (poly(ethylene glycol) molecule which having an amino terminal. These substrates were then immersed into a solution of fluorescent markers (rhodamine::red, fluoresceine::green), which attached themselves to the PEG molecule. The substrates were seeded with human fibroblast cells to observe cell response and behaviour.

RESULTS: Since PEG is a highly protein and cell resistant molecule, [1-3] cells were not expected to attach to these regions. However, results showed the fibroblasts aligning themselves according to these chemical patterns and attaching to the regions of the PEG and the fluorescent markers. Thus, the cells seemed to prefer the regions of the fluorescent patterns over the alkanethiol regions. *[Fig. 1]*

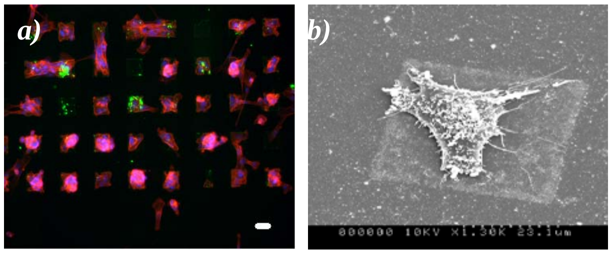

Fig. 1: Cells cover the pattern (about 50µm in size). Pattern is layered with the COOH thiol, PEG molecule and the green fluorescent marker (fluoresceine). a) As observed under the fluorescent microscope, Scale bar 50 µm and b) S.E.M picture.

CONCLUSION: In this project, no adhesive proteins were employed and yet the cells responded strongly to the patterns. The cells clearly preferred the patterned areas (ie. areas covered with the MHA thiol, PEG and a fluorescent marker together (rhodamine::red/ fluoresceine::green)), as opposed to the alkanethiolate coated areas. In conclusion, the methods presented here demonstrated a fast, cheap and simplistic technique for fabricating chemically patterned surfaces. The synthetic strategy used to generate these SAMs presented a surface that was well defined and stable at a molecular scale. The chemical patterns were created step-by-step, with each individual molecule and the patterns were built up on the substrate within a few days. This project introduces a novel method, which would form the basis of a new and interesting technique of chemical patterning for cell biology.

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Cell Response to Microfabricated Surface Rigidity Patterns

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INTRODUCTION: Lo et al. [1] demonstrated the ability of cells to react to a gradient in surface rigidity by orientation and migration towards a stiffer substrate. This reaction was termed durotaxis. To explore this phenomenon further a better control over rigidity gradient and position of rigidity defining features is needed. We developed a method to fabricate substrates with micrometric rigidity landscape and investigate cellular sensing mechanism at a single cell level.

METHODS: Rat calvaria bone cells isolated from neonatal rats by serial digestion were used.
Polyacrylamide gel substrates were created using a modification of Pelham and Wang [2] by incorporating a microfabricated grooved surface underneath the top gel to achieve a surface rigidity pattern. When a curved structure (CSEM,Neuchatel, Switzerland) is used to create a bottom gel, the resulting double gel provides a defined rigidity gradient.

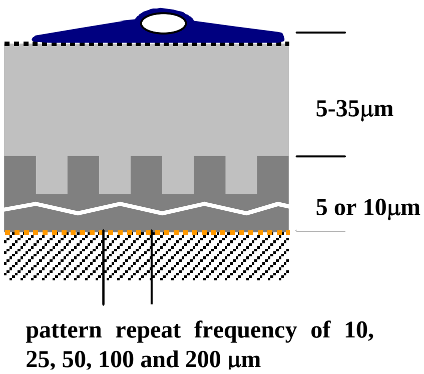

Fig.1: Fibronectin coated polyacrylamide "double gel", with a softer top layer, polymerized over a groove patterned stiffer bottom layer.

Force measurements were performed by tracking the changes of positions of beads incorporated into top gel of substrates and using a Fourier transform traction cytometry [3].

RESULTS: Polyacrylamide double gels provide a surface with defined linear patterns of differences in surface rigidity, with various repeat frequencies (10-200µm) depending on the width of an underlining grooved pattern. The rigidity step value corresponds with a depth of bottom gel grooves.

Cells align in parallel with step changes in substrate rigidity and elongate. This response was observed for burrowed grooves of both 10 and 5µm depth and for repeat frequency of 25µm and higher. After 24h culture, approx. 80% of total cell area is in touch with a 'stiffer' substrate. These results are confirmed with live video microscopy, tracking the cell movements over 24h. Repeated alignment with burrowed steps was observed. Differences in cell area and elongation seen in case of 10% elasticity gradients further support cell preference for a stiffer substrate and its ability to discriminate it.

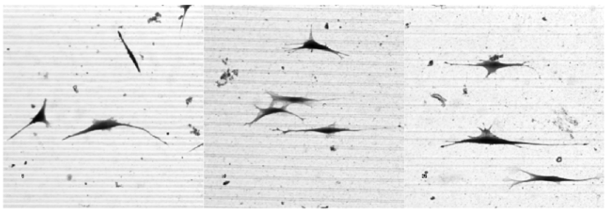

Fig.2: Reaction of cells to spatial frequency of 10, 25 and 50µm pattern repeat. Surface rigidity difference < 5%.

Fluorescence microscopy of vinculin shows that cells prefer to form focal adhesions on 'stiffer' substrates even in case of cells spread over several features of a rigidity pattern.

Force measurements also show that the direction of maximum force is parallel with a rigidity step, corresponding with a position of underlying grooves. Beads displacements seem to be bigger on 'stiffer' parts of the substrate.

DISCUSSION & CONCLUSIONS: We were able to demonstrate a new method to create substrate with rigidity patterns of micrometric features and to provide an otherwise uniform environment for cell adhesion. Results show that cells are sufficiently sensitive to discriminate 5% step changes in surface rigidity and react by alignment and elongation. This is consistent with cells applying maximum forces in parallel with linear rigidity features, in the direction of maximum effective stiffness. There seems to be a spatial sensitivity limit of a pattern repeat frequency 25µm.

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Stem Cells and Orthopaedic Disease

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INTRODUCTION: The promise of stem cell therapy has become an issue of intense public debate, yet there are few true stem cell products in clinical application. This presentation explores strategies to exemplify the potential for stem cell technology, using orthopaedic disease as an example. Hence the focus is more the strategic issues rather than bone biology per-se. There are interlocking, but in principle, separable, debates over the source of stem cells, allograft versus autograft models, and the ethics of embryo-derived cell types and therapeutic cloning. We are investigating in parallel, the potential for both embryo-derived and adult-derived stem cells for bone repair, both as cells alone products and as constructs of bone forming cells and scaffold.

METHODS: For in vitro differentiation, human ES, MSC and HEK cells were cultured and differentiated as described in Sotille et al [1]. For in vivo differentiation in chambers, ES cells were grown as embryoid bodies, disagregated, and exposed to differentiation medium[1] with and without the addition of osteogenic factors (50uM ascorbic acid phosphate, 10mM B-glycerophosphate and 100nM dexamethazone) for a period of 4 days. Cells were resuspended and injected into chambers (10^6 cells), which were surgically transplanted into the peritoneal cavity of nude mice. Chambers were removed and analysed after 79 days. HEF1 cells were generated by retroviral transfection with a telomerase (hTERT) construct as described in Xu et al [2]. To generate the M2 line, for tumour protection, the hES line H9 was electroporated with a construct encoding the galactosyl transferase gene under transcriptional control of the hTERT promoter using established methods.

RESULTS: Human ES cells differentiated in vitro give rise to mineralising osteoblasts in response to OS factors with a similar time course to MSCs[1]. We hypothesised that in the process of osteogenesis, hES cells pass through a stage in which they share properties of MSCs. Telomerisation of differentiating H9 ES cells yielded a cell line, HEF1, that shared cell surface markers with MSCs and was also responsive to OS factors[2]. Human ES cells also give rise to bone in vivo, when placed in chambers implanted in the peritoneal cavity.

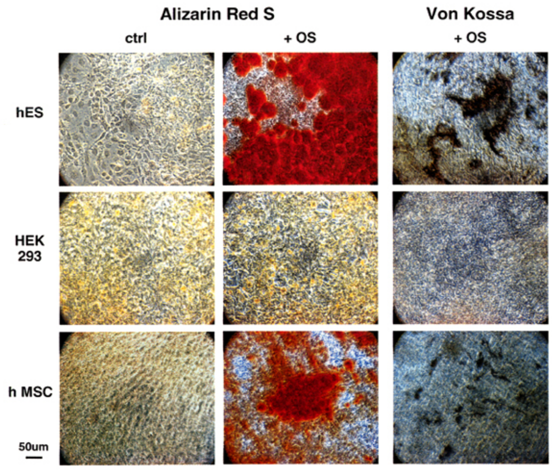

Fig. 1: Human ES, MSC and HEK cells in the absence (-OS) and presence (+OS) of osteogenic factors stained with alizarin red and von kossa for mineralisation

DISCUSSION & CONCLUSIONS: The strategy we describe for the development of stem cell products for bone repair is conceptually simple: to first demonstrate the capacity of stem cells to give rise to mineralising osteoblasts in vitro, then in vivo, then to participate in the repair of a relatively simple calvarial lesion (data not shown). We are now comparing the capacity of ES and MSC-derived cells to repair non-union fractures in a long bone model and to investigate the interactions of cells and scaffolds. To address the issue of scale up we have begun to characterise intermediate cell types generated by telomerisation of differentiating hES cells. Further characterisation of telomerised lines may provide novel markers for committed bone precursors in wild type populations of both embryonic and adult origin.

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Utilisation of Silane Modified Surfaces to Control the Osteogenic Differentiation of Human Mesenchymal Stem Cells

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INTRODUCTION: The ability to control, or influence, the generic plasticity of bone marrow derived mesenchymal stem cells (MSC), is an area that is still relatively unexplored. The aim of this study was to establish how changes in surface chemistry (characterised silane modified surfaces), both with and without the appropriate biological stimuli, affected the behaviour and osteogenic potential of human MSC in vitro over a 28 day time period.

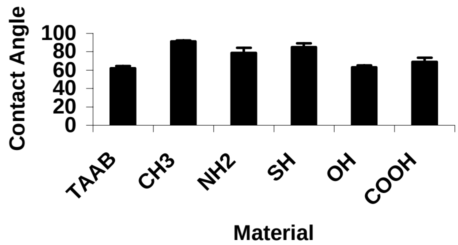
METHODS: Glass coverslips (13mm diameter, Borosilicate Glass Co. UK) were dipped into 5% NaOH solution for 1 hour followed by concentrated HNO_3 for 1 hour. All the coverslips were then rinsed with ultra pure water and with 100% ethanol, dried at 120^{o}C, and stored in a vacuum desiccator prior to the introduction of different functional groups on the surface. TAAB glass was used as a control surface. –CH3, -NH2, -SH, -OH and –COOH groups were introduced using previously described methods[1] and characterized using water contact angle measurements.

Human MSC (Biowhittaker UK), were re-suspended to a concentration of $5x10^4$ cells/ml, and cultured in contact with the samples for 1,7, 14, 21 and 28 days in both basal MSC media (Biowhittaker UK) and osteogenic supplemented media (DMEM + 10% FCS, 100mM ascorbate-2-phosphate, 100nM dexamethasone and 10mM β-glycerphosphate). Viable cell adhesion was quantified using a commercially available lactate dehydrogenase assay (LDH, Promega UK). Real time PCR was used to evaluate the expression of β-actin, ornithine decarboxylase, osteocalcin, osteopontin, osteonectin, collagen I and CBFA-1. Cellular expression of collagen I, osteocalcin, CBFA1 and calcified extra-cellular matrix was qualitatively evaluated by fluorescent immunohistochemistry and von Kossa staining.

RESULTS: Dynamic contact angle measurements using water as the solvent for glass and silane modified surfaces are shown in Figure 1.

The surface energy results supported the contact angle results. The TAAB, -OH, -COOH have the highest surface energies with values of 53.39, 52.89 and 49.51mJ/m^2, respectively. The -NH_2 and -SH had lower values of 43.52 and 39.60mJ/m^2 respectively, and the -CH_3 had the lowest value of 35.53mJ/m^2.

Fig. 1: Water contact angle measurements



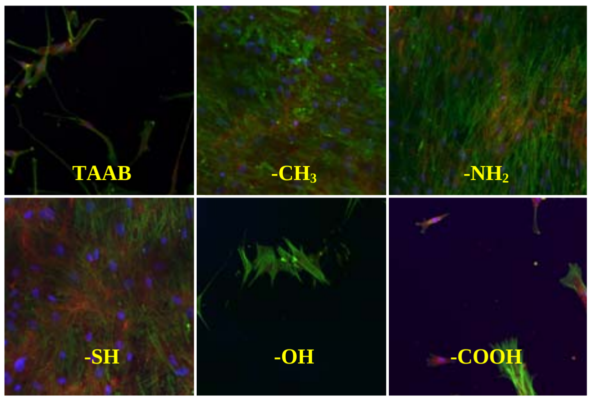


Fig. 2. 14 day collagen I expression (red) in osteogenic supplemented medium.

DISCUSSION & CONCLUSIONS: Changes in surface chemistry did affect the behaviour of the MSC and subsequent osteogenic differentiation in vitro. –TAAB, -OH and –COOH surfaces did not maintain cell adhesion under osteogenic conditions. –CH_3, -SH and –NH_2 provided a favourable surface for osteogenic differentiation in all culture conditions.

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Bioreactor Culture of Cartilage from Mesenchymal Populations

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INTRODUCTION: Cartilage degeneration results in severe pain or disability for millions of individuals worldwide. However, the potential for cartilage to self-regenerate is limited. Cartilage is composed of only one cell type, is avascular and has a relatively simple composition and structure, thus cartilage tissue engineering has tremendous potential. Therefore to address this clinical need, we have adopted a tissue engineering approach to the generation of cartilage ex vivo from mesenchymal cell populations encapsulated in alginate, a natural polysaccharide that favours chondrogenesis, and cultured within a rotating-wall bioreactor and a perfused bioreactor system.

METHODS: Alginate (2% solution, phosphate 300mM) and chitosan (Ca^{2+} 50mM) semi-permeable polysaccharide capsules were generated by a one-step method as described by Leveque *et al* [1]. Human femoral head and bone marrow samples were obtained from haematologically normal patients undergoing routine total hip replacement surgery. Marrow aspirates were washed in α-MEM and centrifuged at 1100rpm. Marrow cells were resuspended in α-MEM with 10% FCS. Primary chondrocytes were isolated from articular cartilage by enzymatic digestion and grown in 10% α-MEM supplemented with 100uM Ascorbate-2-Phosphate. Cells were grown in monolayer and when confluent 2-4 x 10^5 cells were encapsulated within the polysaccharide capsules with the addition of TGF-β3. Marrow cells and chondrocytes were also co-cultured within polysaccharide capsules in a ratio of 2:1 respectively [2]. Capsules were subsequently placed in either a Synthecon rotating-wall bioreactor, perfused at a flow rate of 1ml/hour or held in static conditions for 28 days at which point they were harvested for biochemistry and histology.

RESULTS: Alcian Blue and Sirius Red staining indicated a more ordered, structured and even cell distribution within capsules from the rotating bioreactor system in comparison with perfused and static conditions. In addition, only alginate beads that were cultured in static conditions with mixed cell populations revealed positive staining for both collagen and proteoglycan, with areas that closely resembled the formation of osteoid. Cell viability, assessed using the fluorescent dye Cell Tracker Green, indicated a higher proportion of metabolically active cells in capsules from rotating-wall conditions in comparison with perfused or static. Immunohistochemistry indicated the expression of type II collagen, SOX9 and C-MYC in samples from all conditions after 28 days. C-MYC is implicated in cell proliferation and differentiation and type II collagen and SOX9 are cartilage-specific markers. Biochemical analysis revealed significantly increased ($p < 0.05$) protein synthesis in samples encapsulated with mixed cell populations compared with alginate samples that were encapsulated with single cell populations. There was also a significant increase in protein synthesis in samples that were cultured in the rotating-wall bioreactor in comparison with perfused or static conditions. DNA and cell proliferation was significantly increased in the rotating-wall compared with perfused or static for the bone marrow cultures. Interestingly in chondrocyte cultures perfused conditions were found to result in significantly higher DNA than rotating-wall and static. Increased DNA and cell proliferation was observed in static conditions for mixed cell population samples.

DISCUSSION & CONCLUSIONS: The current studies outline a tissue engineering approach utilising progenitor populations, bioreactors and appropriate stimuli to promote the formation of cartilage within a unique innovative polysaccharide capsule structure. These studies indicate the potential of rotating-wall bioreactor systems to promote cartilage formation and also the potential of the encapsulation of mixed cell populations. Understanding the conditions required for the generation of functional 3D cartilage constructs using such bioreactor systems carries significant clinical potential.

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ACKNOWLEDGEMENTS: PhD Studentship funding was supported by Research into Ageing.

Mechanical Conditioning Influences Mesenchymal Stem Cell Chondrogenesis

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INTRODUCTION: Cell-based repair of articular cartilage defects with autologous chondrocytes are associated with problems of cell source, chondrocyte proliferation and de-differentiation, and donor site morbidity [1,2]. Mesenchymal stem cells (MSCs) present an attractive alternative, provided controlled chondrogenic differentiation can be achieved [3]. The present work explores chondrogenesis in MSCs and the modulation of this phenotype by mechanical conditioning in a controlled bioreactor.

METHODS: Human MSCs, (CD105, CD44 (+) CD34, CD45 (-)) expanded by serial passage in monolayer culture were tested for multi-lineage commitment by parallel osteogenic, chondrogenic and adipogenic cultures. Chondrogenesis was induced in cell-pellet and cell-alginate cultures (1×10^7 cells/ml) by serum-free media supplemented with dexamethasone and 10ng/ml TGF-β_3. Chondrogenic differentiation was analysed in a temporal fashion by the expression of cartilage-specific markers; collagen type II, collagen type X, aggrecan and *Sox*-9 using real-time QPCR. For mechanical conditioning experiments, cell-seeded constructs were placed in a bioreactor and exposed to intermittent dynamic uniaxial compression (c+/c-) (loading/unloading 1.5 hr/4.5 hr respectively, strain amplitude 15%, frequency 1Hz) in combination with 10ng/ml TGF-β_3 (t+/t-) for a period of 8 days.

RESULTS: Chondrogenesis in both pellet and cell-alginate cultures was described by collagen types II and X being progressively up-regulated over a 10 day period. *Sox-9* showed a peak at day 8 followed by a decline and aggrecan a reduction by day 2 followed by recovery at day 8. With intermittent dynamic mechanical loading, collagen type X was significantly up-regulated with dynamic loading in the presence and absence of TGF-β_3 (fig 1), whilst aggrecan was found to be reduced by a combination of treatment stimuli when compared to TGF-β_3 or dynamic loading alone (fig 1). No significant difference was found in collagen type II or *Sox-9* gene expression.

DISCUSSION & CONCLUSIONS: Mesenchymal stem cell expression of the cartilage-specific extracellular matrix proteins collagen type X and aggrecan is sensitive to dynamic mechanical loading. Moreover, alginate represents a suitable scaffold material for tissue engineering applications using MSCs, by sustaining viability, chondrogenesis and enabling mechanical loading *in vitro*.

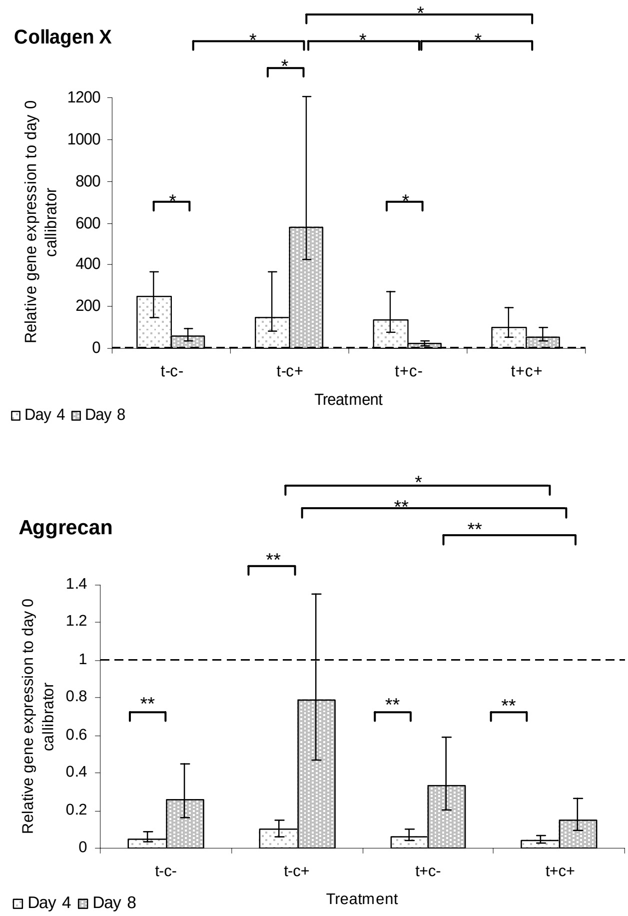

Fig. 1 Average (median) quantitative gene expression for collagen type X and aggrecan in the presence (+) and absence (-) of mechanical loading (c) and TGF-β_3 supplementation (t). Error bars represent semi-interquartile range. Statistical significance by Kruskal-Wallis with Dunn's post-hoc testing ($p<0.05$, ** $p<0.001$).*

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ACKNOWLEDGEMENTS: This work was undertaken with a grant from the Engineering and Physical Sciences Research Council, U.K.

Differentiation of Mesenchymal Stem Cells (MSCS) to NP-Like Cells in Chitosan/Glycerol Phosphate : Implications for Tissue Engineering of The Intervertebral Disc (IVD)

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INTRODUCTION: IVD degeneration is a major cause of low back pain. Surgery has limited success and novel technologies, such as tissue engineering to regenerate the IVD are being developed. One approach is to use a hydrogel seeded with cells, to replace the area most affected functionally by degeneration, the gelatinous nucleus pulposus (NP). NP cells can not be used because removal from a healthy disc causes degeneration and NP cells removed from a degenerate disc behave abnormally. The cells of the NP are chondrocyte- like and an attractive cell source is MSCs, which have the capacity to differentiate down the chondrocytic lineage. However, there is not a method available to differentiate MSCs in monolayer. Instead 3D cultures, such as alginate beads are usually used, which have been demonstrated to support chondrocytic differentiation of MSCs. In this study, we have investigated whether the novel thermoreversible hydrogel chitosan- glycerol phosphate (C/Gp), can support MSC differentiation and compared it to the alginate culture system. C/Gp has the potential to be used for non-invasive injection into the IVD and possesses *in situ* gelation, characteristics that can not be achieved using alginate. These are important features for tissue engineering of the IVD when attempting to replace the NP of the degenerate IVD with a hydrogel seeded with differentiated MSCs. The hypothesis for this study is that MSCs can differentiate into chondrocyte-like NP cells within C/Gp layer systems.

METHODS: MSCs were directly isolated from bone marrow and expanded in monolayer, before transferring to a novel hydrogel layer culture system. Layers were seeded at 1, 5, 10 or 20 million cells/ml and cultured for 1 or 2 weeks. 1.2% (w/v) alginate layers were set by polymerisation with 102mM calcium chloride. C/Gp layers composed of 3% (w/v) chitosan with 0.5M Gp were set in 10 minutes at 42˚C. Cell viability was assessed using live/dead staining. Relative gene expression was assessed by RNA extraction, reverse transcription and real- time PCR for the chondrocytic marker genes type II collagen, SOX9, and aggrecan, the fibroblastic marker type I collagen and endogenous control 18S. Data was analysed using the $2^{-\Delta\Delta ct}$ method.

RESULTS: The live/dead assay showed that MSCs were viable in alginate layers, although this was affected by time in culture and cell density. At 1, 5, or 10 million cells/ml viability reduced from about 85 to 75% after 3 weeks of culture. However, at 20 million cells/ml viability was reduced from 58 to 52 % after 3 weeks of culture. Real- time PCR demonstrated that alginate layers induced chondrocytic differentiation of MSCs compared to monolayer. The relative gene expression was affected both by time in culture and cell density. After 2 weeks of culture, the markers began to decrease to similar levels for all cell densities. The highest gene expression of chondrocytic markers was seen at 5 million cells/ml after 1 week of culture. Nuclear staining of MSCs within the C/Gp layers demonstrated that the cells were dispersed throughout the gel at all densities, although this was more uniform at lower densities and cell clumping increased with density. The live/dead assay for MSCs in optimised C/Gp layers at 5 million cells/ml demonstrated that MSC viability fell from 90% after 2 days of culture to 75% after 3 weeks. Real- time PCR demonstrated that MSCs in C/Gp layers strongly expressed chondrocytic markers. For 1, 5, or 10 million cells/ml this required 2 weeks of culture and cell density had little effect on gene expression levels.

DISCUSSION & CONCLUSIONS: We have shown for the first time that human MSCs can distribute within and be cultured with high viability in C/Gp layers, at a similar level to alginate. In addition, we have shown that this novel hydrogel causes differentiation of MSCs to NP-like cells *in vitro*, after 2 weeks of culture as demonstrated by a change in expression of phenotypic marker genes. The 'smart' properties of C/Gp, coupled with its ability to induce NP differentiation of MSCs, suggest great potential for C/Gp in tissue engineering of the IVD.

The Proliferation of hMSCs on PLA Scaffolds

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INTRODUCTION: Biodegradable synthetic polymers including poly(lactic acid) (PLA) are suitable for biocompatible scaffold constructs but are known to undergo *in vitro* degradation[1]. This may limit their potential for use in long-term cultures or loading regimes. This investigation determines whether it is advantageous to culture cells on scaffolds prior to mechanical compression.

METHODS: Human MSCS (hMSCs) were obtained commercially from Poietics™. P4 cells were seeded at 1.5×10^6 cells onto cylindrical PLA scaffolds (ϕ 9mm x 4mm). The constructs were cultured for either 3 hours, 4 days or 7 days in D-MEM supplemented with 10% fetal calf serum (FCS) with slow rotation. Scaffolds were then divided into 8 sections and a cell proliferation assay was performed on each section using the Promega CellTiter 96® AQueous One Solution (n=5) and cell distribution was also determined with 16µm cryo-sections stained with haematoxylin. SEM was used to image scaffold integrity.

RESULTS: There was no significant proliferation of the MSCs on the scaffolds during culture. The distribution of cells throughout the scaffolds after the initial 3 hour seeding period and subsequent cultures remained similar and the cells were mainly found in the top half of the scaffolds, shown in Figure 1. This was also confirmed by haematoxylin staining of scaffolds cultured for 3 hours shown in Figure 2A. SEM images showed that during the 7 day culture period the scaffold integrity decreased, shown in Figures 2B-2D.

DISCUSSION & CONCLUSIONS: This data suggests that hMSCs do not proliferate during 7 days of culture on PLA scaffolds. This is consistent with an initial lag phase in cell proliferation observed in monolayer cultures[2,3]. The cells appear to remain in the upper half of the scaffold which suggests that the seeding methods used do not result in uniform distribution and that the cells do not migrate throughout the scaffold. This data suggests that there are no advantages in culturing the constructs prior to loading due to a lack of cell proliferation and degradation of the scaffolds which may ultimately affect their mechanical properties.

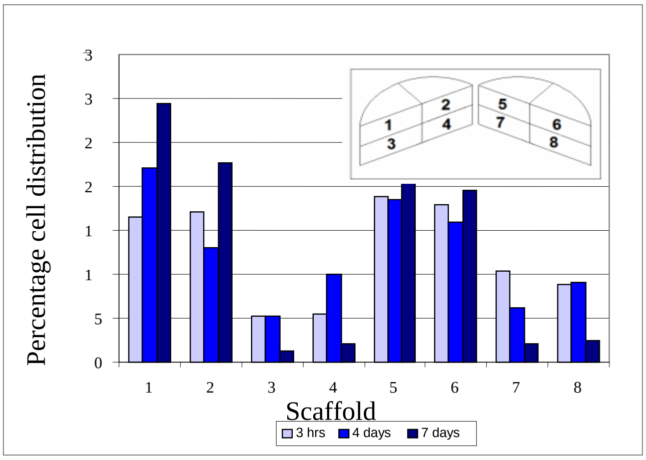

Figure 1: The percentage distribution of P4 MSCs in PLA scaffolds after 3 hrs, 4 days and 7 days

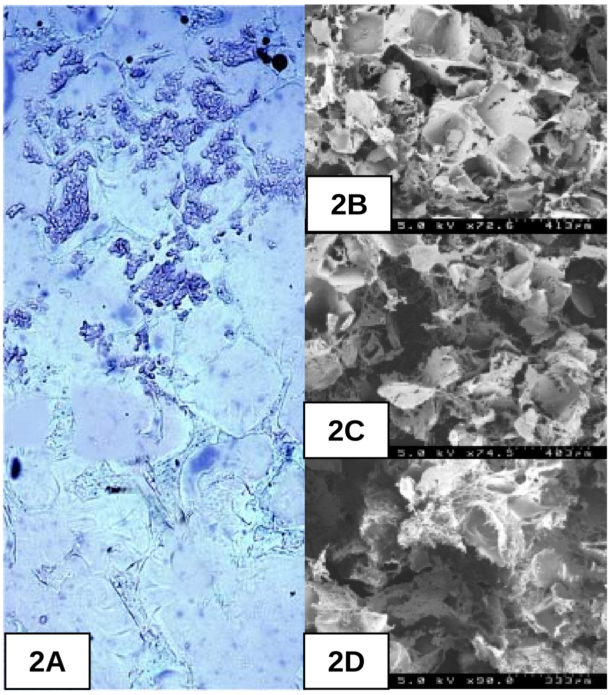

Figure 2:16µm cryo-section stained with haematoxylin after 3 hrs (2A), and SEM images showing scaffold integrity after 3 hrs (2B), 4 days (2C) and 7 days (2D)

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ACKNOWLEDGEMENTS: I would like to thank the EPSRC: GR/S11510/01 and the Welcome Trust: 067743/Z/02/Z for funding. I would also like to thank everyone in the group for their help and Karen Walker at the SEM unit

Ultrasonic Modification of Acellular Tendon to Enhance Recellularisation

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INTRODUCTION: Previous studies have shown that porcine patella tendons can be successfully decellularised using 0.1% (w/v) SDS giving an acellular scaffold with the biochemical and mechanical properties of native tendon(1). However whilst these scaffolds were able to support cell growth, cells were unable to penetrate the scaffold. The aim of this study was to investigate the use of ultrasonic energy to modify acellular porcine patella tendons as a method for enhancing subsequent recellularisation by autologous cells *in vivo* and/or *in vitro*.

METHODS: Porcine patella tendons were harvested within 24 hours of death and decellularised as previously described(1).

Ultrasonication: Ultrasonication was carried out using a Progen Scientific ultra sonicating probe. Acellular scaffolds (n=3) were sutured to a sterile stainless steel grid and immersed in ice cold sterile PBS. The scaffold was subjected to ultrasonication at powers of between 90 and 456 Watts with pulse times of between 1 and 3 seconds for a total of 1 minute. Changes to the scaffold architecture were assessed by routine histology.

Assessment of Scaffold: Fresh, acellular and sonicated scaffolds (n=6) were split in two along their long axis and subjected to uniaxial tensile loading to failure. The denatured collagen content was assessed by pre incubation using α-chymotrypsin and determination of hydroxyproline using the method of Edwards & O'Brien(2). Statistical significance was assessed by one-way analysis of variance (ANOVA).

Recellularisation of scaffold: Primary human tenocytes were seeded on the surface of the sonicated acellular scaffolds at 1×10^5 cells.cm^2 and cultured at 37°C in 5% CO_2 in air. Two scaffolds were removed every 7 days for routine haematoxylin and eosin staining and assessment with Live/Dead stain (Molecular Probes).

RESULTS: Following ultrasonication it was shown that at powers of 380W and above the tendon structure was altered dramatically with many breaks in the collagen bundles. Similarly ultrasonication with 365W with 2 and 3 second pulse times led to large holes within the scaffold. When the acellular scaffold was subjected to ultrasonication at 365W with a 1 second pulse time a pronounced opening of the spaces collagen bundles and surrounding endotenon was observed with no evidence of damage to the collagen bundles themselves (Fig 1). This protocol was used for further studies. There were no significant differences in the denatured hydroxyproline content of the scaffold following decellularisation (0.347±0.157)or after ultrasonication (0.379±0.098) compared to fresh tissue (0.254±0.128). There was no significant difference in the ultimate force of the scaffold following decellularisation and ultrasonication (364.75±86.03N) when compared to fresh tissue (390.8±169.3N). Primary human tenocytes the cells were seen to grow in monolayer on the surface of the scaffold after 1 week of static culture and cells were able to penetrate to the centre of the scaffold after just three weeks of culture (Fig 1). However there was considerable matrix reorganisation at the centre of the scaffold. Use of Live/Dead stain showed approximately 50% cell viability in the centre of the scaffold.

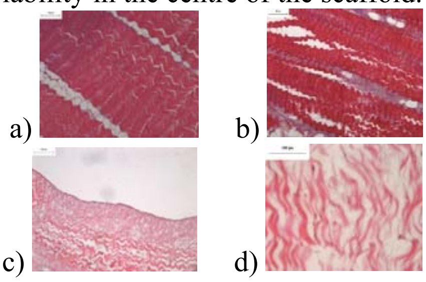

Figure 1: H&E staining of a) acellular scaffold b) 365W sonicated acellular scaffold c) reseeded scaffold after 1 week and d) reseeded scaffold following 3 weeks in static culture.

DISCUSSION&CONCLUSIONS:
Ultrasonication has successfully been used to enhance recellularisation of acellular tendon scaffolds without adversely affecting the biomechanical properties of the scaffold. Upto 50% of the cells in the centre of the scaffold were viable and tissue remodelling was observed. Ultrasonication may also enhance the capacity of acellular tendon, and other tissue scaffolds for recellularisation and regeneration *in vivo.*

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Tissue Engineering In Relation To Skin

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INTRODUCTION:

The ability to culture large areas of sheets of keratinocytes from small biopsies led to the early application of skin tissue engineering to the treatment of major thermal burns in the early 1980s. This led to anecdotal and widespread use of such grafts to different clinical scenarios, including chronic ulcers, surgical excisions, mastoid cavities etc. The graft performance was however poorer in terms of take and cosmesis than standard surgical procedures, so much effort has gone into improving the tissue engineered end product. The remaining compelling indication is in the treatment of major burns, but there are lessons to be learned for current tissue engineering developments from the whole experience. These are that measurable benefit is not the same as clinical efficacy and that controlled trials against standard procedures must be performed to establish the true position of any tissue engineered product.

Characterising Mechanisms of Regeneration for Future Applications in Tissue Engineering

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INTRODUCTION: The proper replacement and restoration of tissue mass after organ damage or injury in higher vertebrate adult animals is critical to the architecture and function of the organ. If the replacement occurs with scar tissue, this often results in adverse effects on function and growth as well as an undesirable cosmetic appearance. We have previously shown that MRL/MpJ mice have a capacity for regeneration instead of scar formation, following an ear punch wound[1,2]. Since regeneration of tissue mass may in part be brought about by apoptosis of primitive cells in MRL/MpJ regeneration following punch injury to the ear, the role of the Bcl-2 family of proteins and the caspases was investigated. Cartilage regeneration in the ear was also studied as was wounding in the backs of these animals.

METHODS: A histological analysis was conducted up to 4 months post-wounding, not only on 2mm punch wounds to the ear but also 4mm wounds to the skin on the backs of the same animals. To investigate the changes in tissue architecture leading to ear wound closure, we investigated whether the type of trauma (clinical biopsy v. crude punch) applied to the ears of different strains of mice including MRL/MpJ influenced the rate of wound healing. Wound healing in the backs of the same animals was also investigated. Tissue samples were wax embedded and H&E and Masson's Trichrome stains were performed. Glycosaminglycan deposition in the regenerating ear was assessed using Alcian blue.

RESULTS: MRL/MpJ mouse ear wounds heal faster than control strains with enhanced blastema formation and markedly thickened tip epithelium. The reduced inflammatory infiltrate seen with the biopsy as opposed to the crude punch, correlated with a faster, more regenerative repair process with reduced scarring. Interestingly, in the excisional back wounds, none of these regenerative features were observed and both the C57BL/6 control and MRL/MpJ healed with scarring. Analysis of apoptosis was undertaken assessing the expression of various members of the Bcl-2 family of proteins. By day 5 post-wounding Bcl-2, Bcl-x, Bax, Bak, Bim and Bid are all expressed but there was little difference of expression between the control and MRL/MpJ strains. By day 14 post-wounding however, in the MRL/MpJ strain, in both the ear and back wounds there was altered expression of the pro-apoptotic family members Bax, Bim and Bid. There was also altered cartilage deposition in ears between the two strains post-wounding (Figure 1).

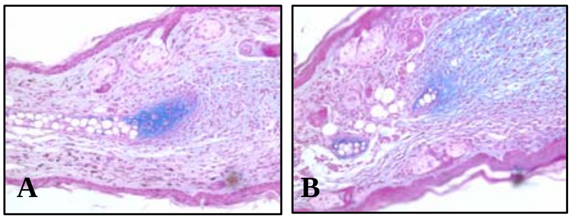

Fig. 1: Mouse ear cartilaginous structures at 28 days post-wounding. A C57BL/6 ear section is depicted with repairing cartilage (A) and regenerating cartilage "islands" in the MRL/MpJ blastemal mesenchyme (B).

DISCUSSION & CONCLUSIONS: The thickened tip epithelium in the regenerating blastema of the MRL/MpJ ear may be the source of a number of growth and differentiation factors. By diffusion into the underlying mesenchyme, these factors may affect proliferation and differentiation of putative progenitor cells to establish a "progress zone" type of organisation. In the backs of the same mice, wounds heal by simple repair and result in scar formation. This difference in the regenerative capacity may be due to physical dimensions. The ear structure is thinner and surrounded on both sides by epithelia whereas the back skin is devoid of cartilage and under greater tensile strain. Ongoing studies using microarray technologies are now focusing on characterisation of the signalling molecules and cascades involved in the regenerative mechanisms observed in the mouse ear. Identification of such signals could lead to their manipulation and use in a novel tissue engineered skin substitute with scar–free integration.

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Anti-Inflammatory Peptide Approaches for Preventing Vascular Inflammation

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INTRODUCTION: Inflammation is implicated in the development of atherosclerotic lesions and restenosis [1]. It is proposed that by inhibiting the local inflammatory process e.g. using a drug-eluting stent platform, neointima formation will be reduced. Melanocyte stimulating hormone (MSH) peptides are potent inhibitors of inflammation, and act via the melanocortin-1 receptor (MC1R) [2]. Therefore, the aim of this work was to investigate whether the MC1R was expressed on porcine vascular smooth muscle (VSM) cells and endothelial cells. Work also looked at investigating whether MSH peptides can inhibit inflammatory signaling, measured via inhibition of the NF-κB transcription factor and upregulation of the E-selectin adhesion molecule.

METHODS: The presence of the MC1R in porcine endothelial and VSM cells was detected using immunolabelling. The inhibitory potential of α-MSH was tested by stimulating both cell types with recombinant porcine TNF-α (pTNF-α) +/- α-MSH (10^{-9} M) and forskolin (10^{-4} M) as well as α-MSH alone and forskolin alone for 60 minutes. Cells were then immunolabeled for the p65 subunit of NF-κB. Inactive NF-κB was quantified by counting the total number of cells with a cytoplasmic location and active NF-κB by counting cells with a nuclear localization. In addition, E-selectin expression was assessed by flow cytometry (Guava PCA) using porcine endothelial cells grown in culture for 3 days and pre-incubated with α-MSH (10^{-8} M/10^{-10} M/10^{-12} M) for 15 minutes prior to stimulation with pTNF-α (2 ng/ml) for 24 hours.

RESULTS:

MC1R Immunolabelling: Porcine endothelial and VSM cells were observed to immunolabel positively for the MC1R on the cell membrane.

NF-κB Activation: Unstimulated porcine endothelial cells showed a heterogeneous NF-κB distribution and unstimulated porcine VSM cells exhibited cytoplasmic NF-κB localization. TNF-α caused rapid nuclear translocation (activation) of NF-κB in both cell types. α-MSH was found to maximally inhibit TNF-α stimulated NF-κB activation by 55 ± 5 % (VSM cells) and 52 ± 8 % (PE cells) at 10^{-9} M (n=3, p>0.05). α-MSH alone (without TNF-α) did not alter NF-κB activity. Addition of forskolin also inhibited TNF-α stimulated NF-κB activation.

E-selectin upregulation: α-MSH inhibited TNF-α stimulated E-selectin upregulation in a dose responsive manner from 10^{-8} M to 10^{-12} M (Figure 1). Complete inhibition was observed at 10^{-8} M.

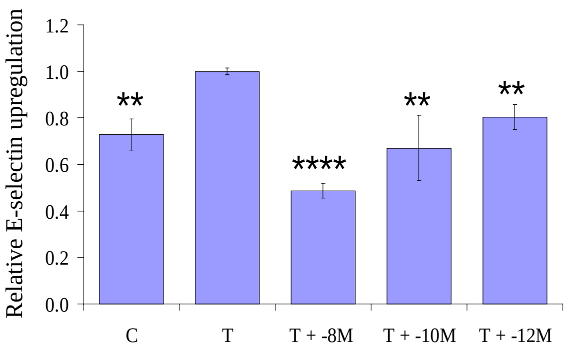

Fig. 1: α-MSH inhibits TNF-α stimulated E-selectin upregulation. C, Control (unstimulated); T, pTNF-α (2 ng/ml); M, α-MSH (10^{-8}/10^{-10}/10^{-12}M). n=3. **p>0.01, ****p>0.0001

DISCUSSION & CONCLUSIONS: We report on the positive expression of MC1R on porcine endothelial and VSM cells. MSH peptides decreased inflammatory signaling and adhesion molecule upregulation in TNF-α stimulated VSM and endothelial cells. Work is currently underway to: (1) confirm the immunolabelling data obtained using transient transfection with an NF-κB dependant luciferase reporter construct; (2) extend the adhesion molecule work studying ICAM-1 and P-selectin on endothelial cells and ICAM-1 on VSM cells. This work suggests that MSH may be of potential therapeutic value in the prevention of vascular inflammation. Future work includes looking at the effect of MSH on cellular apoptosis and proliferation.

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Deposition of Elastic Fibres in a Murine Cutaneous Wound Healing Model

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INTRODUCTION: The ability of the skin to extend and recoil is mediated by an elastic fibre network comprising elastin molecules deposited on a microfibrillar scaffold. Studies have demonstrated reduced tensile strength in scar tissue following cutaneous wounding, possibly due to decreased amounts of elastic fibres[1]. The dermal component of artificial skin substitutes also lacks an organised elastic fibre network, which may contribute to excessive contraction and scarring post-grafting[2]. This study aimed to document the temporal and spatial distribution of elastic fibres following incisional and excisional cutaneous wounding in mice.

METHODS: Incisional wounds (1 cm) or excisional wounds (6 mm punch biopsy) were made on the dorsum of male Balb/c mice and harvested at 1 day to 6 months post-wounding before processing for immunolocalisation of tropoelastin and histological analysis using Miller's stain for elastin. Wound area and elastin content of skin sections were assessed by image analysis.

RESULTS: By 7 days post-wounding, incisional wounds had healed and elastin was first detected. Immunolocalisation of tropoelastin displayed a peak at 56 days post-wounding, whereas histological analysis with Miller's stain demonstrated a peak at 70 days post-wounding. Incisional wounds showed significantly increased elastin levels at 6 months post-wounding compared with normal uninjured skin. Macroscopically, excisional wounds had closed by 5 days post-wounding. Tropoelastin was first present 7 days after wounding as detected by immunolocalisation, whereas histology staining showed elastin appeared later at 21 days post-wounding. Tropoelastin levels peaked at 3 months post-wounding, while histology staining indicated that elastin levels remained elevated at 6 months post-wounding. Elastin was first observed in both wound types in the lower dermis as fine fibres close to the panniculus carnosus muscle, although elastic fibres were absent from this area in uninjured skin. In the upper dermis, tropoelastin and elastin levels increased to three times those in normal skin.

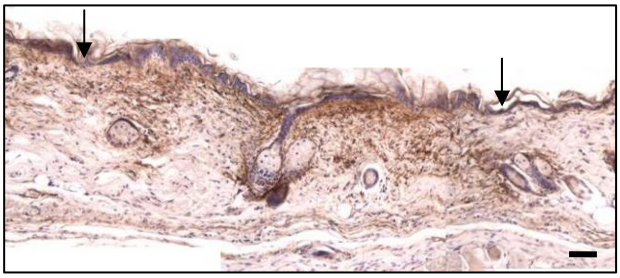

Fig. 1: Tropoelastin present in murine skin 8 weeks post-incisional wounding. Brown DAB stain indicates immunolocalisation of tropoelastin in the wound area. Arrows indicate wound margins. Bar=100 µm.

DISCUSSION & CONCLUSIONS: Findings from this study suggest that precursor tropoelastin, as assessed by immunolocalisation, is present in both incisional and excisional wounds earlier than elastin detected by Miller's stain. Present studies are investigating changes in tropoelastin gene expression and the expression of other elastic fibre components in wound samples. These findings suggest that reduced tensile strength in scar tissue is not due to decreased levels of elastic fibres in the dermis, but more likely abnormal elastic fibre network architecture. Manipulation of elastic fibre synthesis, deposition and organisation may lead to better tissue restoration and improved skin substitutes.

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Profile of Pulmonary Smooth Muscle Cells and Their Response to Blockade and Stimulations

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INTRODUCTION: The tone of the pulmonary arteries is the summation of the activity of each smooth muscle cell (SMC) within a vessel wall and its interaction with the endothelial cells and extracellular matrix (including collagen). There are reported phenotypic differences between SMC in the inner & outer layers of pulmonary artery walls[1]. To tissue engineer a blood vessel a basic understanding of differences between inner and outer SMC's in terms of attachment and contraction of 3D matrix is essential. We hypothesised that they will differ in their ability to contract a 3D collagen gel. Using a Culture Force Monitor (CFM) we sought to: (1) quantify the contractile ability of SMC derived from inner and outer normal and hypoxic arteries, harvested from piglet models, over 24 hours, (2) test the effect of cytoskeletal blockade on force retention in collagen gels and (3) quantify cellular contractile response to agonist or antagonist stimulation.

METHODS: Piglets were exposed to hypoxia (50KPa) for 3-14 days then sacrificed at day 14. Large intrapulmonary arteries were dissected and SMC derived from inner & outer layers were then cultured in DMEM/F12 medium with 10% FCS. 5 ml rectangular Collagen gels2 (rat tail collagen type I, 10x minimal essential medium, sodium hydroxide) were prepared in a sterilised silicone polymer mould and seeded with 5 million cells (passage 3-6). The gel was allowed to set with 2 A-frames (layered polyethylene mesh with a stainless steel frame) on either side and then suspended in DMEM with 10% FCS. One A-frame was connected to a fixed point in the CFM while the other is connected to a transducer. Real time contractile force generated (1 per second) and cellular response were recorded over 24 hours (at 37 ^{o}C, 5% CO^2).

RESULTS: Normal Outer SMC's generated highest peak force (mean 450 dynes). Hypoxic outer SMC's showed a significant ($p<0.005$) decrease in their peak force generation (mean 190 dynes). Normal Inner SMC's generated lower peak force compared to normal outer SMC's (mean 320 dynes to 450 dynes (approximately 30% decrease). Hypoxic inner SMC's also demonstrated a slight decrease in their contractile ability (mean 290 dynes compared to 390 dynes (approximately 25% decrease). Using agonist U46619 we demonstrated a significant increase in the contraction force generated, while an antagonist SNP, a SMC relaxant, resulted in significant loss of the contraction ability of these cells.

DISCUSSION AND CONCLUSIONS: We demonstrated that SMC's derived from the normal outer layer of pulmonary vessels generate a significantly greater peak force compared to SMC's from the inner layers of these vessels. However on exposure to hypoxia, the outer SMCs, had a significant reduction in their contraction capabilities. Hypoxia had no effect on contraction of inner wall SMC's by comparison. The cells also showed an appropriate response when stimulated with known agonists and antagonists. This has previously been demonstrated in 2D or whole tissue and validates the 3D model used in our study. Hypoxia induced hypertension causes the outer SMCs to lose their tone maintaining capabilities in vivo, a feature of pulmonary hypertensive vessels. They also lay down significant matrix, which increase vessel wall stiffness. Our findings clearly demonstrate significant differences in peak force generation and contraction between SMC's resident in the inner and outer walls of pulmonary arteries. The pulmonary arteries need to be structurally and mechanically stable for function. Our findings have implications for functional Tissue Engineering of blood vessel's as different layers of SMC's will need to be engineered or sourced to generated specific range of force and contraction within a mechanically stable construct.

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Cord Blood Derived Endothelial Progenitor Cells for Vascular Tissue Engineering: Enhanced Knowledge of Functional Properties

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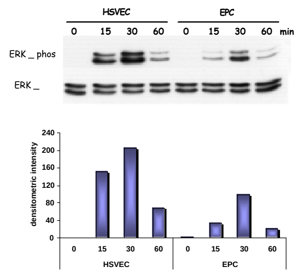
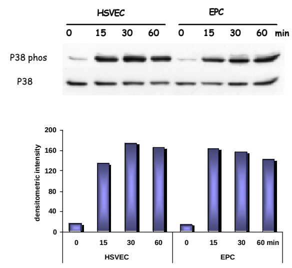
INTRODUCTION: Tissue engineering of small diameter blood vessel substitute for use in femoro-popliteal and coronary bypasses surgery is still a challenge today. Because autologous grafts are not always available, a shift in the focus of research towards reconstructing the endothelial cell (EC) living of synthetic vascular prosthesis wall was an innovation designed to improve patency rates. However, the procedure of obtaining ECs from the patient has disadvantages and limits. Thus, alternative sources of ECs, such as endothelial progenitor cells (EPCs) derived from umbilical cord blood have perspectives for therapeutic applications among which cardiovascular tissue engineering. Before envisaging the use of EPCs in such a way, it is first essential to investigate their in vitro behavior compared with mature vessel wall cells. The aim of our study was thus to explore features of EPCs : tissue factor (TF) biological activity and mitogen – activated protein kinase (MAPK) phosphorylation after activation.

METHODS: CD34+ mononuclear cells were isolated from cord blood by a magnetic beads separation, plated onto gelatin-coated wells and cultured under endothelial conditions [1]. Their EC characterization was assessed by CD31, von Willebrand factor (vWF), VE-cadherin, KDR and Flt1, dil-Ac-LDL, using immunocytochemistry and flow cytometry. For the comparison primary human saphenous vein endothelial cells (HSVEC) were isolated from vein remnants provided by a cardiovascular surgery department, harvested [2] and grown in a complete culture medium. The ECs were activated i) in static conditions with IL-1α for measuring MAPK phosphorylation (western blot analysis of cell lysates according to [3]) and TF activity (EC-associated procoagulant activity (PCA) was determined in a one-stage clotting assay by the acceleration of clotting time of recalcified normal citrated platelet – poor plasma); ii) by fluid shear stress (parallel flow chamber, 16 dynes/cm^2) followed by MAPK phosphorylation.

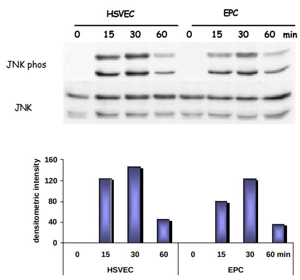
RESULTS: CD34+ mononuclear cells differentiate into cells with a typical endothelial phenotype: ECs derived from CD34+ cells are positive for all endothelial markers. IL-1α

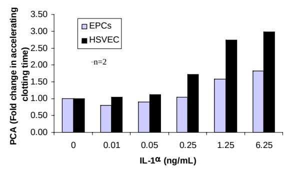
provoked on both cell types MAPK phosphorylation (Fig1-A, B, C). Stimulation with IL-1α generated a dose-dependent PCA response as compared to unstimulated cells (Fold change in accelerating clotting time): fig 2. Shear stress activated p38 phosphorylation after 10 min of





stimulus in both cell types, followed by a decrease





at 30 min.

Fig. 1-A *Fig. 1-B*

Fig. 1-C *Fig. 2*

DISCUSSION & CONCLUSIONS: This study is the first to check PCA on EPCs as well as MAPK participation in transmitting hemodynamic forces to cytoplasmic pathways. Whether shear stress attenuates IL-1α - induced TF expression on EPC surface is in progress. Cord blood derived EPCs are known for exceptional growth characteristics [1,4] and demonstrate phenotype of ECs. EPCs seem to be a promising cell source with regard to vascular tissue engineering.

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Current Developments in Cell-Based Cartilage Repair Systems

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An review of the literature provides no evidence so far for normal regeneration of hyaline cartilage in animal cartilage repair models and still today's treatments for cartilage resurfacing are less than satisfactory, and rarely restore full function or return the tissue to its native normal state. However, cell biologists, bioengineers and surgeons work closely together developing biomedical orthopaedics with a combined knowledge of using biocompatible, biomimetic, biomechanic suitable scaffolds seeded with chondrogeneic cells and loaded with bioactive molecules that promote time relapsed cellular differentiation and/or maturation.

Subsequently, we will see more and more of different cell-containing resorbable scaffolds to be used for arthroscopic implantation. Some scaffolds with cells cultured for several weeks (mature grafts) while other just seeded with cells 1-2 days prior to surgery (immature grafts) or even just at implantation time. The cartilage repair technology with in vitro expanded autologous chondrocytes needs to be further improved. The maintenance of the original phenotype by isolated chondrocytes grown in vitro is an important requisite for their use and handling in a future transarthroscopic technique in articular cartilage resurfacing. The future research in cartilage repair will be directed more and more between biology and materials science working with matrices containing growth factors guiding the implanted chondrogeneic cells to produce a restoration of the injured cartilage, as near as possible a full regeneration.

Research in regenerative biology involves the cell and molecular biology, developmental cell biology, immunology, and polymer chemistry. This new direction in medicine will use three strategies: transplantation of cells to form new tissue in the transplant site, implantation of bio artificial tissues constructed in vitro, and induction of regeneration in vivo from healthy tissues next to an injury [41].

However, regarding the future for cartilage repair in the new century, the idea is to transplant stem/progenitor cells, or their differentiated products, into a cartilage lesion site where they may form new tissue, or the cells could be used to construct a bio artificial tissue in vitro to replace the original tissue or organ. Bio artificial tissues are made by seeding stem or differentiated cells into a natural or artificial biomaterial scaffold shaped in the appropriate form, then implanting or pasting the construct into the defects of the damaged cartilage. Theoretically, the use of stem cells is preferable to the use of differentiated cells harvested directly from a donor because stem cells have the potential for unlimited growth and a rich supply. Such so-called uncommitted cells are capable of a broad range of chondrogeneic expression and could provide a regenerative tissue that recreates the embryonic lineage transitions originally involved in joint tissue formation. However, the recent research described in this talk shows that the use of true committed chondrocytes is still reasonable but more research is needed to know how to make use of them in a more efficient way.

The presentation will include a discussion on the actural state of cartilage repair and speculations for the future cartilage tissue engineering with cells in scaffolds, biocomposites and/or hybrid-semi-biological approaches.

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ATDC5: an Ideal Cell Line for Development of Tissue Engineering Strategies Aimed at Cartilage Generation

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INTRODUCTION: Pivotal in the advancement of clinical approaches for cartilage regeneration will be the development of robust models of cartilage formation, which can also aid in delineating the steps of chondrogenesis. This study was therefore aimed at engineering facile, reproducible three-dimensional (3D) models of cartilage generation utilising ATDC5 cells, a murine chondrocytic cell line widely used as a monolayer culture system to study chondrogenic differentiation, in comparison to the clinically suitable options of human articular chondrocytes and adult mesenchymal stem cells from human bone marrow.

METHODS: Femoral heads and bone marrow samples from patients undergoing routine hip replacement surgeries were used to isolate articular chondrocytes and Stro-1+ mesenchymal stem cells respectively. Proliferating ATDC5 cells, confluent human articular chondrocytes and Stro-1+ cells, grown as monolayer cultures, were harvested for micromass pellet cultures under chondrogenic conditions (10 ng/ml TGF-β3, 1X ITS, 10^{-8}M dexamethasone, 100 μM ascorbate-2-phosphate) and dynamic seeding onto polyglycolic acid (PGA) fleece within a 'high aspect ratio vessel' rotating bioreactor. In addition, micromass pellet cultures of ATDC5 cells were harvested at 7, 14, 21 and 28 days to study the process of chondrogenic differentiation in response to insulin and TGF-β3. After a culture period ranging between 21 and 28 days, explants were analysed for chondrogenic differentiation by histology (Alcian blue/ Sirius red {A/S}, Safranin O and Alkaline phosphatase {ALP} staining), immunohistochemistry and RT-PCR, for protein and gene expression of typical chondrogenic markers – sox-9, aggrecan and type II collagen.

RESULTS: Chondrogenic differentiation, evident by chondrocytes expressing typical chondrogenic genes and proteins, and lodged in distinct lacunae embedded in a cartilaginous matrix of proteoglycans and type II collagen, was observed in ATDC5 and articular chondrocyte pellets at 21 days, and at 28 days in pellets of Stro-1+ cells. The ATDC5 pellet culture time-course illustrated a gradual progression from an aggregation of cells at day 7, to the initiation of matrix synthesis and development of chondrocytic phenotype at day 14, followed by differentiation as pre-hypertrophic chondrocytes synthesizing proteoglycan and type II collagen-rich matrix at day 21 and maintenance of the pre-hypertrophic phenotype in response to TGF-β3 at day 28 (Fig. 1). Explants of ATDC5 cells cultured on PGA fleece in the bioreactor for 21 days were reminiscent of cartilaginous structures composed of numerous pre-hypertrophic chondrocytes, staining for typical chondrocytic proteins, lodged in distinct lacunae embedded in proteoglycan and type II collagen matrix (Fig. 2). In comparison, 21-day articular chondrocyte and Stro-1+ explants exhibited a mesh-work of PGA fibres supporting chondrocytic cells, in combination with discrete islands of chondrocytic cells embedded in proteoglycan-rich matrix.

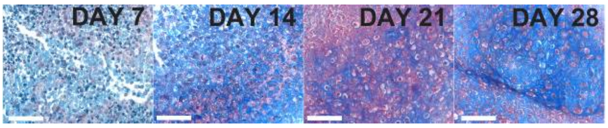

Fig. 1: Chondrogenic differentiation in A/S-stained ATDC5 pellets. Scale bar 100 μm

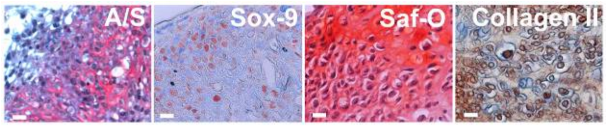

Fig. 2: Chondrogenic differentiation in tissue engineered ATDC5 constructs. Scale bar 20 μm

DISCUSSION & CONCLUSIONS: The study has highlighted suitability of ATDC5 cells in 3D environs, in comparison to human primary cells, to delineate the steps of chondrogenic differentiation and generate cartilaginous structures exhibiting morphology, gene and protein expression profiles reflective of the *in vivo* scenario. Although cell lines cannot replace human primary cell models, our study has shown that they can furnish important information concerning seeding densities, time frames, biocompatibility and judicious use of growth factors and differentiating agents, thereby significantly reducing time and cost associated with the tissue engineering of primary human cells.

ACKNOWLEDGEMENTS: We would like to thank the BBSRC for fellowship support to R. Tare and research support to R.O.C. Oreffo.

Mechanical Compression Activates Chondrocyte Calcium Signalling in a Cycle Dependent Manner Involving the Release of ATP

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INTRODUCTION: The process of mechanotransduction is essential for the health and homeostasis of the articular cartilage. However, the underlying signalling pathways are poorly understood. This study examines the influence of cyclic mechanical compression on chondrocyte Ca^{2+} signalling and the involvement of mechanosensitive release of cellular ATP.

METHODS: The study used a well-characterised model consisting of isolated bovine articular chondrocytes cultured within agarose constructs [1]. After 24hrs in culture, constructs were incubated in Fluo-4 AM (60min, 5µM; Molecular Probes) and mounted in a custom made compression rig [1] on a confocal microscope (Perkin Elmer). Cyclic compression at 1Hz between 0% and 10% strain was applied for 1, 10, 100 or 300 cycles. This was followed by a 5min period in which images of Fluo-4 labelled cells, approximately 20 per field of view, were captured every 4sec using a x20 objective. Separate constructs were held at 10% static compression and subjected to an identical 5min imaging period in the compressed state. Control constructs remained unstrained. In further studies, constructs were bathed in apyrase (10 units/ml) during 1Hz cyclic compression for 10 cycles and throughout the subsequent 5min imaging period. Unstrained controls were also bathed in apyrase. Student t-tests ($p<0.05$) were used to compare the percentage of cells showing Ca^{2+} transients in samples of individual constructs (n=8-15).

RESULTS AND DISCUSSION: In unstrained agarose constructs, a sub-population of approximately 50% of cells exhibited spontaneous Ca^{2+} transients. Static 10% compression produced no change in Ca^{2+} signalling. Previous studies have shown increased signalling at 20% compression [1] indicating that the effect may be strain magnitude dependent. Cyclic compression for 1, 10 or 100 cycles was followed by a statistically significant increase in the percentage of cells exhibiting Ca^{2+} transients (Fig 1). However, 10 or 100 cycles did not induce additional responses compared to 1 cycle, suggesting a redundancy of signal transduction. The stimulatory effect was maintained for up to 5 minutes after the end of compression suggesting the involvement of a paracrine - autocrine mechanism. This was confirmed by the finding that the ATP scavenger, apyrase, abolished the up regulation of Ca^{2+} signalling produced by cyclic compression. After 300 cycles, Ca^{2+} signalling was not significantly different to that in unstrained controls, possibly associated with ATP receptor desensitisation.

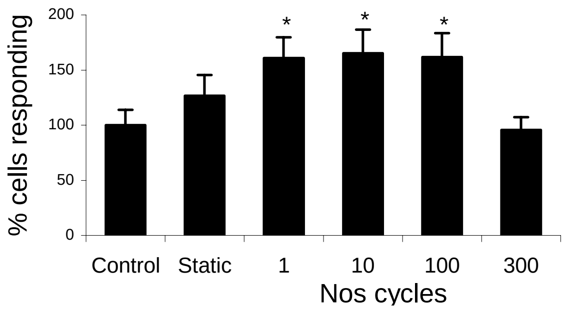

Fig. 1. Percentage of chondrocytes exhibiting Ca^{2+} transients over a 5 min period either during static compression or following 1Hz cyclic compression. Mean values are normalized to unstrained controls. Error bars indicate SEM, n=6-10 constructs.

CONCLUSION: In conclusion, cyclic compression activates Ca^{2+} signalling via mechanosensitive release of ATP as part of a chondrocyte mechanotransduction pathway. Furthermore, Ca^{2+} signalling is modulated by the number of cycles or duration of cyclic compression. This provides a potential mechanism through which chondrocytes may differentiate between different loading regimes.

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Human Osteoarthritic Chondrocytes are Capable of Forming Cartilage Matrix on Hyalograft C under Hypoxic Conditions

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INTRODUCTION: Articular cartilage consists of chondrocytes surrounded by an extracellular matrix (ECM) made of collagens (mainly collagen type II) and proteoglycans. Chondrocytes isolated from tissue can be expanded in culture, but rapidly lose their round morphology and chondrogenic capacity. They stop synthesizing collagen type II and dedifferentiate into fibroblastic-like cells with high collagen type I expression. Culture conditions such as 3-D culture systems, low oxygen tension and anabolic growth factors are parameters that have been shown to partially restore the chondrogenic phenotype of dedifferentiated cells. This study examined the potential of expanded aged human osteoarthritic chondrocytes to produce a cartilage ECM by combining some of the above parameters. Hyalograft-C (FIDIA Advanced Biopolymers, Italy) was used as a culture template for the chondrocytes in the presence of growth factors in either normal or hypoxic conditions.

METHODS: Chondrocytes were enzymatically isolated from osteoarthritic knee cartilage from joint replacement surgery. Cells were plated at a density of 20,000 cells/cm^2 and expanded for 2 passages (DMEM supplemented with 10% FCS) before seeding on 1cm^2 Hyalograft C at densities of $2.5\text{x}10^5$, $5\text{x}10^5$, $1\text{x}10^6$ and $2\text{x}10^6$. Constructs were cultured under normal (21% O_2) or hypoxic (5% O_2) conditions in chondrogenic medium (DMEM, 10% FCS, ascorbic acid , dexamethasone ITS+1 and TGFb-3). Samples were analysed at 7, 14 and 21 days for gene expression by quantitative RT-PCR and at 21 days for histology (safranin-O staining) and immunochemistry (collagen type I and type II).

RESULTS: Histological evaluation of the Hyalograft/cell constructs at day 21 revealed evenly spread, attached chondrocytes at all densities both in hypoxia and in normoxia. Constructs seeded at $2\text{x}10^6$cells/cm^2 cultured in hypoxia had a hyaline-like morphology, whilst normoxic cultures appeared more fibrous (*Fig. 1*). Furthermore, hypoxic cultures exhibited higher collagen type II (*Fig. 1*) and lower collagen type I immunostaining in their ECM. High seeding density combined with hypoxic culture for 21 days maximised the expression of the col2a1 gene, while levels of col1a1 were significantly suppressed. SOX9 mRNA expression levels were not altered under any of the conditions.

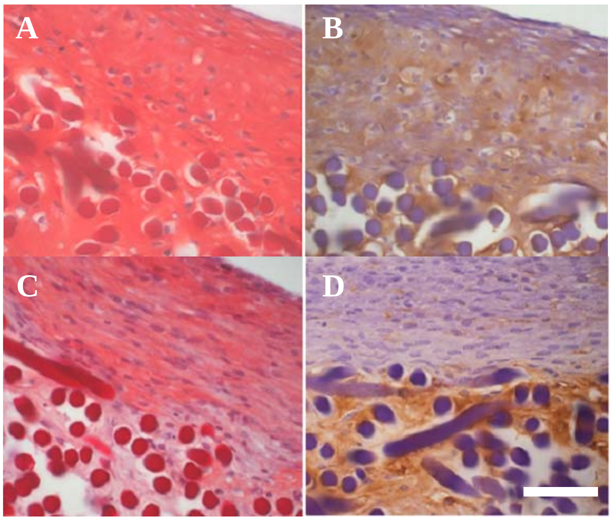

Fig. 1: Hyalograft/chondrocyte constructs (Day 21) stained with safranin-O (A, C) or immunolabelled for collagen type II (brown) (B, D) in hypoxic (A, B) and normoxic (C, D) conditions. All sections were counterstained with haematoxylin. Scale bar = 100um

DISCUSSION: Previous studies have shown that embryonic chondrocytes expanded on biomaterials and bovine chondrocytes cultured using bioreactors were capable of regaining their chondrogenic phenotype and produced a cartilagenous ECM. In this study the results show that the potential of expanded primary aged human osteoarthritic chondrocytes to produce hyaline-like ECM is greatly enhanced by hypoxia. Together with high cell seeding density, hypoxia played an important role in controlling the gene expression and ECM production of the primary osteoarthritic chondrocytes. The ECM produced was rich in glycosaminoglycan and collagen type II and this appeared to occur independently of any change in SOX9 gene expression.

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A Non-Viral Gene Delivery System for Tissue Engineering

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INTRODUCTION: The combination of gene delivery with tissue engineering can offer a new approach to growing new tissue. A plasmid DNA can be incorporated into a polymer scaffold and a sustained release may lead to the transfection of a large number of cells which can enhance tissue development[1]. The success of gene therapy is dependent on the efficient and safe delivery of the encoding DNA. In this study DNA was complexed with a polyamidoamine cationic polymer of the methylene-bisacrylamide/dimethylethylenediamine (MBA-DMEDA) series and then the non-viral gene delivery device is embedded in a scaffold. Both freeze dried complex & polymer were placed in an autoclave and the bioactive scaffold was made by using supercritical fluid technology. Assessment of transfection & physico-chemical properties was carried out before and after lyophilising, in order to investigate whether the delicate 3-D architecture of the complex is preserved.

METHODS: The cationic polymer was synthesised following the method described by Ferruti[2]. Gel electrophoresis & Photon Correlation Spectroscopy (PCS) were used to look at the physico-chemical properties. Transfection activities of the complexes were assessed on A549 cells using a plasmid that contains the firefly luciferase reporter gene (gWIZLuc). Luciferase detection was performed 48h after transfection. Recoverable cellular protein was measured using the Bradford assay and results were expressed as Arbitrary Light Units (ALU) per mg recoverable cellular protein.

RESULTS: The results of the transfection activity are displayed in Fig 1. Transfection levels of the complex MH1 (poly(MBA-DMEDA)) – gWIZLuc in a 5:1 ratio were greater than the negative control (naked DNA). Levels were still lower than the commercial cationic lipid Lipofectamine®. Following freeze-drying a significant loss of transfection efficiency was observed (MH1 0%). Increasing concentrations (5% - 15% w/v) of the cryoprotectant trehalose were added to the complex-solution prior to freeze-drying[3]. After re-hydration transfection activities were measured. Transfection levels could be restored.

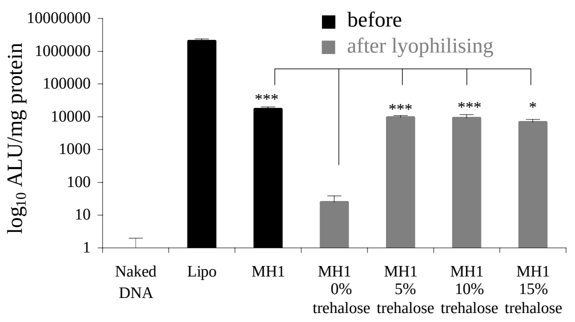

*Fig. 1: Level of luciferase activity in A549 cells 48h after exposure to MH1/gWIZLuc complexes. (Statistical significance using the Tukey–Kramer multiple comparison post-test ***p<0.001, * p<0.05)*

The physico-chemical properties were investigated with PCS & gel electrophoresis and correlated to transfection efficiency. The average particle size of the polymer-DNA complex is ±120nm (Fig 2). After freeze-drying a significant increase in size was observed. The size following lyophilising could be reduced by the addition of trehalose.

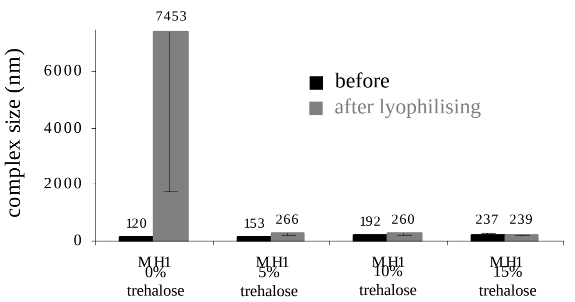

Fig. 2: Particle diameter of MH1/plasmid complexes before & after lyophilising with or without trehalose

DISCUSSION & CONCLUSIONS: Dried complexes that exhibited good transfection activity upon re-hydration had sizes comparable to non-lyophilised controls. The polymer/DNA complex required trehalose, a cryoprotectant, to maintain efficient levels of transfection after lyophilisation.

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ACKNOWLEDGEMENTS: The authors would like to thank the BBSRC for funding.

European Cells and Materials Vol. 10. Suppl. 2, 2005 (page 26) ISSN 1473-2262

In Vivo Gene Delivery and Transfection with Biomimetic Biomaterials

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INTRODUCTION: The ability to deliver a specific gene to cells *in vivo* without toxicity and with high transfection efficiency is a common goal for many tissue engineers. For bone tissue engineering, the ideal construct contains the patient's own stem cells, a bio-degradable scaffold for support and bone conduction, and osteoinductive growth factors. We have previously reported gene transfection in a polysaccharide capsule(1). Here we present demonstrations of plasmid release, functionality and high transfection efficiency in a bone defect model using biomimetic constructs.

METHODS: Plasmid DNA expressing *E. coli* ß-galactosidase gene was entrapped in porous calcium carbonate (vaterite) microspheres. Release was measured by OD_{260}.

Plasmid was added to the water phase of the microemulsion prior to synthesis as described by Walsh et al.(2). Vaterite spheres were prepared with entrapped GFP expression vector and pelleted with CHO K1 cells for 7 days. Cell pellets were disaggregated, fixed in 4% paraformaldehyde, mounted in Movelat and viewed by confocal laser scanning microscope.

Vaterite microspheres with entrapped *E.coli lacZ* expression vector were centrifuged with CHO K1 cells and sodium alginate, loaded onto a chick bone defect and sealed with drops of chitosan. Chick bones (n=3) were implanted subcutaneously into nude mice. At 7 days the bones were harvested and fixed for immunochemistry.

RESULTS: Plasmid release was followed over 7 days and showed that from 4 days significant amounts of plasmid are released (**Figure 1**)

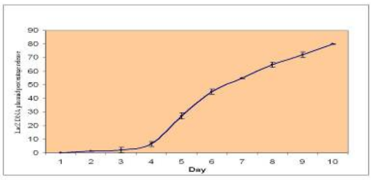

Figure 1*: Cumulative release profile of plasmid DNA. [n=3];*

Demonstration that released plasmid is intact, competent and able to transfect cells was achieved by pelleting spheres and CHO K1 cells (**Figure 2a**)

Histochemical analysis of *in vivo* chick bone defects filled with vaterite, cells and plasmid shows significant uptake of plasmid as shown by strong staining for β-gal (**Figure 2b**).

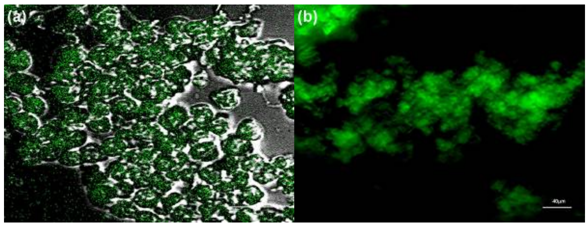

Figure 2*: (a) CHO cells in pellet culture expressing GFP; (b) Immunostaining with* β*-gal mAb, 2nd antibody conjugated to FITC and imaged by confocal laser scanning microscopy*

DISCUSSION & CONCLUSIONS: Plasmid DNA was entrapped within vaterite microspheres and released over 7 days. Vaterite is a labile polymorph of calcium carbonate and in culture medium microspheres will degrade and transform to calcite rapidly. We believe dissolution of the outer structure occurs with internal structural fracture, thereby releasing DNA integrated within crystals and DNA entrapped between crystals respectively. Within a cell pellet of CHO K1 cells, high levels of GFP expression are observed; GFP expression was not observed in monolayer culture with DNA microspheres overlaying CHO K1 cells at a similar time point (data not shown). This study provides further evidence for the efficacy of biomimetic approaches in gene delivery.

In a chick bone defect, CHO K1 cells are viable, have been transfected, and express lacZ. The applicability and potential of this approach is significant and evaluation of primary cells combined with bone specific growth factor genes is currently underway in this group

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ACKNOWLEDGEMENTS: We thank the BBRSC for funding this work. We thank Graham Packham for provision of pcDNA3.1(-)myc-his-lacz.

The Role of Computational Modeling in Tissue Engineering

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INTRODUCTION: Usually in tissue engineering the following phases can be distinguished: First, cells are isolated from the patient and expanded in culture to obtain sufficient numbers. Then the cells are seeded in a scaffold. Subsequently, during culture in a bioreactor, the cells are conditioned to synthesize extra-cellular matrix components, resulting in neo-tissue formation. Eventually, the newly formed construct has to acquire adequate mechanical properties, in order to be re-implanted into the patient.

However, in general the mechanical properties of constructs do not meet these functional requirements. In addition there is a definite lack of control over the functional development of the tissue. Current research is mainly characterized by a strong experimental basis. The use of computational methods is relatively limited, while modelling can potentially provide an important contribution to the optimization of bioreactors and culture protocols. In addition, modelling can prove useful in the reduction of the number of experiments and the interpretation of the results.

A computational approach will be proposed that enables the modelling of the functional development of tissue engineered material. The proposed approach was developed for articular cartilage, but can easily be applied to other tissues.

METHODS: In particular, use was made of the finite element method. The method has been applied to establish relationships between mechanical stimulation by dynamic compression and transport, of for example nutrients, within cartilage constructs. It was shown that compression induced fluid flow can affect the transport of large solutes but not that of small solutes, which has implications for matrix synthesis.

A microstructural homogenization approach was used to determine how the synthesis of extra-cellular matrix at the cellular level, translates in the evolving mechanical properties of the newly formed cartilage as a whole. The results indicate that the matrix distribution at the cellular level may be of less importance than its molecular organization.

With respect to the kinetics of cartilage formation, it is known that cellular energy metabolism is closely associated with the synthesis of extra-cellular matrix. Therefore, using the model, the cellular uptake of glucose and oxygen and the production of lactate were characterized on the basis of experimental data. It was shown that cellular uptake was influenced by both the initial glucose concentration in the culture medium and the cell density.

Matrix synthesis and proliferation can be stimulated in an uncoupled manner, by applying different intervals of dynamic compression. In order to interpret and predict cell behaviour, models were developed for the temporal regulation of chondrocyte proliferation and biosynthesis, in response to varying dynamic compression regimens. The models were able to provide a reasonable overall representation of experimental results for different loading regimens. However, for specific loading cases the predictive value was limited.

Subsequently, cellular uptake data were used to predict, numerically, the nutrient supply in different bioreactor setups and to evaluate the effect of mixing, perfusion and geometry. The results indicate that transport limitations are not insurmountable, providing that the bioreactor environment is well homogenized and oxygenated.

At the present stage, additional identification and quantification of chondrocyte behaviour is necessary, with respect to utilization, biosynthesis and mechanotransduction. In addition, criteria with respect to matrix synthesis have to be established.

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[2]B.G. Sengers, C.C. van Donkelaar, C.W.J. Oomens, F.P.T. Baaijens (2004) *The local matrix distribution and the functional development of tissue engineered cartilage, a finite element study,* Ann Biomed Eng **32**(12), 1718-1727.

In vivo measurements and theoretical modelling of oxygen partial pressure in outer layers of human skin

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INTRODUCTION: Tissue oxygenation is a key factor ensuring normal cell functions and viability. Oxygen sensitive microelectrodes are developed and applied to investigate the spatial variation of pO_2 in superficial layers of human skin in relation to local microvessels. The first part of the study focuses on *in vivo* measurements of spatial and temporal variations of pO_2 following localized ischaemia-reperfusion [1]. The second part of the study gives insight into oxygen transport mechanism using a three dimensional theoretical model that represents cutaneous microcirculation and multi layer structure of the skin [2].

METHODS: Microelectrodes are made using platinum-iridium wire (ϕ=25μm) electrochemically etched to a slender profile with tip diameter less than 1μm. The tip of the finished electrodes is approximately 3–8μm in diameter, giving a theoretical spatial resolution of 20–50μm. Finger nail fold is used for measurement and a layer of oxygen free paraffin oil is applied on top of the skin. Micromanipulator is used to guide the electrode under an optical microscope. pO_2 at different depths and at different locations in relate to papillary loops are recorded. Local ischaemia is introduced by compression of skin using special shaped microelectrodes, and temporal changes in skin pO_2 are measured.

Furthermore, a theoretical model based on anatomical arrangement of the skin is built which takes into consideration of 3-dimensional multi-layered structure of the skin and cutaneous microcirculation. Tissue oxygen uptake from the blood and from the air is considered. pO_2 distribution in skin is simulated based on mass conservation equations and the Hill equation for the nonlinear oxygen dissociation from hemoglobin.

RESULTS: pO_2 is found to increase from the surface of the skin to the depth just above the sub-papillary plexus (Fig. 1). Temporal decay in pO_2 following tissue compression and rise in pO_2 following pressure release can be described using mono-exponential functions. The time constant for the exponential decay is consistently greater than that for the exponential rises. Significant decrease in pO_2 is seen following arrest of the local micro-circulation, and when blood flow is restored, pO_2 increases to 23% greater than the control (Fig. 2).

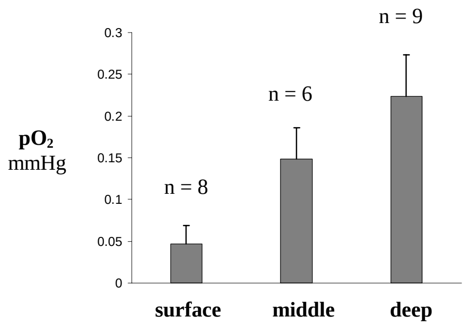

Figure 1. Changes in pO_2 with depth into the skin.

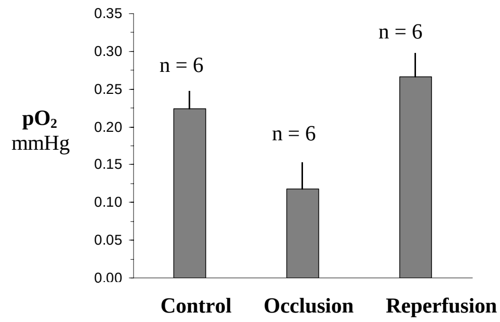

Figure 2. pO_2 in localized ischaemia-reperfusion.

Predictions from the numerical model confirm above experimental findings. Furthermore, the model gives quantitative insight into relative contribution to tissue oxygen uptake from the blood and the air under different microcirculatory conditions.

DISCUSSION & CONCLUSIONS: The difference in pO_2 change with the time following tissue compression and pressure release reveals different dynamic mechanisms involved in the two transient phases. The elevated steady state pO_2 following reperfusion indicates localised reactive hyperaemia. Theoretical model provides quantitative insight into oxygen uptake by the skin and tissue pO_2 distribution.

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[1] Wang, et al (2003) *J Physiol*, 549: 855-863.

[2] Wang (2005) *Microcirculation*, 12: 195-207.

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Mechanical Behaviour of Primary Human Skeletal Muscle Cells and Isolated Non-Myogenic Cells Within a 3D- Construct

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INTRODUCTION: An understanding of the mechanical and mechano-molecular responses that occur during the differentiation of primary human myoblasts in 3D-culture is critical for understanding growth and progress towards producing a tissue-engineered muscle construct[1]. In an effort to characterise the mechanical behaviour of myoblasts within a 3D construct, previous work by Cheema et al. (2003) utilised a cell line of immortalised skeletal myoblasts (c2c12 cells). The aim of this study was to determine the mechanical response of primary human skeletal muscle (PHSM) cells. Further, to isolate myoblasts from PHSM cells and establish the force generated by those cells.

METHODS:

Masseter muscle biopsies were obtained from consented healthy patients undergoing orthognathic surgery at the Eastman Dental institute. The expanded PHSM cells were subsequently defined as a co-culture of CD56+ve (adult human myoblasts) and CD56-ve cells. Primary human myoblasts were isolated using microbead-immunomagnetic selection (CD56+ve). The efficacy of this technique was verified by immunostaining. The co culture of primary human muscle cells, isolated CD56+ve cells and CD56-ve cells respectively were seeded in collagen constructs (1million/ml) and the force generated by the cells was quantified over 24 hours.

RESULTS:

The mechanical response of PHSM cells in a 3D construct reveals an average peak force generation of 50 dynes (*Fig.1a.*). PHSM myoblasts (CD56+ve) and non myogenic (CD56-ve cells) were successfully isolated (*Fig. 2.*) and the non-myogenic cells also generated an average peak force of 50 dynes over 24 hours (*Fig. 1b.*).

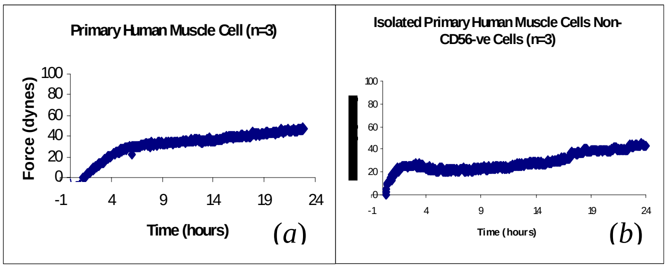

Fig. 1: Contraction profiles of (a) primary human skeletal muscle cells and (b) isolated non-myogenic cells.

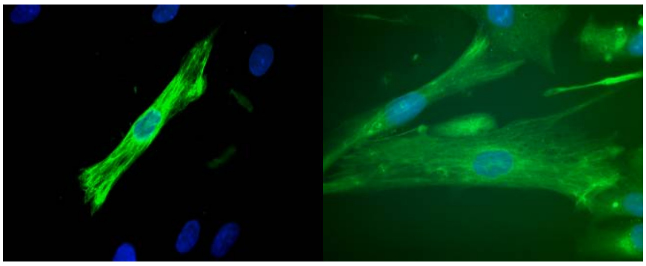

Fig. 2: Immunostaining confirming the isolation of human myoblasts (CD56+ cells) from primary human skeletal muscle (green immunostaining).

DISCUSSION & CONCLUSIONS:
We have shown that there are no differences in force generation and contraction of 3D collagen constructs by PHSM's and CD56-ve cells. The mechanical characterisation of isolated primary human CD56+ cells and future differentiation of these cells into myotubes within a 3D construct will further understanding of muscle growth and regeneration and aid in defining parameters for functional muscle engineering.

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Cartilage Tissue Engineering Using Modified Poly-(L-lactide) Microspheres in an Intermittent Stirred Flow Bioreactor under Hypoxic Conditions

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INTRODUCTION: The successful production of 3-D cartilage tissue is often compromised by the formation of a necrotic core within the supporting biomaterial scaffold. This is generally attributable to mass transfer limitations of nutrients and gases to and from the scaffold. We have investigated using a combination of modified bioresorbable microspheres and chondrocytes initially freely suspended in an intermittent stirred flow bioreactor to allow the formation of 3-D nascent cartilage aggregates. The effect of reduced oxygen tension on chondrocyte proliferation and matrix deposition within the aggregates was also studied.

METHODS: Three types of PLLA microspheres were used in this study; (1) Virgin PLLA spheres, (2) PLLA spheres surface modified with an RGD motif and (3) PLLA spheres containing time release growth factor TGF-β1. These were compared with commercially available polystyrene (PS) microspheres as a control material (Nunc Biosilon). Pendulum stirred bioreactors (Techne, USA) were used with media volumes of 125ml and, for each microsphere type, operated with intermittent flow (50rpm for 5mins, static for 15 mins) for 7 and 14 days at 21% (normoxic) and 10% (hypoxic) oxygen concentrations in a controlled environment chamber. Human articular chondrocytes (OUMS-127) were used at a concentration of 2E+05 cells/ml in all experiments. Samples of engineered tissue removed from the bioreactors at 7 and 14 days were assessed qualitatively in terms of size, morphology (SEM) and viability using a LIVE/DEAD assay (L-3224, Molecular Probes). Quantitative measurements were made for proliferation (LDH assay), s-GAG content (Blyscan assay) and collagen content (SirCol assay). Cell proliferation potential and expression of selected chondrogenic markers were quantified using RT-PCR and SYBR green (Biorad U.K.). Expressions of all markers were normalised to an endogenous β-actin control at each time point.

RESULTS: Representative images for cell aggregation and viability on PS spheres are shown in Fig.1 and comparative performance of alternative microsphere types are shown in Fig.2 for aggregate size and s-GAG production. RT-PCR for selected chondrogenic markers are shown in Fig. 3 for aggregates using PS spheres.

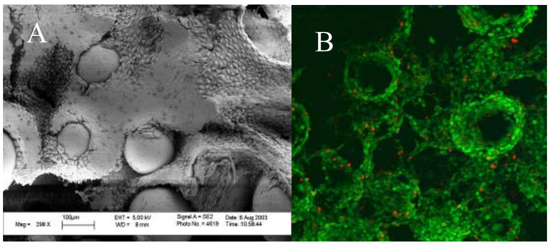

Fig. 1: PS spheres at 14d; SEM (A) and CLSM for viability (B)

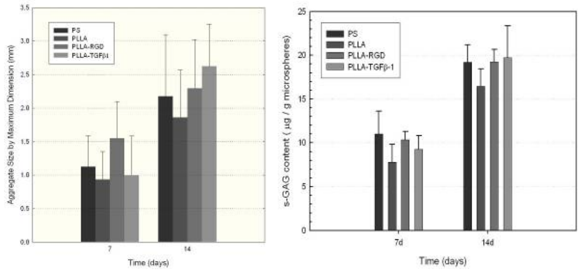

Fig. 2: Comparison of aggregate size and s-GAG production of chondrocytes grown on four types of microsphere for 7 and 14d

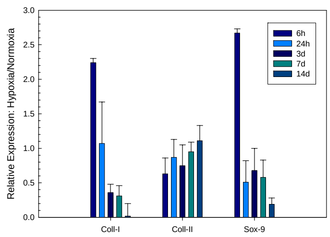

Fig 3. RT-PCR expression of Collagen types I & II and SOX-9 in hypoxic/normoxic conditions at five time points.

DISCUSSION & CONCLUSIONS: Our studies suggest that flow intermittency and hypoxia promote the formation of large viable cellular aggregates of cell seeded micropspheres. Incorporation of surface receptors and TGF-β1 with PLLA spheres is advantageous in terms of aggregate size and deposition of extracellular matrix components.

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In Situ Quantification of Subpopulation Behavior in Heterogeneous Cell Populations: Implications for Tissue Regeneration

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INTRODUCTION: Advances in quantitative molecular- and cellular-biology highlight the need for correspondingly quantitative tissue-biology. We present a novel, widely applicable and non-invasive means to quantify behavior of subpopulations in mixed-cell cultures, using adhesion patterns within mesenchymal populations as an illustration. Relationships between the underlying statistical distributions of cell areas on various surfaces are considered using a combination of graphical and nonparametric statistical analysis and it is shown that activity can be accurately quantified in tissue subpopulations smaller than 1% by cell number using this method. Presence and behavior of a number of specific mesenchymal subpopulations are outlined, and implications for the translation from cell- to tissue-biology are discussed.

METHODS: We seeded fluorescently labelled STRO-1 selected and unselected and MG63 samples on tissue culture plastic and fibronectin coated surfaces. Cells were then fixed and randomly photographed. A large number of individual cell areas were then obtained per sample using retrospective image analysis. The distributions of cell areas for each cell type and surface were investigated by calculating percentile points, which provide a simple way to elucidate structure. We then constructed Percentile-Percentile (P-P) plots, which constitute a sensitive non-parametric means to compare relationships between underlying statistical distributions in different sets of data. In particular, if two datasets have distributions which are significantly different then the P-P plot will be nonlinear with the degree of nonlinearity relating directly to the degree of distributional disparity between the two datasets. Changes in the distribution of cell areas upon surface modification were used to determine the presence and activity of subpopulations with varying responses to fibronectin. Differences in the shapes of the distributions of cell areas from different mesenchymal fractions were used to further elucidate differences in composition between them.

RESULTS: We detected the presence and activity of significant subpopulations in both STRO-1 selected and unselected mesenchymal fractions. Crucially, we identified the presence and activity of morphologically indistinct subpopulations. The continuous cell line MG63 was used as a negative control in which no subpopulations were present or active.

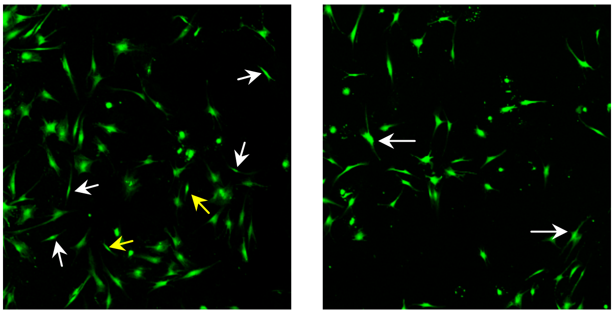

Fig. 1: Significant, morphologically distinct subpopulations are identified in both STRO-1 selected (right) and unselected (left) fractions. Yellow arrows highlight a morphologically indistinct subset of the white arrowed STRO-1$^+$ subpopulation which behave in a significantly different manner.

DISCUSSION & CONCLUSIONS*:* We present a simple method which uses fluorescence labeling in combination with image analysis and mathematical data processing to detect the presence and activity of cell subpopulations retrospectively, in a non-invasive manner. Although we consider cell area in response to surface modification, recent advances in fluorescence cell- and protein-labeling mean that this method can be used to characterize behavior using a wide variety of biochemical-, protein- or alternative phenotypic-markers within any mixed cell population. This has significant implications to advance understanding of *in situ* tissue regeneration.

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Investigation of Tenocytes Proliferation under Perfusion in Microchannels Chitosan Scaffolds by Optical Coherence Tomography

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[2]*Institute of Bioscience &Technology, Cranfield University, Silsoe, UK*

INTRODUCTION: We produced a three-dimensional environment suitable for tenocyte proliferation and subsequent production of a well organized uniaxially orientated collagen matrix, one of the key issues in tendon engineering. To encourage this production, tenocytes were stimulated by continuous fluid flow in a perfusion bioreactor. To this specific purpose, chitosan microchannels in scaffolds were designed to provide micro tubular structures where cells were in contact with a continuous laminar flow. We expected that the flow would have helped aligning cells and extracellular matrix along the channel. The structural changes occurring in the scaffolds were monitored by optical coherence tomography (OCT) given its ability to acquire 3D image in a non-destructive manner[1].

METHODS: Primary pig tenocytes were grown from pig explants obtained from the local abattoir and cultured in DMEM, supplemented with 10% FCS and 1% L-Glutamine. Chitosan gel (2%) was cast inside moulds containing needles arrays, which formed the template for the microchannels. After freeze-drying, the resulting scaffolds were microporous and possessed several microchannel parallelly orientated and regularly spaced. Different channel sizes (250 μm to 500 μm) were prepared by varying the type of needles used. $1x10^6$ cells were seeded into each scaffold. Two weeks of static culture in growth medium allowed cell proliferation on the inner surface of the microchannels. The scaffolds were then cultured in a differentiation medium (5 %FCS, 100 mg/mL acid ascorbic) and split into two groups. One group was in static culture, whereas group 2 was cultured in a perfusion bioreactor[2] with a 0.1 ml/min flow rate for one week. A bench-top OCT system equipped with a broadband light source centred at 1300 nm providing an imaging resolution at ~10 μm was utilised in this study.

RESULTS: OCT revealed clearly the microchannel structure in the chitosan scaffold (Figure 1). Cell proliferation and ECM generation changed the size and morphology of the microchannels. These changes were not only shown in OCT images, but were also verified directly by scattering intensity profile (Figure 2). The empty channel had a low intensity response due to background noise, whilst cell plus extra cellular matrix (ECM) filled channel produced a distinct scattering signal from that of chitosan. The channels subjected to perfusion exhibited broader and higher intensity peak.

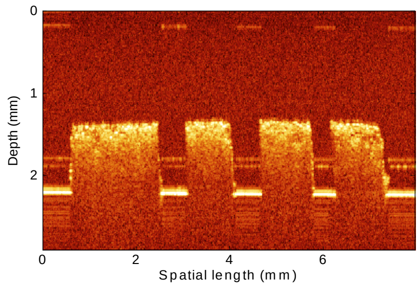

Fig. 1: OCT scan of a blank chitosan scaffold with microchannels.

DISCUSSION & CONCLUSIONS: Tenocytes proliferation and ECM formation have occurred in the microchannels, and these features were increased by continuous perfusion. The resulting microstructural variations were displayed adequately by OCT.

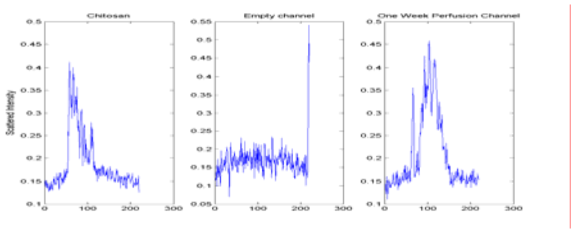

Fig. 2: Mean scattering intensity profile of chitosan scaffold (left), blank channel (middle) and cultured channel (right).

REFERENCES: [1] W. Tan, A. Sendemir-urkemez et al (2004) *Tissue Engineering* **10**:1747-56. [2]Freyria, Yang et al (2005) *Tissue Engineering* **11**: 674-684.

ACKNOWLEDGEMENTS: This work was supported by BBSRC grants BBS/B/04277 and BBS/B/04242.

Mechanical Characterisation and Stimulation of Corneal Stroma Equivalents

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[2] *Birmingham and Midland Eye Centre, City Hospital, Birmingham, GB*

INTRODUCTION: Annually there are over 2000 corneal transplant in UK. One of the problems facing the transplant services is a lack of donated corneas. Tissue engineering has the potential to be used to grow corneal tissue for transplantation. One of the major difficulties to be overcome in tissue engineering cornea is to replicate the native tissues mechanical strength. Little work has been done to characterise the mechanical properties of these corneal equivalents or to examine the effect of mechanical stimulation on them.

METHODS: Human keratocytes were suspended within a collagen solution to create corneal stroma equivalent samples. Collagen gels without cells were used as control samples. The mechanical properties of all the samples were characterised using a newly developed indentation system. This consists of a sample holder with an indentation element and an image acquisition system as shown in figure 1. The circular holder clamps the sample around its outer circumference and a ball of known weight and size was placed on top of it causing a deformation. The image acquisition system, consisting of a long focal distance objective microscope connected to a computer-linked CCD camera, records side-view images of the deformation profile from outside the incubator through a glass window. A theoretical model [1] was derived to calculate the samples mechanical properties from the deformation profile.

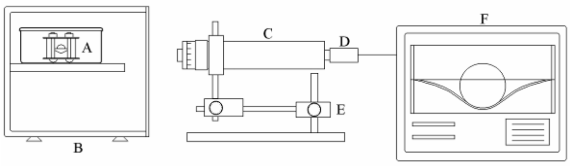

Fig. 1: Diagram of instrument system: (A) Sample holder and ball; (B) incubator at 37℃ 5% CO_2; (C) long focal microscope; (D) CCD camera; (E) X-Y translation stage; (F) image analysis system.

Even more recently we have developed a new method of mechanically stimulating the samples via the application of magnetic force. This system consists of a similar ball and holder set up as used for mechanical characterisation. A magnet connected to a translation stage is moved up and down vertically above the ball. When the magnet approached the ball, it applied an upward force to the ball thus offset partially the balls weight on the sample, reducing the sample deformation. The deformation returns to normal when the magnet was moved away from the ball. Such magnetic driving force resulted in a vertical up-down motion of the ball that stimulated the samples.

RESULTS: Values of the Young's modulus have been obtained for corneal equivalents up to 4 weeks in culture. It can be seen from figure 2 that there is little change over time in the mechanical properties of samples without cells but a small increase in the stiffness of the samples with human keratocytes. Remodelling of the collagen gel by cells may be responsible for this phenomenon. Experiments to determine the effect of mechanical stimulation on the mechanical properties on the corneal equivalents are on-going and values for the Young's modulus of these samples should be determined in the near future.

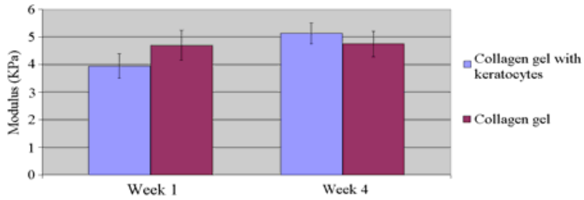

Fig. 2: Young's Modulus of collagen hydrogels after one weeks and four weeks incubation.

DISCUSSION & CONCLUSIONS: It can be seen that our novel indentation system allows non-destructive, online real-time mechanical characterisation and stimulation of corneal stroma equivalents under physiological conditions, with no risk of damage to the instrument. This new technique may provide a powerful tool to constantly monitor and stimulate engineering tissues in culturing process.

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Induction of 3-D Cell-Cell Interaction via Cell Surface Chemical Modification

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INTRODUCTION: Tissue engineering aims to regenerate or grow functional tissues in the laboratory or to enhance tissue repair within a patient ([1]). The transformation of cultured cells into 3D tissues is reliant on forming intimate cell-cell contacts and this is not readily achievable without allowing them to form randomly on polymer scaffolds. We have recently shown that sodium periodate can be used to engineer cell surface to create unnatural aldehyde groups. These can be used as chemical "handles" to modify the cell surface with biotin moieties ([2]). Then by the addition of multibinder material like avidin to modified cells would cause them to be cross-linked into 3-D aggregates. Hence, optimization of the different factors affecting cell surface engineering such as concentration and time of treatment of sodium periodate, Avidin, cell density and proof cell-cell interaction is due to specific cross linking between biotin and avidin are keys for aggregate formation. This would provide a valuable tool for tissue formation ([2]).

METHODS: 3T3 fibroblasts and L6 myoblasts were suspended and treated with various concentration of sodium periodate in the dark at 4°C for different treatment times, then biotinylated with 5mM biotin hydrazide at room temperature for 90 minutes. To assess the extent of biotinylation, cells were saturated with FITC-avidin and fluorescence measured using flow cytometry. Using optimum oxidation conditions and subsequent biotinylation, aggregate formation was studied by incubating cells in spinner flasks using different Avidin concentrations, incubation times and cell number. Resulting aggregates were imaged using phase contrast microscopy and their surface area measured using Leica Qwin software.
Secondly, the ability of our system to aggregate different cell lines due to biotin-avidin specific cross linking was studied using biotinylated and untreated 3T3 fibroblasts with preaggregated L6 myoblasts stained with green and red cell-tracker respectively then analysis with confocal microscope were done over 2 hours of mixing.

RESULTS: Our data demonstrate that cell surface oxidation by sodium periodate is a concentration-dependent process, with a maximal effect at 1mM under the conditions employed. This reaction is also very rapid, peaking by 5 minutes, and did not affect cell number or viability. Incubating biotinylated cells with Avidin resulted in the formation of large multicellular aggregates, with optimum conditions being 10µg/mL Avidin, an incubation time of 1 hour with 20x106 cells. Fig. 1 shows that using our Biotin-avidin technology has the ability to achieve 3-D cell-cell contact between cells with engineered cell surface only even after 15 minutes of mixing and continued to give a complete 3-D heterotypic aggregation.

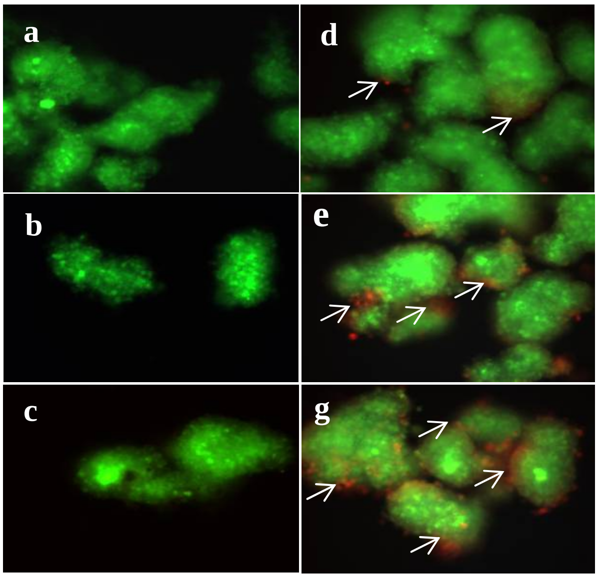

Fig. 1: Effect of biotin-avidin technology on heterotypic cells aggregation: a,b &c represent untreated 3T3 mixed with L6 aggregates after 15, 60 and 120 minutes respectively. d,e &g represent biotinylated 3T3 mixed with L6 aggregates after 15, 60 and 120 minutes respectively. Arrows shows the sites of attachments.

DISCUSSION & CONCLUSIONS: These results show that cell surface modification can be used to rapidly form 3D multicellular aggregates, with aggregate size controlled by the conditions employed. This technique has the potential to aggregate any cell type and has the potential to generate functional tissue form a population of single or two different cell types without the need to seed on to a scaffold.

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Plastic Compression of Collagen: Development and Assessment of A New Biomaterial in Nerve Repair

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INTRODUCTION: Nerve reconstruction is a surgical challenge. Current neural conduits provide sub-optimal clinical results. We have developed a device from a composite material comprising a fibronectin core and a collagen outer layer, using collagen made by a new technique called plastic compression. This collagen element has the strength to improve the mechanical properties of nerve repair devices which is an important design consideration.[1] Preliminary studies have been performed to asses the suitability of the new collagen material for use in nerve repair conduits.

METHODS: A 6ml gel was made from type I rat tail collagen (First Link), substituted with 10xMEM and 10 % EBSS. This was neutralized with NaOH and left to set for 30 minutes in a rectangular metal mould. The gel was removed from the mould and compressed, leaving a flat sheet of collagen. This material can be rolled to create an implantable conduit. In order to investigate the ability of the compressed collagen to support neuronal growth, flat sheets were used in this experiment.

To assess stability, a 6ml flat sheet was divided and incubated at 4˚C and 37˚C in EBSS for 2 weeks. At the end of the incubation period, the material was degraded with collagenase to quantify the protein remaining in the material. The rate of protein release from the material into the solution was assessed using a BCA assay.

Dissociated dorsal root ganglia (DRG) from 250g Sprague Dawley rats were seeded upon the surface of the collagen material and maintained in culture for 4 days at 37˚C, 5% CO_2. Controls were equivalent cultures grown on shear aggregated fibronectin mats, an established substrate for neuronal growth.[2]

The constructs were fixed in 4% paraformaldehyde, then mouse anti-β_3 tubulin, (1:300 for 1h, Sigma) was used to detect neurones. The secondary antibody was TRITC conjugated anti-mouse IgG, (1:100, 45 min). Labelled cells growing on the materials were visualized using fluorescent microscopy.

RESULTS:

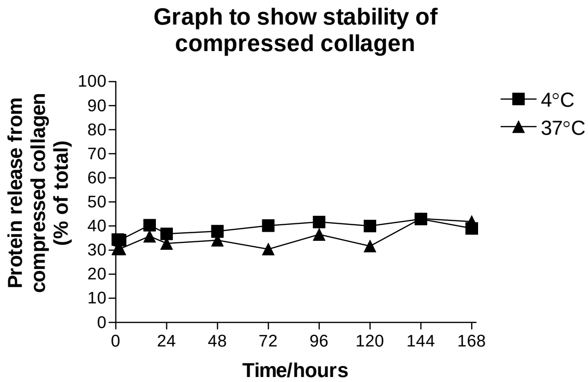

Fig 1: Protein loss from compressed collagen sheets incubated for 7 days in EBSS.

After incubation for 1h at 4 and 37˚C, protein loss from the material was 34 and 31% respectively. There was minimal further loss in the remaining 7 days.
Culture studies showed that both the compressed collagen and fibronectin control supported neurite outgrowth from the DRGs .

DISCUSSION: This novel biomaterial shows potential for use in a neural conduit. It is sufficiently stable to be incorporated into composite devices and supports neural growth.

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Confocal Microscopy of Chondrocyte-Seeded Chitosan Hydrogels

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INTRODUCTION: Cartilage is a type of connective tissue that has very little potential for spontaneous natural repair if damaged. The use of tissue engineering for the treatment of articular cartilage has been investigated for a number of years. However, due to its unique biomechanical properties and functional requirements, no man-made system has been able to imitate cartilage. Chitosan is a natural, abundant polysaccharide, biocompatible and biodegradable. It has the potential to fulfill many of the requirements of a cartilage tissue engineering scaffold material. Chitosan has previously been studied with chondrocytes but live cells have not been observed within the scaffolds.

METHODS: Chitosan hydrogels were produced by dissolving the chitosan powder in weak acetic acid solution, vacuum filtering and then cross-linked to 2.5% with Glutaraldehyde. This solution was then oven dried at 40°C to form the xerogel. The xerogel was swollen in distilled water, treated with NaOH and $NaBH_4$, and sterilized before being seeded with articular chondrocytes. These chondrocytes were obtained by the sequential enzymatic digestion (pronase & collagenase) of bovine articular cartilage explants.

RESULTS:

Figure 1

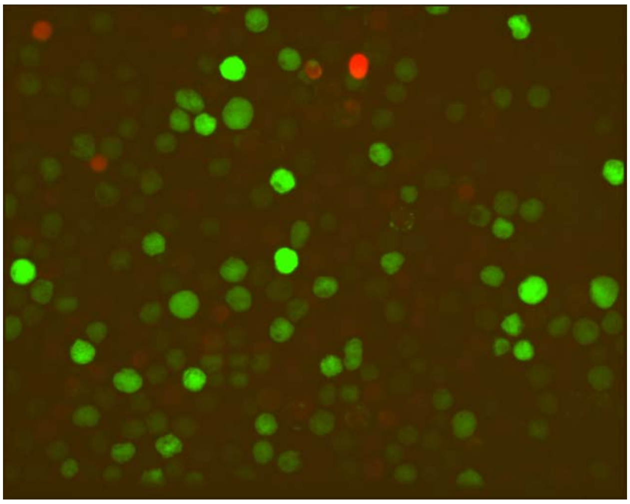

Figure 2

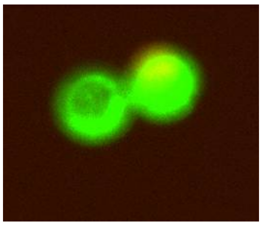

Fig 1 & 2: Confocal micrographs of chondrocytes seeded within chitosan hydrogels on Day 3, at x60 and x20 magnification, respectively. Live and dead cells stained as green and red, respectively.

DISCUSSION & CONCLUSIONS: A procedure was developed to form chitosan hydrogels under relatively mild conditions and then seed them with 10 million bovine chondrocytes per scaffold. It was seen that the quality of the hydrogel was subject to chitosan weight, cross-linking density, oven temperature and type, and the pre-treatment regime. The cylindrical scaffolds formed were then were observed under the confocal microscope over 9 days. The cells were seen to move readily into the scaffolds, to remain viably and maintain their rounded morphology up to day 9. Cell counts showed that there were more cells in the centre of the scaffolds compared to the edges and that the total number of cells within the scaffold increased up to day 3. At higher magnifications (see fig. 2) cellular organelles were visible, including vacuoles and nuclei, suggesting metabolic activity. This suggests that chitosan hydrogels have the potential to support and maintain articular chondrocytes for cartilage tissue engineering.

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Isolation of S-Phase Osteoblasts: Focal Contact Quantification on Nano-Pitted Substrates

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INTRODUCTION: Cell-substrate interactions have always played a pivotal role in determining the performance and ultimate acceptance of a foreign material placed within an *in vivo* environment. Man-made structures, which can induce cell attachment and maintain differentiation of e.g. osteoblasts, while possessing suitable mechanical properties can be viewed as the paradigm of tissue engineering. Integrin receptors are shown to cluster together at discrete foci, resulting in anchoring complexes, known as focal adhesions (FAs). FA's are linked to many mechanotransductive pathways and hence cell proliferation and differentiation. However, with the viewing of FA's, there are problems associated with cell cycle phase and FA rearrangement. Thus, to eliminate the discrepancies obtained in focal contact number and size, it is favourable to quantify FA formation within a synchronised population of cells. Bromodeoxyuridine (BrdU), a thymidine analogue, was used to label S-phase primary osteoblasts cultured on nano-pitted polycarbonate (PC) after serum starvation and then feeding to induce S-phase in the cell population. Incorporation of BrdU into the nucleus of cells undergoing increased DNA synthesis was subsequently fluorescently labeled at the same time as FA detection.

METHODS: Primary human osteoblasts (HOB) were cultured on the control (flat) and test materials (100 nm diameter, 300 nm centre-centre spacing originally produced by electron beam lithography in orthogonal, near orthogonal, hexagonal and random symmetries) injection moulded in PC. The cells were serum starved for 4 days before adding fresh media to the culture system. Cells were left for 17 hours after feeding before the addition of 10 μM BrdU, in which they were incubated for 3 hours. Subsequently, DNase treatment and immuno-labelling were used to view S-phase nuclei and FA's. Images were processed using ImageJ.

RESULTS: Osteoblasts were shown to form extensive arrays of focal contacts with the underlying substrate. These were shown to be both of the dot and dash variety, indicating a dynamic system of focal complex formation and maturation. Focal contacts were seen to be increased in osteoblasts cultured on near-orthogonal and random nano-patterns while numbers of cells in S-phase were decreased on flat and orthogonal nano-pitted substrates (Figure 1).

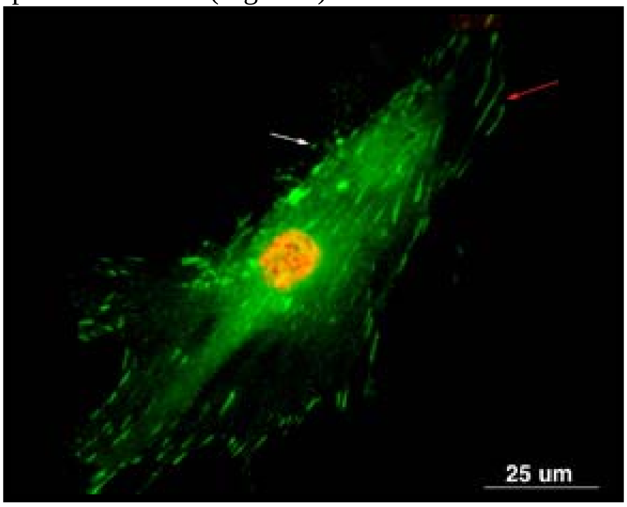

Fig. 1: Typical S-phase Osteoblasts cultured on nano-pitted polycaprolactone. Dot FA's (white arrow) and dash FA's (red arrow) are evident.

DISCUSSION & CONCLUSIONS: Osteoblast focal contact formation is influenced by nano-topography, Highly ordered arrays of nano-pits such as orthogonal and hexagonal patterns result in decreased cellular adhesion and reduced focal contact formation**.** It is anticipated that the above technique will yield invaluable information as to how specific cells respond and adhere to various substrates.

ACKNOWLEDGEMENTS: This work was supported by a grant from the AO Research Foundation. MJD is supported by a BBSRC David Phillips Fellowship. NG is supported by a Royal Society of Edinburgh Fellowship.

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Characterisation of The Regenerative Ability of The MRL/MPJ Mouse in Order to Re-Innervate and Promote Scar-Free Healing in Engineered Skin Equivalents

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INTRODUCTION: Understanding morphogenesis of tissues is key to advancing cell and tissue based therapies collectively known as regenerative medicine. We have identified a mutant mouse model that may help us understand mammalian scar free skin regeneration. The MRL/MpJ mouse displays an unusual trait amongst mammals in that it has the ability to perfectly regenerate skin and cartilaginous components without scar tissue formation. Following tissue loss due to an ear punch wound, histological analysis has revealed the formation of a blastema-like structure 14 days post-wounding, showing many parallels with regenerating amphibian limbs and mammalian limb embryogenesis. [1] We aim to identify the differential expression of candidate regenerative genes and their translated protein products responsible for the formation of a blastema-like structure and regeneration of peripheral nerves in the MRL/MpJ model.

METHODS: Mice wounded with a 2mm biopsy punch to the centre of each ear and a 4mm biopsy punch to the back, were harvested up to 112 days post-wounding. Immunohistochemical co-localisation with antibodies directed against PAN-Neurofilament (NF) and the endothelial marker CD31 was used to visualise both nerves and blood vessels at the wound site. To detect differential expression of key genes, RNA probes were created for *in situ* hybridisation to sections of regenerating ears. Candidate growth factors and developmental markers such as FGF8, FGF10, Hoxb13, Hoxa9, Hoxa13 and Msx1 may potentially regulate blastema formation within the mouse ear, and this is currently under investigation.

RESULTS: Immunohistochemical analysis has shown the murine ear to be highly innervated, but more interestingly, from 10 days post wounding, the MRL/MpJ mouse exhibits a greater capacity for peripheral nerve regeneration compared to the poorer healing C57BL/6 and BALB/c strains. Co-localisation of CD31 with PAN-Neurofilament has revealed that the pattern of re-vascularisation within the blastema-like structure is closely associated with the regenerating nerve network. Furthermore, nerve regeneration into the blastema precedes blood vessel growth (fig 1).

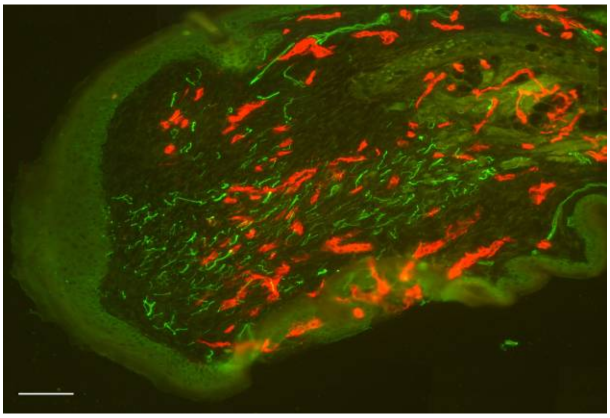

Fig. 1: MRL/MpJ ear blastema 21 days post wounding. CD31 (red), NF (green) (bar = 100µm).

However, in the same animal, an excisional back wound undergoes repair and subsequent scar formation.[2] Unlike the ear wound blastema, we have shown that blood vessels are the first to infiltrate the back wound area and nerve regeneration follows.

DISCUSSION & CONCLUSIONS: Initial results indicate that during regeneration in the ear, the nerve network appears to act as a template for angiogenesis, recapitulating the process of early development. The accelerated nerve regeneration observed in the MRL/MpJ mouse may contribute to its regenerative capacity. The molecular mechanisms involved in this trait are yet to be determined. Further investigations will ascertain if other differentiated structures, such as hair follicles, arise within the blastema region *de novo*. The co-localisation of Schwann cells and regenerating nerves during blastema formation is also underway. The ultimate goal is to harness the molecular signalling pathways responsible for the generation of the blastema-like structure and subsequent nerve regeneration for use within an engineered skin substitute.

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Rapid Fabrication Tissue Engineering by Plastic Compression

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INTRODUCTION:

Development of 3D connective tissues *in vitro* is heavily dependent upon remodelling of the matrix, in particular collagen by resident cells. This process is often difficult to control, slow and costly. This is the main question inherent in 3D tissue bioreactor operations, namely to get cells to 'fabricate' collagen matrices rapidly without the constraints of cell perfusion, spatial control and matrix density. We have tackled this by attempting to pre-fabricate the collagen template without relying on any cell activity at all. We have developed a process of 3D collagen tissue prefabrication by plastic compression (PC) of hyper hydrated native collagen gels[1].

METHODS:

Acellular and cell-seeded, hydrated type I collagen gels were made, as previously described[2], and routinely compacted by a combination of compression and blotting. The rate of compaction was controlled by the force applied and the extent of fluid removal to a porous 'sink'. Cellular collagen gels were made, using dermal fibroblasts and horse bone marrow stromal cells. These constructs were compressed using standardised protocol and rolled to form tight spiral rods. These were then cultured with or without tension in a bioreactor, for periods of up to 30 days.

RESULTS:

Highly structured and mechanically strong collagen constructs were made by using plastic compression. This fabrication process was cell independent. Where cellular constructs were fabricated, these retained good cell viability after undergoing plastic compression, with cells surviving 30 days in culture.

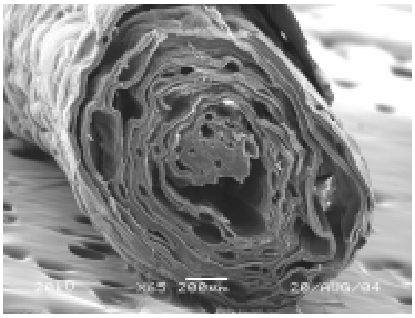

Figure 1. Shows rolling of the collagen sheet to give many adjacent spiral layers This is an SEM across the end of the construct (bar = 200 µm)

DISCUSSION & CONCLUSIONS:

The PC fabrication process will rapidly and efficiently make tissue-like matrices for cell culture. During the plastic compression process, a dense, strong matrix is formed, without the need for any cell activity. These plastic compressed constructs retain many features crucial for tissue-engineering purposes: high-density matrix protein, in this case collagen, biomimetic structure and functional mechanical strength with good cell viability.

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ACKNOWLEDGEMENTS:

We are grateful to the BBSRC for the funding for this work.

The Role of Poly-L-Ornithine, Poly-L-Lysine and Extracellular Matrix Proteins on the proliferation and Function of Pancreatic Insulin-Producing β Cells by Complex Alginate Microencapsulation

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INTRODUCTION: The properties of alginate bead surface need to be further modified in order to modulate the proliferation or function of encapsulated cells. In this study, we investigated the role of PLL, PLO and extracellular proteins on the surface coating of alginate beads as a microcapsule of insulin-producing β cells from rat insulinoma (Rin-m5F cells).

METHODS: Rin-m5F cells were obtained from ATCC. The alginate/cell mixture was extruded as drops through an 18-gauge drawing-up needle into 10 volumes of 1% CaCl2 or 0.5 % BaCl2 supplemented with 10mM HEPES buffer, pH 7.4. The alginate beads that entrapped Rin-m5F cells were coated with poly-L-lysine (PLL), poly-L-ornithine (PLO), CN, FN or LN respectively for 15 minutes. They were coated with another layer of 0.04% alginate for 5 min.

Alamar blue assay was used to estimate cell proliferation. Surface analysis was carried out using atomic force microscopy (AFM). The interaction between FITC-labelled CN and alginate beads was uncovered using a Leica confocal microscope. Accumulated insulin secretion in medium was measured using ultrasensitive rat insulin ELISA.

RESULTS: Barium alginate beads are more stable and provided similar biocompatibility for Rin-m5F cells compared to calcium alginate beads, which showed progressive rupture after culture for a couple of days. 0.1 % (w/v) of PLL or PLO enhanced the proliferation of encapsulated Rin-m5F cells when barium alginate beads were coated using them (Fig.1). Surface topographic images detected by AFM techniques showed the process of protein covering changed the barium alginate polymeric surface (Fig.2, A&B). Using confocal microscopy, we directly showed that barium alginate beads provided spacious porosity to allow FITC-labelled CN infuse (Fig.2, C). Laminin and collagen type I increased accumulated insulin release, while fibronectin increased cell proliferation. Indeed, The effect of ECM proteins on cell morphology could be identified up to 5 hours' culture.

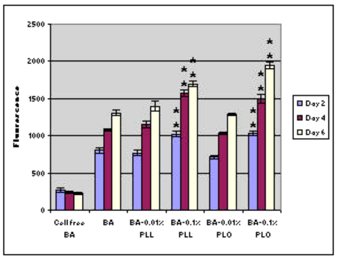

*Fig. 1: Effect of PLL and PLO on proliferation of Rin-m5F cells entrapped in alginate microcapsules. ** P<0.01 vs. BA.*

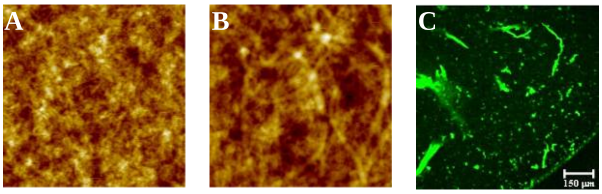

Fig.2: Effect of PLO and CN on physical properties of BA complex. A and B: AFM images. A: BA layer. B: BA layer coated with 0.1 μg/μL Collagen Type I. C: Infusion of FITC-CN into a BA bead.

CONCLUSIONS: For Rin-m5F cells in complex 3D alginate beads, 0.1% of PLL and PLO have an effect on proliferation, while CN and LN have an effect on insulin production. These effects might occur through surface modification and cell-surface interaction. These data provide advanced understanding of the role of PLL, PLO and particular ECM proteins in barium alginate microcapsules when insulin-producing cells were encapsulated and cultured.

ACKNOWLEDGEMENTS: We thank EPSRC and University of Nottingham for funding.

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Extracellular Matrix Proteinsh an Effect on Cell Adhesion and Proliferation but No Significant Differentiation of Adult Rat Pancreatic Duct Epithelial Cells (ARIP))

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INTRODUCTION: Research suggests that adult pancreatic stem cells/ progenitor cells could differentiate into insulin-producing cells or cultivated islet-like clusters. ARIP cells, an adult rat pancreatic ductal epithelial cell line, were used as a model to explore the possibility of insulin-producing cell differentiation.

METHODS: ARIP cells were from ATCC and maintained in F12K medium supplemented with 10% FCS. Tissue culture (TC) surface was coated with Collagen Type I (CN), Fibronectin (FN), Laminin (LN) and Vitronectin (VN) respectively. 3000 cells/cm2 of cell suspension was cultured for 6 hours. Cells were stained with 2uM of Calcein AM. Fluorescent images were captured from 4 random areas surrounding each centre of a well. Individual cell features were analysed using a Leica Qwin program.

Insulin content was estimated using an Ultra Sensitive Rat Insulin ELISA Kit. DNA content was measured using Hoechst 33258 assay. Gene expression was analysed using RT-PCR.

RESULTS: Four types of extracellular matrix proteins enhanced cell adhesion of ARIP cells. The individual cell area and perimeter of ARIP cells were increased by extracellular matrix proteins, suggesting the enhance of cell adhesion. Examination of the surface coverage of ARIP cells in the presence of ECM protein coated surfaces and defined serum-free medium showed that laminin significantly enhanced cell surface coverage after 96 hours' culture. Insulin content (ng/mg DNA) studies were subsequently carried out using GLP-1, ECM proteins in defined serum free medium conditions. Results using these constituents singly were inconclusive; in combination, however, results showed that the insulin content was only enhanced in the presence of collagen. The insulin gene, however, did not show distinct expression corresponding to the above insulin content.

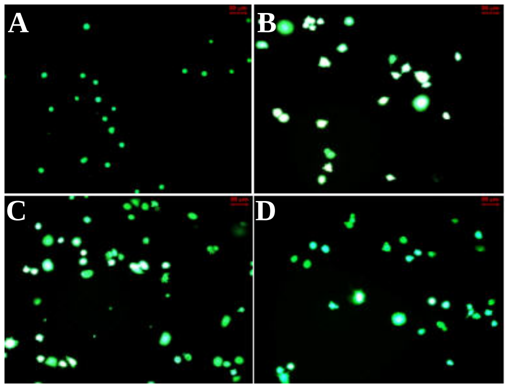

FIG.1: ARIP cells cultured on different surfaces for 6 hours. A.Control. B. 10µg/cm2 CN surface. C. 10µg/cm2 FN surface. D. 10µg/cm2 LN surface.

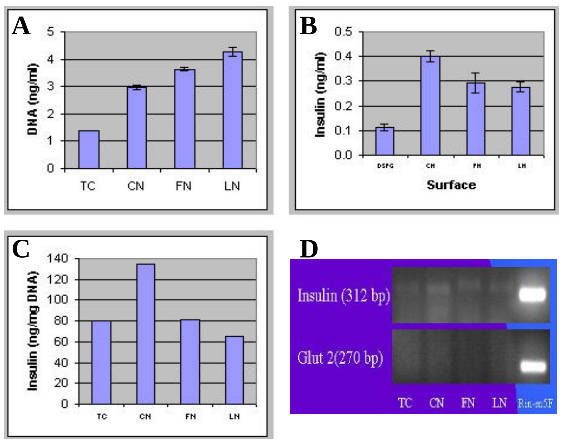

Fig.2: Insulin content and gene expression. ARIP cells were cultured for 48 hours in F12K medium supplemented with ITS, BSA nicotinamide and GLP-1. Each value=mean±STD from triple tests. A. DNA content. B. Insulin content. C. Insulin content/DNA content. D Gene expression.

CONCLUSIONS: Cell morphology, adhesion and proliferation of ARIP cells were affected by ECM proteins. The insulin-producing cell differentiation of ARIP cells is however not affected by ECM and/or GLP-1 significantly.

Attachment and Functionality of Primary Rat Hepatocytes Cultured on Plasma Polymerised Allylamine for 24 Hours

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INTRODUCTION: Successful cell culture and tissue engineering relies on good attachment of cells to their substratum. Many cells including hepatocytes attach poorly to synthetic substrata and favour attachment to extracellular matrix (ECM) proteins such as collagen. Collagen coating is the conventional method employed to improve adhesion of primary hepatocytes to surfaces during cell culture[1]. Plasma polymers are increasingly used in cell culture and tissue engineering[2]. In this work, plasma polymerised allylamine (ppAAm) was examined as an alternative coating method to collagen type I gel to promote primary rat hepatocyte (PRH) attachment to glass.

METHODS: Standard glass cover slips were used in this work after cleaning with 7X and sonicating in distilled water. ppAAm was deposited following the method described by Barry et al[2]. A thin layer of collagen type I gel coating was applied as described in Pearson et al[3].

PRH were isolated using a modified two-step collagenase perfusion method[4] and plated at 1 x 10^5 cells/cm^2 in serum supplemented Leibovitz-15 medium (L-15). The medium was changed to serum free L-15 after 1 hour of attachment. The attachment and functionality of the hepatocytes cultured on ppAAm coated glass was compared to untreated glass, collagen type I gel and collagen type I gel on ppAAm after 24 hours in culture.

X-ray photoelectron spectroscopy (XPS) was carried out to analyse the chemical composition of ppAAm coating. The attachment of PRH to glass was assessed using phase contrast microscopy and total cellular protein measurements. The 7-Ethoxyresorufin-O-deethylase (EROD) assay was performed to determine the cytochrome P450 (CYP1A1/2 and CYP1B1) activity of hepatocytes and secreted albumin was measured using enzyme linked immunosorbent assay (ELISA).

RESULTS: The XPS results indicated that the ppAAm has a nitrogen containing surface where the nitrogen is present in the form of amine, imine and amide functionalities[5]. Fig. 1. shows that the attachment of PRH to glass was significantly improved by coating with ppAAm, collagen type I gel or collagen type I gel on ppAAm. This was consistent with total cellular protein measurements[5]. The functionality of PRH plated on ppAAm, when measured using EROD activity and albumin secretion (Table 1.), was similar to the functionality observed in hepatocytes plated on collagen type I gel or collagen type I gel on pAAm[5].

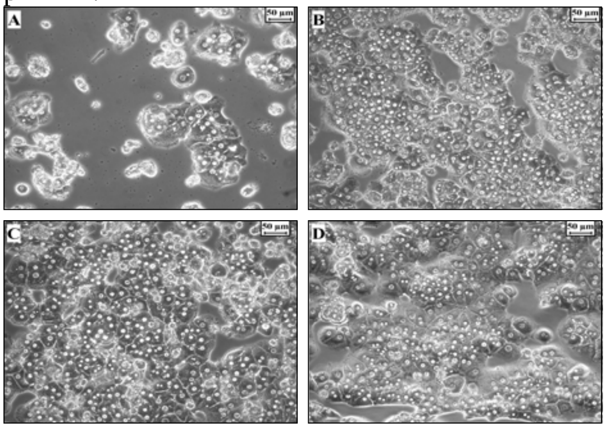

Fig. 1: Attachment of PRH to (A) untreated glass, (B) collagen I gel, (C) ppAAm, and (D) collagen I gel on ppAAm coated glass, images were taken 24 hours after seeding.

Table 1. EROD activity (resorufin pmol/min/mg protein) and albumin secretion (µg/mg protein) of PRH (as in Fig. 1.) after 24 hours in culture (mean of 3 experiments ± SEM).

	A	B	C	D
EROD	18 ± 5	29 ± 11	49 ± 5	35± 10
Albumin	23 ± 2	43 ± 11	46 ± 11	46 ± 9

DISCUSSION & CONCLUSIONS: The ppAAm coating was shown to improve the attachment of hepatocytes to glass while maintaining similar functionality to cells cultured on collagen type I gel. The ppAAm can replace the complicated and variable collagen coating processes, at least for short-term PRH cultures.

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A Gene Therapeutic Approach to Scar Reduction at the Site of Cutaneous Wounding

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INTRODUCTION: Cutaneous wound healing models suggest that TGFβ1 and TGFβ2 amplify the degree of tissue scarring while the relative increase of TGFβ3 to TGFβ1 supports scar-free healing. Topically applied recombinant TGFβ3 protein significantly reduces scar formation but the process is costly and inefficient. An alternative strategy would be to genetically modify syngeneic dermal fibroblasts to express high levels of TGFβ3 *ex vivo*, injecting these into the wound site to act as TGFβ3 secretor vessels within the local environment.

In order to design vectors capable of combining efficient infection and enrichment of high expressing dermal fibroblasts with necessary long-term regulatory elements we chose to use bicistronic retroviral vectors. Conventionally IRES sequences have been employed for multiple gene expression however, these are associated with reduced 3'-gene expression leading to misrepresentation of transduced cell populations. The Foot and Mouth Disease Virus (FMDV) 2A sequence offers an alternative, providing equal gene expression through post translational protease cleavage. However, cleavage results in a residual 19 amino acids at the C terminus of the upstream protein and a proline attaching to the N terminus of the downstream protein. Consequently, by construction of bicistronic vector systems incorporating 2A sequences, equal gene expression would be attainable providing neither of the flanking gene products are affected by the 2A residuals sequences.

METHODS: Bicistronic retroviral vectors were constructed expressing murine TGFβ3 and the selection marker genes GFP or tCD34 linked by either IRES or 2A sequences. Gene expression was initially verified by transient transfection of 293T cells. Media collected from transfected cells was treated with HCl to allow active TGFβ3 protein to be released from its latent pro-peptide form. Treated media was then assayed for the presence of TGFβ3 by ELISA, or applied to mink lung epithelial cells stably expressing a Luciferase reporter under the control of the TGFβ3 responsive plasminogen activator inhibitor-1 promoter (MLEC/PAI-Luc). Marker gene expression was confirmed by FACS sort analysis. Once dual expression had been verified, retrovirus was produced from 293T cells transfected with our bicistronic retroviral expression vector and retroviral packaging plasmid. Virus containing supernatant was then used to transduce 3T3 cells and primary Murine Dermal Fibroblasts (MDF). As previously described, media was analysed for functional, active TGFβ3 and cells assessed for marker gene expression. Transduced cells expressing tCD34 where enriched by treatment with CD34 specific antibody conjugated magnetic beads, producing enriched populations of TGFβ3 expressing cells.

RESULTS: The plasmid rKat.TGFβ3.IRES.GFP was capable of expressing GFP and TGFβ3 protein post-transfection in 293T cells providing a 78% GFP positive population of cells secreting 5.33ng/10^6 cells active TGFβ3. Furthermore, 3T3 cells transduced with rKat.TGFβ3.IRES.GFP secreted 9.93ng/ 10^6 cells of TGFβ3 protein in a 24 hour period, whilst transduced MDFs secreted 13.32ng/10^6 cells. Transfection using the plasmid rKat.TGFβ3.2A.tCD34 provided multiple gene expression producing an 11% tCD34 positive population secreting 0.21ng/10^6cells active TGFβ3 but the levels obtained were significantly lower than those with the IRES construct. Through enrichment the percentage of tCD34 positive transduced cells was increased from 11% to 83%.

DISCUSSION & CONCLUSIONS: We have generated and compared bicistronic vectors expressing TGFβ3 and a selection marker. Although the IRES system gave significantly higher levels of secreted TGFβ3, the 2A construct has the added ability of equi-molar gene expression. We are presently further refining the 2A system with the aim of replacing the marker gene with a prodrug inducible suicide gene that could be required for removal of MDF cells after wound healing is complete.

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Perfusion-Enhancement of Molecular Transport within 3D Scaffolds

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INTRODUCTION: Hydrogels have demonstrated their usefulness in studying the response of isolated chondrocytes to a range of stimuli, while excluding the influence of other factors present in native cartilage. Examples of such studies involve the effects of mechanical stimulation on metabolism [2] and cell deformation[1,3]. It is clear, however, that the diffusion of oxygen and soluble nutrients to cells centrally-located within the 3D constructs is inadequate to maintain both cell viability and proliferation. Indeed, GAG deposition by chondrocytes and mineralised matrix deposition by osteoblasts have both been reported to curtail, approximately 400μm and 240μm from the surface of the 3D constructs, respectively [4].

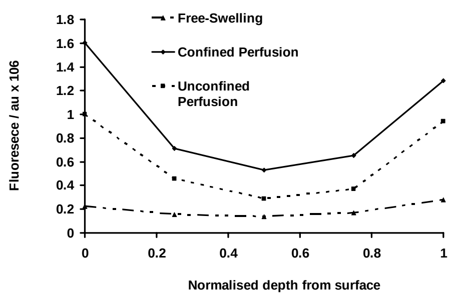
In the present study, bioreactors haven been designed to investigate the influences of two strategies of medium perfusion on cell proliferation and matrix synthesis within agarose constructs. Solute movement within the 3D gels was profiled using confocal microscopy, to test the hypothesis that perfusion-increased fluid flow will enhance molecular transport and dispersion.

METHODS: Two bioreactors, using confined and unconfined configurations, were used to perfuse fluid into 4%(wt/vol) agarose cylinders, of 10mmx3mm cylinders. 0.001% (wt/vol) FITC-Dextran dissolved in PBS was perfused for 24 hours and scanned, using a confocal microscope, Control samples in the free-swelling state were immersed in the same FITC-Dextran solutions for 24 hours.
The agarose cylinders were seeded with $20x10^6$/ml of bovine articular chondrocytes and cultured for 20 days using both perfusion strategies, with the medium refreshed every 3 days. Biochemical assays were used to measure cell proliferation and matrix synthesis and were compared to control groups that had been cultured under free-swelling condition.

RESULTS: Solute concentration gradients, from peripheral regions of the constructs to their central regions, were less significant following perfusion with either bioreactor compared to those that were incubated in the free-swelling condition.
Using the perfusion bioreactors, an increased Dextran (figure 1a) and essential nutrient concentration were achieved at the central regions of the scaffolds.

The perfusion-aided increased media convection resulted in improved cell proliferation (figure 1b) and matrix synthesis (data not shown).

a



b

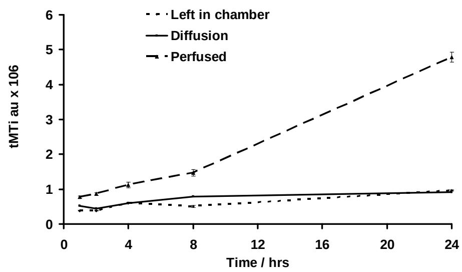


Figure 1: Perfusion a) improves molecular transport into the agarose constructs and b) improves cell proliferation over 20 days

DISCUSSION & CONCLUSIONS: Perfusion enhanced the transport of solutes into agarose constructs, increasing their concentration in the central regions. The improved solute delivery to cells centrally located within the 3D structures was maintained even after the cells began to produce their extracellular matrix, averting the temporal decline of cellular viability at the construct centres (data not shown).

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Chondrocyte Growth in Porous Spider Silk 3D-Scaffolds

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INTRODUCTION: Silk is rediscovered the past decade as a possible biomaterial. As a strong and flexible natural material that biodegrades slowly, it's promising for tissue engineering applications [1, 2]. Porous 3D scaffolds have been made with silk fibroin for these purposes [3]. Spider silk exceeds the mechanical properties of silkworm silk. This explains the many attempts to produce synthetic spider silk. In this research spider cocoon silk is used to create porous 3D scaffolds for cartilage regeneration. Human chondrocytes were seeded on it and cultured for several weeks to establish the scaffolds are not cytotoxic and cells can migrate, attach, grow and express their ECM in the pores of the spider silk matrix.

METHODS: Spider is harder to dissolve than silkworm silk. But after washing a few times in Marseille-soap, the fibres do not only loose their yellow colour but also their protection against strong salts. The fibres can be dissolved in LiBr. After dialysis against water and freeze-drying, the spider silk fibroins can be re-dissolved in formic acid and mixed with NaCl particles. These particles are sieved for a size between 100-200µm and are ten times more abundant than the spider silk fibroin. The typical secondary structure can be regenerated with methanol before the NaCl particles are leached out of the material to leave the pores behind.

Human Chondrocytes were isolated from knee articular cartilage and seeded on the scaffolds. After 1, 3 and 6 weeks of culture cross-sections were made from top to bottom of the scaffold and coloured for cell-viability and expression.

RESULTS: The obtained spider silk scaffolds do not excel in tensile strength, but are quite flexible, very compressible and absorb fluid. The interconnectivity of the pores was proven by an ink-absorption-test and was clear when we saw the diffusion of the chondrocytes through the scaffold. The cells weren't found in every pore, but spread all over the scaffold from top to bottom and attached to the spider silk. The immuno-histochemical colouring of the ECM showed some dedifferentiation, but the cells growing in the pores were still round and shaped chondrocyte-like.

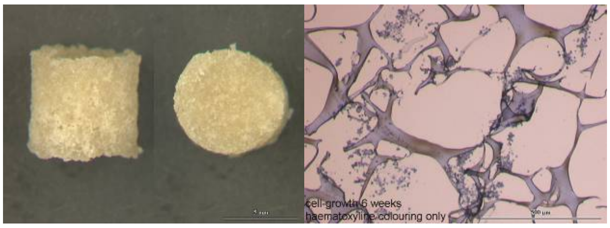

Fig.1: a) a porous, cylindrical scaffold, made with spider silk. B) Chondrocytes growing in the pores..

DISCUSSION & CONCLUSIONS: Even with small amounts of spider silk, it is possible to create a porous 3D scaffold that exists of spider silk fibroin only. As the pores are interconnected and porosity and pore-size and can be controlled, medium, cells and tissue can migrate through the matrix. As cells could be grown in the matrix for at least 6 weeks and their ECM-expression could be detected, it could definitely be used as a plug in cartilage regeneration.

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Interaction of Myofibroblasts with Silk Scaffolds

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INTRODUCTION: High tensile strength, elasticity and good biological compatibility all make silk fibres desirable as scaffolds for engineering tissues[1,2]. However, silks are secreted by numerous species of insects and spiders, and show great variability in their material properties and surface characteristics[3]. Although cells have been grown on a few species and types of silk threads, comparison of cell behaviour on the different silks remains, as yet, largely an unexplored territory. Here, we compare the morphology and alignment of myofibroblasts on four different types of silk threads.

METHODS: Silk mesh preparation: Degummed *Bombyx mori* silk threads and processed wild silk Spidrex® were supplied by Oxford Biomaterials Ltd., and washed with phosphate buffered saline (PBS). *Nephila edulis* egg sack cocoons supplied by Prof. F. Vollrath, Oxford University, were either left untreated or washed with tween and deionised water. All silks were than dried at room temperature, and weighed as coherent random meshes (0.8-1.2mg). Each mesh was autoclaved in a separate container.

Silks morphology and surface topography: Threads were gold coated and examined in Field Emission Gun Scanning Electron Microscope (FEG-SEM).

Silk mesh pre-conditioning: Silk meshes were placed in well plates containing 1ml supplemented growth medium (10% FCS) for varying time intervals at room temperature.

Cell adhesion and morphology: Human colon CCD-18Co myofibroblasts were plated into the wells containing the silks meshes. After 72h incubation, medium was removed from the plates, and meshes were gently washed with PBS. Cells were than stained using Molecular Probes' Vybrant™ cell adhesion assay kit. Meshes were than transferred to glass slides and visualised using standard fluorescence microscopy.

RESULTS: Human colon myofibroblasts adhered to all silk fibres studied to some extent. Cell morphology varied between the different silks studies. Cell growing on Spidrex® were mostly flat and elongated, and aligned along the ribbon-shaped brins. Cells growing on both clean and untreated Nephila edulis cocoon silk, however, presented a round morphology, suggesting a lesser degree of adhesion. Cells grown on Bombyx mori threads presented predominantly rounded but also flat morphologies. Pre-incubation of the meshes in growth medium for one hour did not appear to influence cell attachment or morphology.

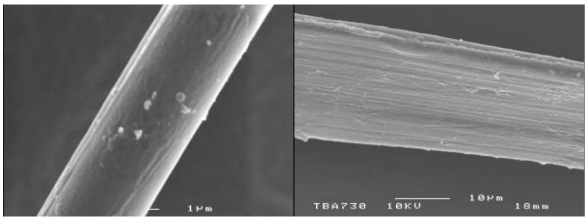

Fig 1: Surface of silk threads: Untreated Nephila edulis (left) and Spidrex (right).

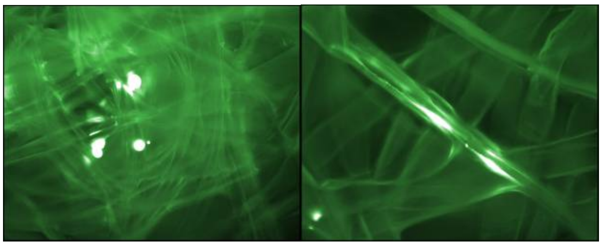

Fig. 2: Morphology of myofibroblasts grown for 72h on Nephila edulis (left) and Spidrex (right) threads.

DISCUSSION & CONCLUSIONS: The morphologies and alignment of human colon myofibroblasts were shown to vary depending on the species of silk scaffolds used. These observed differences might be related to the morphology or surface properties of silk threads, including nano-topography and chemical constitution. Also, it is possible that specific amino acid sequences present at the surface of the silk thread are responsible for the oriented alignment of the cells.

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The design of a rotating bioreactor for use in multi-sample loading regimes

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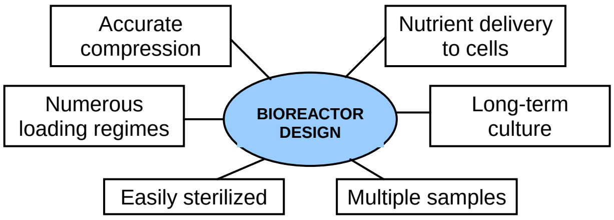
INTRODUCTION: Mechanical forces, spatial environment and biochemical stimuli influence the differentiation of mesenchymal stem cells (MSCs) *in vivo*[1]. These types of stimuli can be applied *in vitro* to MSCs whilst they are incorporated in a 3D scaffold, which can then be placed in a bioreactor. We have designed a novel bioreactor which will enable the application of numerous loading regimes to multiple samples.

METHODS:



RESULTS: Nutrient delivery and long-term culture: To achieve this the bioreactor was designed to hold approximately 700mls of culture media and was fitted with a circular stand to enable the bioreactor to be horizontally rotated at 5 RPM for up to 4 weeks, Figure 1A. Multiple samples: To compress multiple samples simultaneously a flexible holder was designed out of silicone rubber, Figure 1B. Sterilisation: The materials were chosen to enable the bioreactor to be autoclaved. The main body of the bioreactor is made out of glass and the lid is made out of stainless steel. Application of loading regimes: A loading rig was designed, Figure 2, and Labview software was used to enable the application of varying ranges of compressive displacement ranging from 1 – 20% strain when applied to a 4mm height scaffold (40 - 800µm) with varying frequencies and cycle numbers. Accurate compression of scaffolds: The original start position prior to compression was determined over 60 minutes. It showed that pre-conditioning the stepper motor for 30 minutes prior to use reduces the variability, Figure 3.

DISCUSSION & CONCLUSIONS: Most of the design objectives have been achieved. The completed bioreactor is capable of rotating whilst remaining sterile and allows the application of compression loading with various regimes to numerous samples. Initial displacement measurements suggest that the stepper motor has

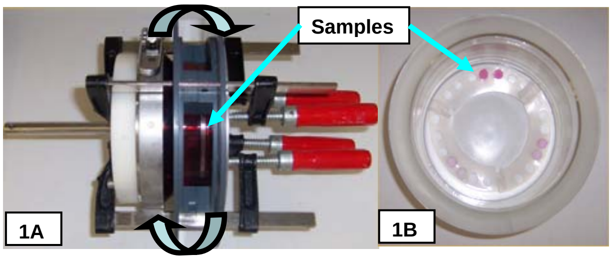


Figure 1A: The bioreactor during rotation 1B: The samples held in a silicone rubber ring

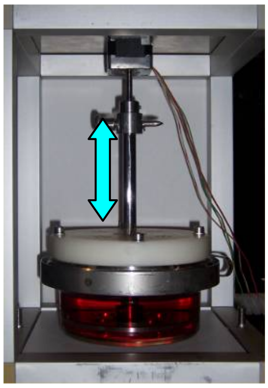


Figure 2: The bioreactor in the loading rig. The arrow shows the direction of the stepper motor

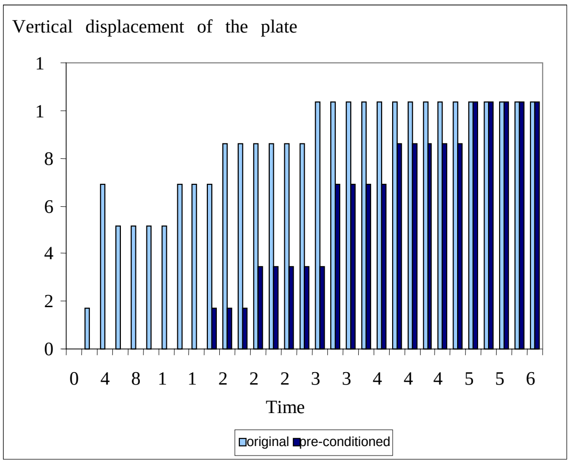


Figure 3: The displacement of the plate over a 60 minute period of compression. Pre-conditioning of the stepper motor for 30 minutes prior to sample loading reduces the variability in the displacement

backlash due to the metric thread of the axis. To overcome this problem a displacement sensor will be added to the system. Further studies are also needed to characterise the shear stresses being applied via the culture media during rotation.

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Superficial and Deep Chondrocyte Subpopulations both Express the Crabtree Effect but Exhibit Differences in Oxygen Consumption Rate

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INTRODUCTION: Chondrocytes from full depth articular cartilage exhibit aerobic glycolysis, but demonstrate enhanced oxygen consumption during periods of low glucose availability[1]. *In vivo*, the superficial subpopulation experiences greater oxygen and glucose levels than the deep zone. We hypothesise that the superficial and deep subpopulations of articular cartilage exhibit different basal oxygen consumption rates and oxidative response to low glucose levels.

METHODS: *Cell isolation.* Bovine articular cartilage was harvested from metacarpalphalageal joints as a thin superficial layer, representing the uppermost 15-20% of uncalcified tissue depth, and the remaining deeper tissue. Chondrocytes were isolated from the extracellular matrix by serial digestion in pronase and collagenase. Viability was assessed to be >95% by trypan blue exclusion. *Oxygen measurement*. 10^6 cells were loaded into each well of a 96-well plate oxygen biosensor (BD Biosciences) and incubated in 310 µL DMEM+16% FCS containing 0.5, 1.3, 2.2, 3.0, 4.6 and 19.8 mM glucose. Additional samples were incubated with 0.5 mM glucose + 5 mM 2-deoxyglucose (Sigma, Poole, UK). The plate was sealed with film and the oxygen concentration $[O_2]$ within the cell suspensions monitored fluometrically for 6h.

RESULTS: A monotonic depletion of oxygen was observed in each well, as illustrated in fig. 1.

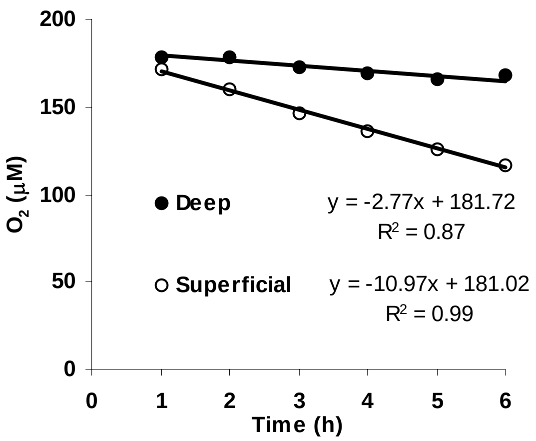

Fig. 1: The decreasing $[O_2]$ in suspensions of either superficial or deep chondrocytes incubated in DMEM+16% FCS containing 0.5 mM glucose.

The oxygen consumption rate of each sample was calculated from the fitted linear relationship (fig 1) and normalised to cell number. The results summarised in figure 2 indicate that the superficial subpopulation, which represented 57-59% of the cell yield, has more than twice the per cell oxygen consumption rate of deep cells. Both the superficial and deep subpopulations exhibited a Crabtree effect, with a 2.2 and 2.1 fold respective increase in oxygen consumption rate as glucose was reduced from 4.6 mM to 0.5 mM. The addition of 5 mM 2-deoxyglucose to low glucose incubations did not suppress oxygen consumption, but instead stimulated it in the deep cells (data not shown).

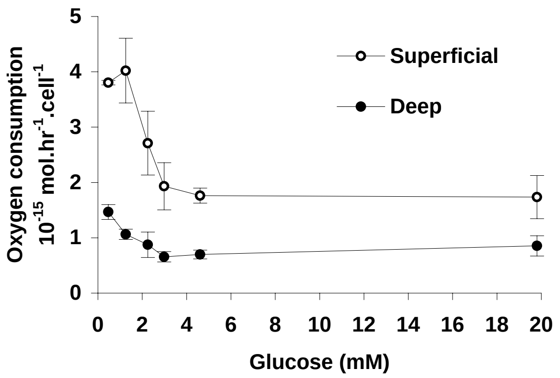

Fig. 2: The increasing oxygen consumption rate of superficial and deep chondrocytes with glucose deprivation. Data represents the mean ± StDev of 3 cell isolations, each tested in duplicate.

DISCUSSION & CONCLUSIONS: That the oxygen consumption of superficial cells is more than double that of deeps cells has important implications for the oxygen concentration within tissue engineered constructs formed using full-depth cells, compared to native tissue or constructs employing a layered approach. The present study has also demonstrated that the Crabtree effect is present in both subpopulations.

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ACKNOWLEDGEMENTS: This work was funded by the research councils of the UK

Designing Flexible Biodegradable Scaffolds for Cardiac Tissue Engineering

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INTRODUCTION: Recently cardiovascular diseases have become the main cause of death not only in the western countries but also in many developing countries [1]. Current research has concentrated on new alternatives such as cellular therapy. The lack of physical support containing growth factors to induce specific differentiation in the cellular therapy suggest that a cardiac construct could provide more clinical benefits if is able to supply such support. Cardiac muscle engineering is focused on obtaining cardiomyocyte constructs by seeding cardiac cells on suitable scaffolds. According to some works additional mechanical stimuli on cell culture systems have been shown to improve protein expression and differentiation in mammalian cells [2]. Our aim is to produce a flexible scaffold able to support mechanical load during tissue regeneration and evaluate the effects on the resulting 3D cardiomyocyte construct.

METHODS: The elastomer Poly-(1,8-octanediol-co-citric acid) [POC] was processed to obtain a porous structure using the salt leaching method before it was completely crosslinked, porosity was increased by modifying the salt concentration [3]. Porosity was evaluated with micro-tomography (μCT) and scanning electron microscopy (SEM). POC films were precoated with Collagen type I, Fibronectin and Laminin at different concentrations to assess the effect on cell attachment and proliferation. The films were stored at room temperature in PBS and then seeded with a cardiac cell line, HL-1 [4]. The monolayer cultures were evaluated for morphology, proliferation and survival. Also, 3D porous scaffolds were seeded with HL-1 and the constructs evaluated for morphology, proliferation and survival.

RESULTS: The scaffolds obtained from the POC polymer resulted flexible interconnected porous structures with a mean pore size of 350 μm., suitable for cardiac tissue engineering (Figure 1). Data showed that unreacted monomers should be washed after the scaffold is processed to avoid a drop in the pH. Two—dimensional culture of HL-1 cells on POC films showed an acceptable attachment to the surface, and this was improved in all the precoated films (Figure 2).

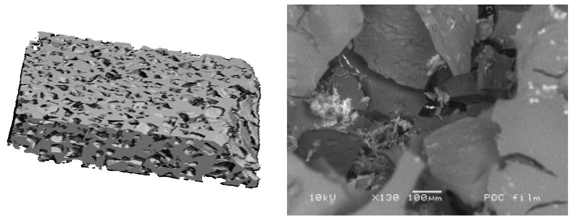

Fig 1: micro-tomography image (left) and SEM image (right) of a Poly- (1,8-octanediol-co-citric acid) [POC] scaffold

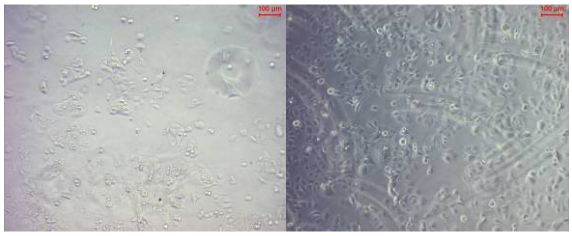

Fig 2: HL-1 cells after 24 hours of culture on POC film (left) and on POC film precoated with Fibronectin (right)

DISCUSSION & CONCLUSIONS: POC is a biodegradable and flexible material that can be processed to obtain elastic porous scaffolds suitable for cardiac engineering applications. Cell culture on POC showed cell attachment but the modification of the surface by the protein coating improved cell adhesion, making it a suitable material for cardiac tissue engineering. The composition of the POC scaffold is being optimized to meet the mechanical and biological parameters needed for this purpose. Further research on the constructs cultured on other systems will be carried out to assess the impact of different culture conditions in this material.

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Changes in Membrane Potential of Human Osteoblasts and Chondrocytes in Response to Novel Magnetic Particle Based Force Application Technique

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. **INTRODUCTION:** This study describes short-term changes in MG63 human osteoblast-like and human chondrocyte cell membrane potential in response to a novel method of applying forces. Magnetic particles were attached to individual cells and an oscillating magnetic field was applied, delivering an approximate force of 30pN to the cell membrane. This study demonstrates a novel type of mechanical force application that elicits similar responses in cells to that seen in previous studies where application of stress to the cells was applied through different mechanisms.

METHODS: Carboxyl ferromagnetic microparticles (4.0-4.5 µm diameter) were coated with RGD. Particles were then adhered to the cell membranes in serum free media for 40 mins at room temp at a concentration of one particle per cell. Magnetic source used in all experiments was a rare earth NdFeB magnet. A time-varying magnetic field was applied to primary chondrocytes using a computer-controlled, linear drive system developed at Keele University (Fig 1). Field application to MG63 cells achieved by manual movement of magnet [1,2]. In both cases, the magnetic field was applied for 10 minutes at 1Hz frequency. Cell membrane potential was measured immediately after exposure to magnetic field/mechanical force. All electrophysiological recordings taken using method previously described [3].

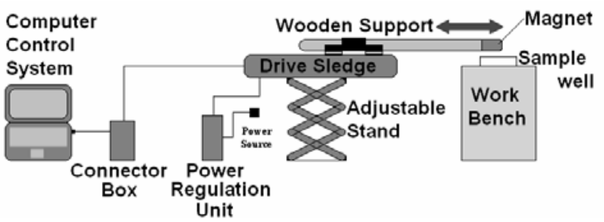

Fig. 1: A schematic representation of the computer controlled magnetic field delivery system.

RESULTS: Application of manually applied dynamic magnetic field to MG63 cells with adhered particles demonstrated a significant 34% ± 7.02 (SE) average increase in membrane hyperpolarisation compared to the average resting potential and a 52% ± 20.79 (SE) compared to recording taken after particle adhesion.

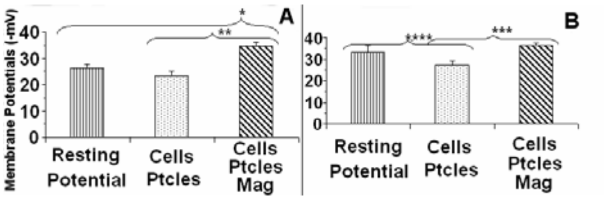

*Fig 2: *p < 0.001 **p < 0.00001 ***p < 0.01 ****p < 0.03. "Resting potential" refers to the initial membrane recordings. "Cells + Ptcles" had particles adhered but were not exposed to a magnetic field. "Cells + Ptcles + Mag" had both particles present and exposed to 1Hz magnetic field for 10 minutes.* ***A.*** *MG63* ***B.*** *Chondrocytes.*

DISCUSSION & CONCLUSIONS: The majority of loading regimes apply force to the entire cell. These techniques are further limited as they often act upon large cell populations and not individual cells. The magnetic particle technique removes these limitations enabling controlled application of forces to a specific region of the cell membrane at a theoretically determined level [1,4]. Coating the particles with RGD enabled the application of mechanical forces specifically to cell integrins. The preliminary findings shown in fig 2 provide an early indication of the efficacy of the magnetic particle technique in the study of early cellular responses to mechanical force application.

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Biomechanics of Plaque Rupture in Carotid Atheroma: Utility of *in vivo* High Resolution MRI

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INTRODUCTION: Stroke is one of the leading causes of death in the developed world. Up to 50% of stroke may be caused by rupture of atheromatous plaque in the carotid artery of the neck. Such strokes are potentially preventable by carotid intervention such as carotid endarterectomy, angioplasty and stenting [1]. Appropriate selection of patients for intervention is a crucial clinical issue and currently based on the severity of carotid luminal stenosis. It has been, however, widely accepted that luminal stenosis alone may not be an adequate predictor of risk [2]. Factors such as plaque morphology and biomechanical stress are increasingly thought to be other important risk factors for plaque rupture [2,3]. To explore the role of biomechanics in identifying plaque vulnerability and to seek a better risk predictor for patient selection, we combined the advances of *in vivo* high resolution magnetic resonance imaging (MRI) with finite element analysis (FEA) and simulated the interaction between blood flow and atheromatous plaque in human carotid artery.

METHODS: A symptomatic patient underwent high-resolution multi-sequence *in vivo* MR imaging of the carotid bifurcation. Each axial MR slice was segmented and contours of lipid core, fibrous cap, vessel lumen and wall were traced to generate boundaries for finite element analysis. The plaque components were modelled as hyperplasic materials and a pulse-pressure loading was simulated on the luminal surface. Plain strain analysis was used for the plaque structural simulation. Maximal Von Mises stress within plaque was obtained for the two groups.

RESULTS: Fig. 1 shows the computed Von Mises stress contour of a carotid plaque in a patient with a 70% degree of stenosis based on *in vivo* MRI plaque geometry. a shows the identification of plaque components from *in vivo* carotid MRI (L: lumen; fibrous cap: yellow arrow; lipid pool: green star(*)). b is the histology sample for the co-registration of plaque characterization. c is the plaque geometry derived from MRI and is used for FEA simulation. D is Von Mises stress contour shows the peak stress concentration on the shoulder region of the plaque (red thick arrow).

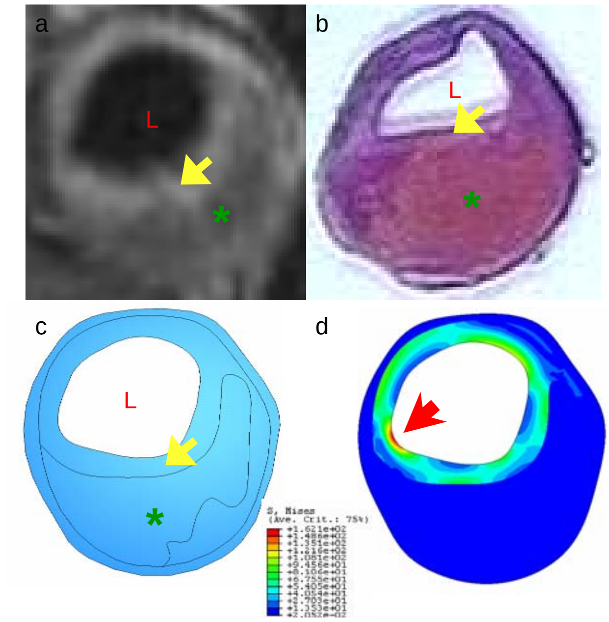

Fig. 1: Stress contour of a carotid plaque in a patient with a 70% degree of stenosis. a: Identification of plaque components from in vivo carotid MRI (L: lumen; fibrous cap: yellow arrow; lipid pool: green star(*)). b: Histology for co-registration of plaque characterisation. c: Plaque geometry for FEA. d: Von Mises stress contour shows the peak stress concentration on the shoulder of the plaque (red thick arrow).

DISCUSSION & CONCLUSIONS: This study illustrates the potential application of state-of-the-art computational modeling techniques in risk stratification of clinical patient groups. The results suggest that high stress concentration and thin fibrous cap at the shoulder regions of the plaque may cause plaque rupture. A combination of high resolution MR of carotid plaque and FEA could potentially act as a useful tool for assessment of risk in patients with carotid atheroma alongside the established quantification of luminal stenosis.

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Co-Expression of SOX 9 and SOX 6 Enhances Collagen 2 Gene Expression

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INTRODUCTION: The manipulation of cultured articular chondrocytes (HACS) for the purposes of cartilage repair is challenging due to loss of phenotype over time *ex vivo*.This loss of chondrogenic morphology and function is a direct result of the down regulation of SOX9. SOX9 is a transcription factor that controls the expression of genes responsible for chondrocyte differentiation, such as collagen II, IX, XI and aggrecan. Col2a1 is one of the most important components of cartilage formation and gene activation requires the presence of SOX9 along with two other accessory proteins, L-SOX5 and SOX6, in a transcription complex known as the SOX trio. Recent evidence suggests that it is not necessary that both L-SOX5 and SOX6 are present in the SOX trio; rather the 2:1 ratio of SOX5/6 to SOX9 is maintained. We have constructed a bicistronic retroviral vector that will allow the co-expression of SOX9 and SOX6 within the same transduced HACS. The human SOX9 and SOX6 genes were cloned either upstream or downstream of an IRES sequence. We would predict that the gene upstream of the IRES sequence will be expressed at a higher level than the downstream gene. In order to distinguish which SOX protein had more precedence we created two constructs; one that expresses the SOX9 gene upstream of the IRES sequence and the SOX6 downstream and the second construct has the SOX6 gene placed upstream of the IRES. We aim to compare the effect on Col2a1 expression these vectors have using a Col2a1 promoter driven luciferase reporter system in chondrocytes.

METHODS: Retroviral vector were generated using the rKat system expressing SOX9 and/or SOX6 bicistronically from the viral LTR.HACS were isolated from tissue obtained following total knee arthroplasties.

The cells were cultured on plastic then transduced with the retroviral vector and grown as a monolayer in DMEM +10% FCS. Relative levels of transcript were determined for SOX6/9 and Col2a1 by RT-PCR. We were also able to compare the effect of increased expression of the transgenes by activation of a Col2a1 promoter driven luciferase system.

RESULTS: From our previous data it is evident that SOX9 enhances Col2a1 expression in cultured chondrocytes. A single cycle of transduction produced a high percentage of GFP positive cells, 95%, 57% and 85% using adenoviral, retroviral and lentiviral vectors respectively. It was also noted that the level of SOX9 transduced cells when using a retroviral vector was increased by a factor of 2 in the presence of growth factors such as IGF-1 and TGF-β3. We present evidence that the co-expression of SOX6 and SOX9 from a single retroviral cassette induces higher levels of expression from the Col2a1 promoter than with either single gene. Furthermore the position effect within our IRES construct is critical.

CONCLUSION: Previous studies have shown that SOX9 plays a crucial role in maintaining chondrogenic phenotype both *in vivo* and *in vitro*. Our data shows that co-expression of SOX6 and SOX9 from a single retroviral cassette can further maintain the stable chondrogenic phenotype characterised by high expression of collagen 2a1.

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Observation of Calvaria Cells on Nanostructure with Pyramid Morphology

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Introduction: The influence of nanotopography on cell adhesion, morphology and proliferation is of major interest in understanding the complexities of cell functions. This study focuses on a method of nanostructuring [1] for in vitro cell engineering called Physical Vapour Deposition [2]. Cells are observed on a nanostructure with pyramid morphology [fig 1]. Physical vapour deposition (PVD) is fundamentally a vaporization coating technique, involving transfer of material on an atomic level. It is an alternative process to electroplating.

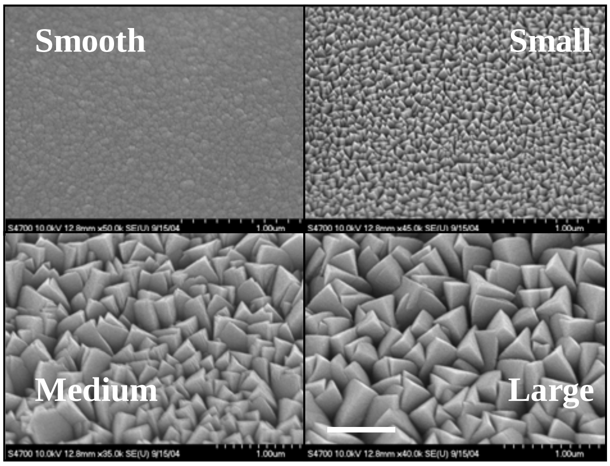

Fig. 1: SEM of pyramid structures made by PA-PV. Small Pyramids 30-60nm Height (H) 50-70nm Distance from Peak to Peak (D) Medium Pyramids 150-300nm (H) 150-250 (D) Large Pyramids 200-400nm (H) 200-300nm (D). Hardness is 7 to 9 Gpa. Scale500nm

Methods: Rat calvaria cells are collected and maintained in DMEM at 37°C in 5% CO_2 until confluent. Cells are then seeded onto structures and grown for 24hrs then washed in PBS, fixed in 4% formaldehyde with 2% sucrose then incubated in permeablising buffer ready for fluorescence.

Immunostaining: Cells are incubated in blocking buffer with 1% BSA/PBS. This is followed by incubation of Cells with the Primary Antibody Monoclonal Anti-Vinculin (Sigma). Washes with 0.5% Tween-20 in PBS is carried out between incubations. The final incubation is with Secondary Antibody Alexa Flur 555 Donkey Anti-Mouse IgG (Molecular Probes). Images of cells are captured using Image-Pro software.

Results:

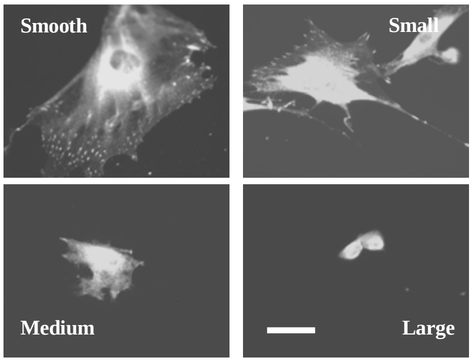

Fig 2: 24hr culture of calvaria cells on smooth, small, medium & large pyramid structures stained for vinculin. Scale 20μm

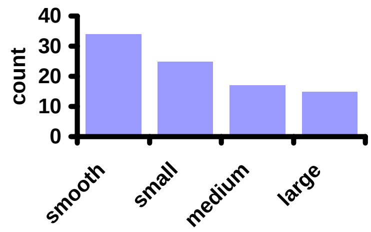

Fig 3: Number of cells on smooth, small, medium & large after 24hr on pyramid structure.

Discussion & Conclusion: Cells [fig 2] on the smooth show a well spread evenly spaced culture with good focal contacts and well defined actin. Nice round evenly spaced nuclei. Cells on small structure show starry shaped cells and although fairly confluent the spaced between the cells are uneven and the contacts not so clear. Cells on medium and large structures show very irregular shapes with little or no focal contact. Cells on large structure show clearly the furry round cells that have not spread. As can be seen clearly from these results the cells respond [fig 3] very differently to the topography of the pyramid structures. The more uneven the surface, from smooth to large, the more uneven and irregular the cell shape becomes with less degree of spreading and contacts to other cells.

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Orientation and Confinement of Cells on Chemically-Patterned Polystyrene Surfaces

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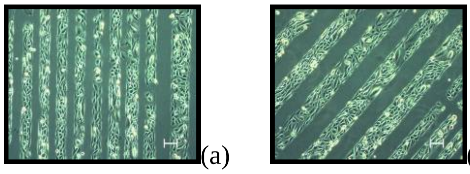
INTRODUCTION: There are many possible applications for devices that study cell behaviour, organisation and cellular interactions. Such applications include medical implants for wound and fracture healing, drug delivery systems, dental implants, artificial prosthesis, biosensors and biochips[1]. It has been reported that 'contact guidance' can promote the alignment of cells on substrate grooves and ridges[2]. This study is aimed to control the alignment and confinement of cells to chemical micro-patterns as a cost-effective alternative to topographic micro-patterns.

METHODS: Polystyrene (PS) surfaces have been patterned using an ultra-violet ozone treatment to investigate the behaviour of Chinese hamster ovarian (CHO)[2] cells grown on surfaces comprising of regions different chemistry (i.e. oxidised and unoxidised)[3]. A Sjostrand transmission electron microscope (TEM) grid was used to produce oxidised strips 55µm and 125µm wide. Additionally, 250 and 400 mesh TEM grids were used to produce an array of oxidised squares of different dimensions.

RESULTS & DISCUSSION: CHO cells attached predominately to the oxidised areas of the surfaces and during the 72h incubation period only a small number of cells spread to the unoxidised polystyrene. Using figures 1a and b, the orientation of the cells was determined by drawing a line through the long axis of each cell and measuring the angle relative to the edge of the surface micro-pattern[4]. Cells in relatively close proximity to the linear oxidised/unoxidised border showed significant axial alignment, with cells attached to the thinner oxidised regions showing overall greater axial alignment (figure 2).

CHO cells can be confined to specific regions of the polymer surface (figures 3a and b). Some treated 'islands' have not been occupied by cells; this may reflect their inability to 'detect' regions of the surface favourable for growth. The confinement of cells had a marked influence on their size and shape (figure 4). Cells attached to larger areas, 75×75 µm, (figure 3a) were found to have a smaller average size than cells attached to the smaller, 56×56 µm areas (figure 3b).



(a) (b)

Fig. 1: CHO cells grown (a) 55µm and (b) 125µm wide oxidised strips. Scale bar = 100 µm.

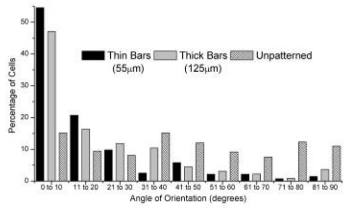
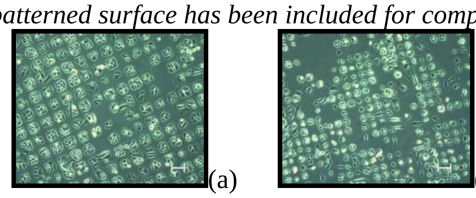


Fig. 2: Histogram showing the orientation of cells attached to patterns consisting of 55µm and 125µm wide oxidised strips. The orientation of cells on an unpatterned surface has been included for comparison.



(a) (b)

Fig. 3: CHO cells grown on PS surfaces patterned with (a) 250 and (b) 400 mesh grids. Scale = 100 µm.

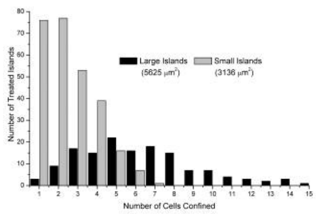


Fig. 4: *Number of islands supporting a number of CHO cells observed from micro-patterns created using 250 and 400 mesh TEM grids after 72 hours incubation.*

CONCLUSIONS:

The orientation, size and morphology of cells can be controlled on UV/Ozone patterned surfaces. Close confinement and orientation of cells can be used to study specific cellular functions and reduce cross-talk in bio-assay applications.

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Adhesion of Primary Human Osteoblast Cells to UV/Ozone Modified Polyethylene

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INTRODUCTION: The effect of material surface chemistry and topography on cell attachment is currently of interest for the development of devices for biomaterials applications[1,2]. It has been found that high-energy surfaces promote rapid cellular adhesion and spreading, whereas low energy surfaces do not[3]. Surface topography has also been found to influence cell orientation and attachment[4]. Previous studies by this research group have shown the effect of UV/ozone treatment of polystyrene (PS) on primary human osteoblast (HOB) cell attachment. The present study examines the attachment, extracellular matrix protein expression and mineralisation of HOB cells on UV/ozone modified polyethylene (PE).

METHODS: PE sheets were cut to fit the internal surfaces of 45mm diameter PS dishes (Nunc) and were modified by UV/ozone treatment. Nunclon tissue culture polystyrene (TCPS) dishes were used as the control surfaces. Surface chemical compositions of treated and untreated surfaces were determined by XPS and topographic changes were examined by AFM under ambient conditions[5]. Primary human osteoblast (HOB) cells were isolated from osteoporotic femur heads and were grown to 70-80% confluence in DMEM supplemented with 10% FCS in 5% CO_2 at 37°C, and plated at 5000 cells/cm^2. 48hrs after plating, the media was changed to α-MEM supplemented with 10μM dexamethasone and 10mM β-glycerophosphate to achieve mineralisation, over the 21 day time period. Cell functionality was assessed by alkaline phosphatase production (ALP) mineralisation, total protein, specific protein assays and proliferation through Alamar blue assay.

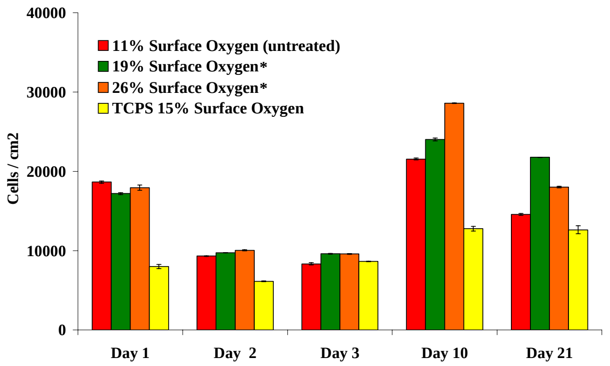

Fig. 1: HOB cell proliferation over the 21 day period for the different levels of treatment. (signifies statistical significance of surface treatment compared to TCPS, $p<0.05$).*

RESULTS: The proliferation data from Alamar blue assays show a significant increase in cell number on the treated surfaces compared to TCPS, as shown in figure 1. The total protein and ALP produced on the oxidised surfaces also increased compared with TCPS. Figure 2 shows a scanning electron micrograph of HOB cells aligning to the grooves and ridges in the PE surface. Through AFM analysis the height of the ridges and the distance between these has been determined to be ~5μm, so the cells are spreading across several, but aligning along the grooves.

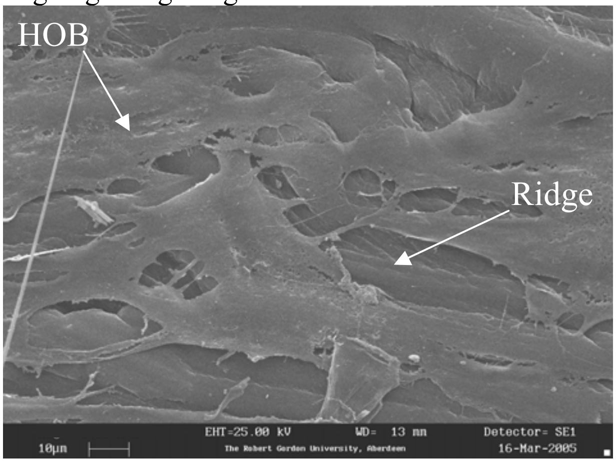

Fig. 2: Scanning electron micrograph showing HOB cell attachment 3 days post-plating on a PE surface with 19% surface oxygen.

DISCUSSION & CONCLUSION: UV/ozone treatment provides a method to introduce controllable chemical functionality on both PS and PE surfaces, which is simple, rapid, and cost effective. These modified surfaces have been shown to increase proliferation, protein expression and ALP production in HOB cells.

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Differentiation of Human Mesenchymal Stem Cells to Nucleus Pulposus Cells Using A Novel Co-Culture Technique

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INTRODUCTION: Low back pain is one of the largest health problems in the western world today and in the majority of cases intervertebral disc degeneration is identified as the main cause. While at present treatments are symptomatic, cell-based tissue engineering methods are being explored as realistic alternatives for tissue regeneration; however the major problem for these strategies in the generation of a suitable population of cells. Adult bone marrow-derived mesenchymal stem cells are undifferentiated, multipotent cells that have the ability to differentiate into a number of cell types, including the chondrocyte-like cells found within the nucleus pulposus of the intervertebral disc. While these cells offer a promising solution to the cell problem, no method exists to differentiate these cells in a monolayer environment.

METHODS: Monolayer human MSCs and NP cells were co-cultured (n=3) for 7 days either with or without contact at the following ratios – 75% NP:25% MSC, 50% NP:50% MSC or 25% NP:75% MSC. Co-culture was conducted in 24-well plates with 0.4µm cell culture inserts used for co-culture without contact. Prior to co-culture MSCs were labelled with a cell-permanent green-fluorescent dye to allow separation following co-culture with contact. Separation was achieved using high-speed cell sorting of fluorescent and non-fluorescent cells. RNA was extracted from co-cultured MSCs and NP cells individually, then reverse transcribed and used in real-time PCR for the following genes – 18S; GAPDH; collagen types I, II and VI; aggrecan; versican; and SOX-9. Data was analysed using the $2^{-\Delta\Delta Ct}$ method.

RESULTS: Real-time PCR revealed significant increases in NP marker gene expression following co-culture with cell-cell contact for 7 days. Furthermore these changes were shown to be affected by cell ratio. In particular, SOX-9, type II collagen and aggrecan showed large increases in mRNA expression, particularly at a ratio of 75% NP:25% MSC (around 700-fold, 6000-fold and 5900-fold respectively). Both type I and type VI collagens showed small, but significant changes in expression, while versican did not show a significant change. As the ratio of NP cells to MSCs decreased, so did the fold change in gene expression, however in all cases MSCs showed increased expression of NP marker genes. In most cases NP cells showed some increase in marker genes, however not to the same extent as MSCs. When cells were cultured without cell-cell contact there were no consistently significant changes in gene expression in either NP cells or MSCs. To confirm the change in expression in MSCs was caused by NP cells, the co-culture with cell-cell contact was repeated using dermal fibroblasts, which did not cause an increase in NP marker genes in MSCs, but did increase elastin expression.

DISCUSSION & CONCLUSIONS: We have shown that co-culture of NP cells and MSCs induces differentiation of MSCs to cells with an NP-like phenotype. Furthermore the novel techniques used in this study have allowed investigation of individual cell populations following co-culture with cell-cell contact and that this cell-cell contact is necessary for MSC differentiation. While increasing the number of NP cells present appeared to improve differentiation we have shown that the presence of even a relatively small number of NP cells is sufficient to induce differentiation in monolayer MSCs. Therefore we have shown that human nucleus pulposus and mesenchymal stem cell co-culture with contact is a viable method for generating a large population of differentiated cells that could be used in cell-based tissue engineering therapies for regeneration of the degenerate intervertebral disc.

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Surface Modification of a Polyether-urethane with RGD-containing Peptides for Enhanced Cell Attachment and Signalling

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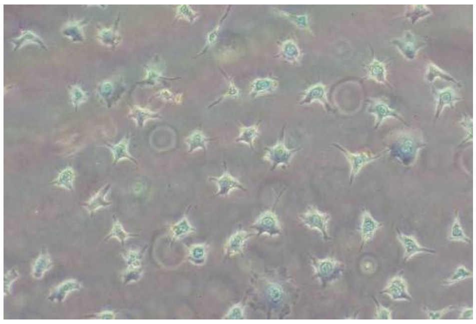
INTRODUCTION: The chemical modification of polyurethane with RGD-containing peptides offers a means of encouraging the adhesion, spreading and proliferation of cells cultured on its surface. This study assesses the efficacy of a modification procedure using surface analysis techniques and preliminary cell culture studies.

METHODS: A commercially available polyurethane was dissolved in dimethylacetamide and spin-coated on to glass discs. Amine groups were introduced to the surface using reversible swelling to sterically trap polyethyleneimine within the polymer network. Reductive amination was used to couple partially oxidised dextran to the amine groups. The Gly-Arg-Gly-Asp-Ser-Pro-Lys (GRGDSPK) domain of fibronectin or a recombinant fragment of fibrillin-1 was then covalently bound to the dextran layer again using reductive amination. The surfaces were characterised by FTIR and fluorescence analysis using fluorescamine. L929 murine fibroblasts were cultured on the surfaces for up to 4 days. Image analysis was used to assess the cell number and an Alamar Blue redox assay was used to quantify the metabolic activity of the cells.

RESULTS & DISCUSSION: Fluorescence analysis and FTIR spectra verified the amination and dextran attachment steps of the modification. The immobilisation of the peptides was verified with FTIR. The cells cultured on the GRGDSPK-modified surface proliferated extensively. The cell counts show that after 4 days there were more cells on this surface than on the positive glass control. The results of the Alamar Blue assay show an increase in the metabolic activity of the cells on this surface over the 4-day time period. The fibrillin-modified surface also supported cell proliferation to a greater extent than on the plain polyurethane (Fig 1). Cell number increased steadily over the 4 days with the quantity after 2.5 days similar to that on the glass control. The results of the Alamar Blue assay show an increase over the 4 days. As the dextran coating appeared to limit cell attachment it is clear that it is the peptides that have caused the high level of proliferation.



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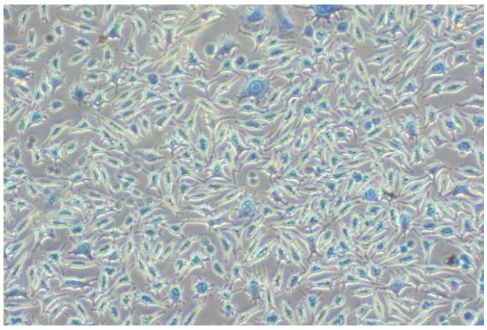

B

Fig. 1: L929 fibroblasts cultured for 4 days on A) plain polyurethane and B) fibrillin-modified polyurethane.

CONCLUSIONS: Both the GRGDSPK and the fibrillin-1 fragment caused an increase in cell adhesion and proliferation compared to the plain polyurethane. Further work will seek to analyse further the changes in cell behaviour including the cell cycle and extracellular matrix production.

ACKNOWLEDGEMENTS: This research was funded by grants from the BBSRC, EPSRC and MRC. We thank Dr Daniel Bax for providing the fibrillin fragments.

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Grafting of PEG-Macromonomers to Plasma Polymers Using Ceric Ion Initiation

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INTRODUCTION: Bioadhesion, the adsorption of proteins, cells, or bacteria to a surface can be extremely detrimental to the performance of medical devices. The consequences can range from fibrous encapsulation of implants to thrombus formation. Prevention of non-specific adsorption is therefore a key characteristic for many biomaterial applications and applying a non-fouling surface treatment can greatly improve the performance and lifespan of some medical devices. Poly (ethylene glycol) [PEG] is currently the most effective chemical modifier at reducing bioadhesion. Plasma polymers provide a thin, conformal, chemically active base on which to graft. It is proposed that grafting PEG onto plasma polymerised surfaces will confer non-fouling properties.

Ceric ion initiation is commonly employed to graft polymers to natural polysaccharides e.g. starch. It is thought that the initiation proceeds through a one-electron oxidation of hydroxyl groups. We have investigated the ceric ion initiated grafting of PEG-macromonomers to plasma polymers with a variety of functional groups including hydroxyl groups. To the best of our knowledge there are no literature reports of ceric initiated grafting from plasma polymers or of ceric initiated grafting of PEG-macromonomers.

METHODS: Radio frequency glow discharge plasma polymerisation of isopropyl alcohol was carried out in a tubular glass reactor to obtain functional surfaces which contain alcohol groups. Substrates used were fluorinated ethylene propylene tape and silicon wafers. Octadiene plasma polymer was used as a control hydrocarbon surface. Characterisation was carried out via X-ray Photoelectron Spectroscopy [XPS] with and without chemical labelling of alcohol groups using trifluoroacetic anhydride.

PEG-dimethacrylate and PEG-diacrylate were dissolved in water and combined with an aqueous solution of cerium prior to the introduction of plasma polymer samples. The influence of reagent concentrations, chemical nature of the plasma polymer and grafting time were investigated. The samples then analysed by XPS

RESULTS: Each monomer produced polymeric coatings that are thoroughly in accord with literature reports.

The success of ceric initiated grafting was demonstrated by the presence of a chemically shifted peak in the C1s narrow scan at 286.5eV binding energy. The intensity of this peak can be directly correlated with the amount of grafted material. In fig. 1 we show the influence of ceric ion concentration on PEG-diacrylate grafting. A higher concentration of ceric ions produces a larger amount of grafted PEG. In the absence of ceric the C1s spectra corresponded with that of the untreated plasma polymer. Polymer graft density is dependant on the plasma polymer chemistry, and concentration of initiator.

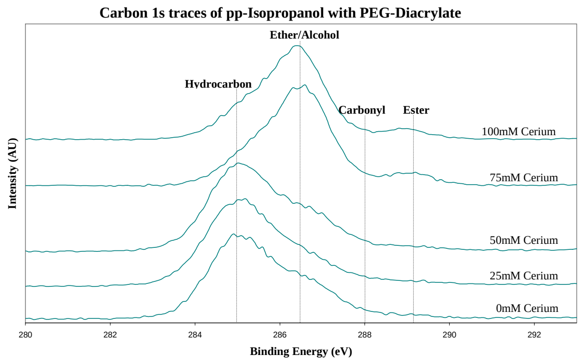

Fig. 1: C1s peaks of pp-isopropanol samples incubated with PEG-diacrylate and cerium ammonium nitrate of varying concentrations.

DISCUSSION & CONCLUSIONS: Ceric ion initiated grafting of PEG-macromonomers onto alcohol terminated plasma polymers is shown to be successful. Grafted PEG should prevent bioadhesion onto surfaces. Work is currently underway to investigate the non-fouling properties of the PEG grafted surfaces using protein adsorption studies.

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Identification of Calcium in Osteoid as a Result of Load Profile Utilising Backscattered Electron Detection in Combination with X-Ray Microanalysis

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INTRODUCTION: The ability to identify calcification of tissue-engineered bone in relation to constructs allows for modelling of tissue development and also monitoring of load profile effects. The effects of chemical agonists, for example Bay K8644, which augment L-type voltage-operated calcium channel (VOCC) opening times, can also be investigated for their effects on mineralisation relative to controls. Field emission scanning electron microscopy (FESEM) can be utilised in backscattered electron (BSE) mode allowing for identification of medium-density materials, for instance calcium, in comparison to low-density organic materials, for example cells and extracellular matrix (ECM)[1&2]. Following identification of medium-density material, appearing in images as higher contrast entities, these areas can be investigated for their chemical composition using energy dispersive X-ray microanalysis (EDX).

METHODS: Primary bone cells from rat were seeded in silicon tubes with 2mm internal diameter and cultured statically for 3 days in supplemented DMEM. Subsequently, cell-seeded tubes were subjected to either load or static conditioning in the presence/absence of Bay K8644 at physiological agonist levels for a further 3 days. Mechanical conditioning was induced via a perfusion-compression bioreactor[3] where constructs were subjected to 1% strain for 1 hour per day at 37°C. Samples were then fixed utilising a standard chemical fixation protocol[4] without post-fixation with osmium tetroxide, dehydrated in an ethanol series and critically point dried (CPD) prior to mounting and coating with 10nm carbon. All imaging and analysis was conducted using a Hitachi S-4100 FESEM equipped with an Autrata-modified YAG single crystal scintillation BSE and Oxford ISIS EDX detector.

RESULTS: Complimentary BSE and EDX was utilised to identify calcium crystals in primary bone cells cultured in tubes, *Figure 1*. Loaded samples exhibited increased amounts of medium-density material distributed throughout, however positive calcium identification was only made in Bay-loaded samples. Loaded cells exhibited rougher morphologies in comparison to smooth, flat non-loaded cells. Furthermore, loaded cells appeared to alter cell-cell contacts resulting in cluster and group formations in comparison to static samples, where cells appeared to be contact inhibited.

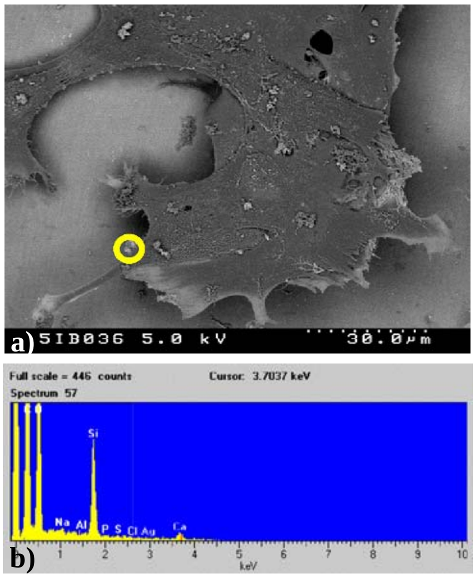

Fig. 1: BSE identification of medium-density materials, a). Highlighted area was then investigated using EDX, b), where calcium was observed to be a primary constituent (peak at 3.7037keV).

DISCUSSION & CONCLUSIONS: BSE imaging in combination with EDX analysis presents a good platform to study bone cell response and mineralization as a result of external mechanical and chemical cues. Furthermore, tubular scaffolds may be used to represent the loading profile found in a single pore of a porous scaffold.

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Cellular Responses to External Mechanical Stimuli When Seeded to 3d Collagen

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INTRODUCTION: Collagen is a naturally occurring visco-elastic protein and widely used biomaterial in Tissue engineering. Mechanical stimulation of cell seeded collagen constructs and its effects on cell orientation, intracellular signalling and molecular responses have been reported in literature[2]. Monitoring of cellular responses to mechanical stimulation include synthesis of active regulatory molecules such as growth factors or hormones, changes in matrix synthesis, cell alignment and enzyme release. The aim of this study was to investigate cellular responses to pre strained, stiffer and more organised collagen bio-artificial matrices.

METHODS: Human Dermal Fibroblast (HDF) as well as Neonatal Human Dermal Fibroblasts (NHDF) cells seeded (1million/ml) collagen constructs were subjected to 0%, 5% and 10% pre-strain using a computer driven tensional loading device (t-CFM) capable of unidirectional loading. force[1] generated by the cells monitored for 24 hours. Furthermore, cells were Fetal Calf Serum (FCS) starved for 1 hour post strain, to stop/control cell attachment and spreading. FCS was then added, at 1 hour, and contraction force was again monitored for 24 hours.

RESULTS: Results showed significant reduction on the contraction force generated by the cells when constructs were pre strained (5% and 10%) compared to non pre-strained (0%). FCS starvation for 1 hour did not alter this effect (Figure 1). However it did introduce a more rapid contraction slope. Furthermore, contraction force generation was increasingly delayed as the pre strain increased and delayed even further when constructs were FCS starved.

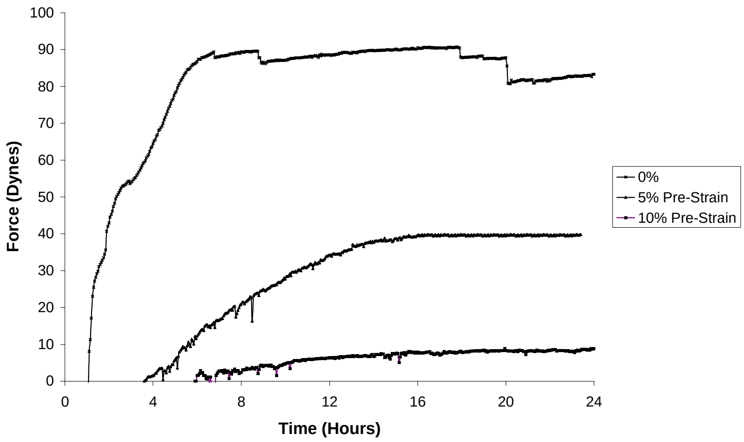

Figure 1. HNFF's contraction profile following pre strain and 1h FCS starvation

DISCUSSION & CONCLUSIONS: The increased stiffness of a Tissue Engineered construct by means of external mechanical stimuli will have to be tailored to account to elicit predictable cellular responses. Furthermore, FCS cell starvation will have to be taken into account on rate as well as initiation time of cellular contractile forces.

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The Use of Thermo-Sensitive Chitosan as an Injectable Carrier for Bone Tissue Engineering

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INTRODUCTION

Due to its biocompatibility, biodegradation properties and potential angiogenic potential, the natural biopolymer chitosan has been used in the biomedical field. In combination with glycerol phosphate (GP – disodium salt), this cationic polyelectrolyte becomes thermosensitive in diluted acids and can undergo gelation around body temperature [1]. This property makes it promising for use in injectable tissue engineered bone, by acting as a delivery vehicle for cells and biomaterial microparticles.

In this study, we monitored the gelation time of 1% chitosan-HCl-GP solutions by adjusting the GP concentration (5-20%), and evaluated the cytotoxicity of the gels by monitoring the growth rate (proliferation) of goat bone marrow derived stem cells (gMSCs).

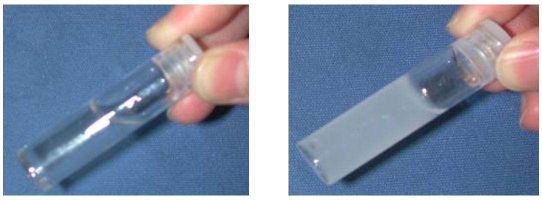
MATERIALS AND METHODS

Chitosan (BMSI, China) with a nominal 90% deacetylation rate (DDA) was dissolved in HCl to form a 1% solution. Subsequently, 5%, 10%, 15% or 20% (w/v) glycerol phosphate (GP) was added drop-wise to form a uniform solution and the pH was measured. The gelation or polymer transition time of the solutions was measured by soaking them in a 37°C water bath.

Cytotoxicity of the gels was evaluated by an extraction test according to ISO10993-5. In short, the chitosan-GP gels (5-20% GP concentration) were immersed in normal culture medium (1 ml per 1.25 cm^2 gel surface area) for 24 hours at 37°C/5% CO_2. The extraction fluid was then removed and placed onto a semi-confluent layer of gMSCs in 12-well plates. Cytotoxicity (cell proliferation) was measured after 24 and 48 hours using the Alamar blue assay. Results were compared to a negative- (cells grown in normal culture medium) and a positive control (cells grown in PVC extract medium).

RESULTS

Before polymer transition (gelation), all chitosan-GP solutions had pH values around the physiological range (table 1). The gelation time of the various chitosan-GP solutions (with various GP concentrations) was time-dependent. All solutions could gel at 37°C, while those with more than 10% GP formed gels from seconds up to a few minutes (table 1). During the gelation process, the gels turned from transparent to opaque (figure 1).



a) 22°C **b) 37°C**

Figure 1. Chitosan (1% w/v)-GP solution before gelation (a) and after gelation (b)

Cytotoxicity of chitosan-GP gels increased with increasing GP concentrations (table 1). Interestingly, extraction fluids from chitosan-GP with GP concentrations of up to 10% enhanced the proliferation of gMSCs 4 to 11-fold (table 1) as compared to the negative control. GP concentrations of 15% gave a growth inhibition to 65% of the control, while 20% GP was highly cytotoxic.

Table 1. Relation of GP concentration in chitosan-GP solutions to pH, gelation time, cell proliferation

GP Concentration (w/v)	5%	10%	15%	20%
pH before Gelation	7.0	7.3	7.5	7.6
Gelation Time at 37°C (min)	720	60	4	0.7
Cell proliferation compared to NC*	1100 %	400 %	65%	0%
Toxicity	none	none	mild	severe

*NC= negative control.

DISCUSSION & CONCLUSIONS

Chitosan with low GP concentrations (5-10%) is not cytotoxic but enhances the growth and proliferation of gMSCs compared to negative control. Nevertheless, long gelation times associated with these GP concentrations are a possible obstacle, which might be solved by adjusting the molecular weight, purification, or increasing the DDA of chitosan. These are the subjects of our future investigations.

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Dynamic compression influences the biochemical response of human mesenchymal stem cells cultured in agarose constructs

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INTRODUCTION: Mesenchymal stem cells have the potential to differentiate into the chondrogenic lineage and may therefore provide an alternative cell source for implantation in tissue engineered repair systems [1]. Mechanical compression is known to modulate the metabolic activity of chondrocytes [2]. Accordingly, the current study examines whether dynamic compression could influence the metabolism of human mesenchymal stem cells cultured in agarose constructs.

METHODS: Human mesenchymal stem cells (hMSC) were expanded in monolayer culture for up 6 passages in high glucose DMEM + 10 % FBS. Cells were subsequently seeded in 3 % agarose type VII (4 x 10^6 cells.ml^{-1}) and incubated under free-swelling conditions for a further 2, 4 or 6 days in 1 ml of a defined culture medium supplemented with 10 ng.ml^{-1} TGFβ_3 (all from Cambrex Bioscience, Wokingham, UK).

In a separate experiment, a fully characterised cell-straining system (Zwick Testing Machines, Leominister, UK) was used to apply dynamic compression to constructs [2-3]. Constructs were subjected to 1.5 hour compression, with 4.5 hour unstrained period, for a total period of 48 hours. Intermittent compression was applied in a dynamic manner, with a compressive strain amplitude of 15 % at 1 Hz up to 2, 4 or 6 days of culture. All constructs were incubated in 1 ml of a defined medium supplemented with 10 ng.ml^{-1} TGFβ_3.

Constructs and medium were subsequently analysed using standard biochemical techniques [2-3]. Total DNA was determined using the Hoescht 33258 method and glycosaminoglycan (GAG) was determined using 1,9-dimethylmethylene blue (DMB) assay.

RESULTS: Figure 1 illustrates the effects of dynamic compression on DNA and GAG content in unstrained and strained constructs and constructs cultured under free-swelling conditions for up to 6 days. Total DNA and GAG levels were similar in free-swelling constructs cultured at each time period. No differences were found for DNA levels in unstrained and strained constructs cultured for 2 or 4 days. The application of dynamic compression resulted in a significant increase in DNA levels after 6 days of culture ($p<0.001$; Fig. 1A). Dynamic compression inhibited GAG content at day 2 and 4 and significantly enhanced GAG levels after 6 days of culture ($p<0.001$; Fig. 1B).

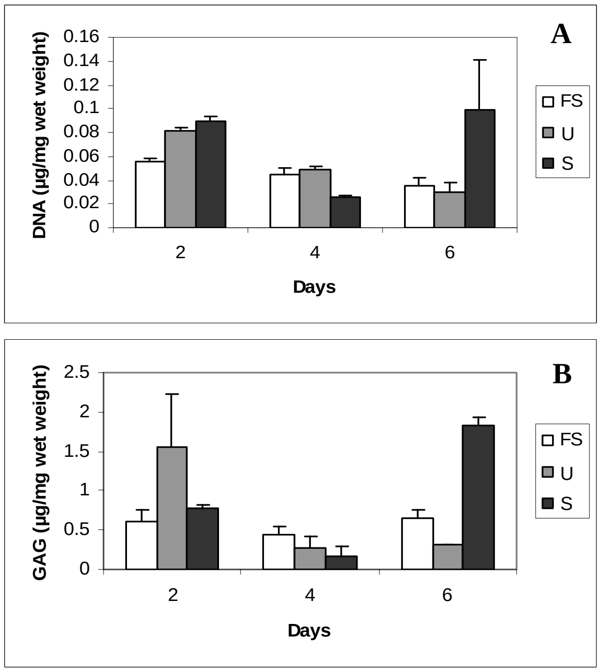

Fig 1: Total DNA (A) and GAG (B) content of constructs cultured under free-swelling (FS) conditions or in unstrained (U) and strained (S) constructs subjected to dynamic compression.

DISCUSSION & CONCLUSIONS: Preliminary results show that hMSC respond to dynamic compression by increasing DNA and GAG levels. This response is dependent on the culture period. Further study will determine if mechanical stimuli will influence the chondrogenic potential of mesenchymal stem cells.

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ACKNOWLEDGEMENTS: This work was supported by the BBRSC.

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Dynamic Compression Counteracts IL-1β Induced INOS and COX-2 Activity by Human Chondrocytes Cultured In Agarose Constructs

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INTRODUCTION: ·NO and PGE_2 are derived from the inducible nitric oxide synthase (iNOS) and cyclo-oxygenase 2 (COX-2) enzymes, which are potentially important pharmacological targets in osteoarthritis (OA) [1]. It is unclear, however, whether dynamic compression acts to abrogate IL-1β mediated ·NO and PGE_2 release via iNOS/COX-2 inter-dependent pathways. The present study tests the hypothesis that dynamic compression can alter iNOS and COX-2 activity in human chondrocytes.

METHODS: Human chondrocytes were seeded in agarose gel and incubated for 48 hrs in radiolabelled medium containing 0 or 10 $ng.ml^{-1}$ of IL-1β + 1 mM L-NIO (NOS inhibitor) or 0 to 2 mM 1400W (iNOS inhibitor) or 0.1 to 100 μM NS-398 (COX-2 inhibitor). In a separate experiment, a fully characterised cell-straining system was used to apply 15 % dynamic compressive strain (1 Hz, 48 hrs) to constructs cultured in radiolabelled medium supplemented with 0 or 10 $ng.ml^{-1}$ IL-1β and/or 1 mM L-NIO or 2 mM 1400W or 100 μM NS-398 [3]. Cell metabolism was quantified using standard biochemical techniques [2-3].

RESULTS: IL-1β significantly enhanced nitrite release, an effect which was reversed by L-NIO and 1400W at all concentrations tested ($p<0.001$). The IL-1β induced PGE_2 release was completely inhibited by NS-398 ($p<0.001$) and partially reversed with L-NIO or 1400W (both $p<0.01$). Nitrite release was unaffected by the presence of NS-398 in IL-1β-stimulated constructs. The IL-1β-induced inhibition of $[^3H]$-thymidine and $^{35}SO_4$ incorporation was partially reversed with L-NIO (both $p<0.01$) or 2 mM 1400W (both $p<0.001$). By contrast, the IL-1β-induced inhibition of $[^3H]$-thymidine and $^{35}SO_4$ incorporation was not influenced by NS-398.

Figure 1 illustrates the percentage change from unstrained control values for nitrite and PGE_2 release for constructs subjected to 15 % dynamic compression. Dynamic compression-induced inhibition of nitrite was increased by IL-1β, when compared to untreated constructs ($p<0.001$, Fig. 2A). L-NIO, NS-398 and 1400W abolished this effect. Dynamic compression did not influence PGE_2 levels in untreated constructs (Fig. 2B).

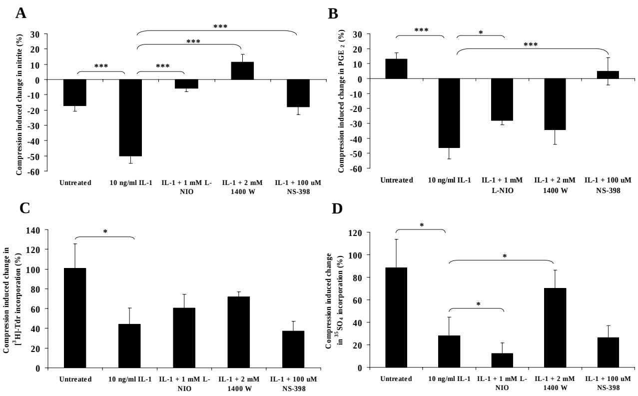

Fig 1. *Effects of L-NIO, 1400W and NS-398 in IL-1β stimulated constructs subjected to dynamic compression.*

The IL-1β-induced PGE_2 release was reversed by dynamic compression. The response to strain was reduced in the presence of L-NIO and abolished with NS-398 but was not influenced by 1400W. The IL-1β-induced inhibition of $[^3H]$-thymidine was not influenced by L-NIO, 1400W or NS-398. Strain-induced stimulation $^{35}SO_4$ incorporation in IL-1β stimulated constructs was reduced in the presence of L-NIO and enhanced by 1400W.

DISCUSSION: The current data demonstrate that IL-1β enhanced ·NO and PGE_2 release via iNOS-driven-COX-2 dependent pathways. This response could be reversed by dynamic compression. These findings will provide new insights in the development of pharmacological or biophysical treatments for cartilage disorders.

ACKNOWLEDGEMENTS
This study was supported by a project grant from The Wellcome Trust, 073972.

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The Mechanical Strength of Collagen Gels Containing Glycosaminoglycans and Populated with Fibroblasts

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INTRODUCTION: Collagen based scaffolds are widely used for tissue engineering strategies; many are designed to biodegrade gradually and become populated with host cells. The effect of infiltration by host cells on the mechanical properties of the scaffolds is unclear. We have used fibroblast populated collagen lattices to study the effects of the presence of 3T3 mouse fibroblasts on the mechanical properties of collagen gels containing glycosaminoglycans (GAGs) crosslinked with the carbodiimide, 1,1-carbonyldiimidazole (CDI), and the polyamine, putrescine (Put).

METHODS: The gel substrates were prepared as described by Osborne et al [1]. Briefly, gels containing 0.3% (w/v) type I collagen ± the GAG, chondroitin-6-sulphate (0.6mg/ml), were allowed to set for 4h before some were treated with 1M CDI and/or 0.5M Put for 18h. Gels were set in a rectangular Perspex casting plate 25mm x 60mm and a polymer mesh (PVC, 38 hole/cm^2) was incorporated at either end of the gel to facilitate gripping the mesh in the Instron tensile testing machine. Load, stress, strain at failure and Young's modulus (Mod on Table 1) were calculated for each gel composition in the absence and presence of 3T3 cells. Where 3T3 cells were added, the cell density was 2.5 x 10^4 cells/cm^2 and cell viability in the gels was determined by confocal microscopy.Both cell seeded and unseeded gels were incubated for 6 days in Dulbecco's medium containing 10% foetal calf serum at 37^{o}C in air/5%CO_2 before being tested.

RESULTS and DISCUSSION: In the absence of cells, incorporation of either GAG and/or the crosslinkers transformed the gel into a stronger stiffer gel, as shown on Table 1A by the increases in the values for the load, stress, and Young's modulus compared with those measured in plain collagen gels. Regardless of chemical composition, the presence of 3T3 cells weakened the gel considerably, and decreased the Young's modulus. This marked effect can be observed by comparing the values for each parameter in gels of the same composition in Tables 1A and B. The mechanical advantages attained by the addition of GAG and crosslinkers to the gels, were overcome by the effect of the fibroblasts. In these gels the process of scaffold degradation, which *in vivo* would allow cell migration and proliferation, predominates over synthesis of new matrix by the cells. It may be possible to modify the balance between these two processes and influence scaffold remodelling.

*Table 1. The mechanical properties of collagen (Coll) gels in the absence (A) and presence of 3T3cells (B) after 6 days incubation. Results are mean±S.D. of n=5. *P<0.05 comparing the treated collagen gels with plain collagen gels in each Table, by ANOVA followed by Dunnett's test. All parameters were statistically different when cell seeded and unseeded gels with the same chemical composition were compared by unpaired Student's t-test.*

A. Gels without cells

P	Coll	GAG/ Coll	Coll + CDI + Put	GAG / Coll + CDI+Put
Load (N)	0.9± 0.1	1.2± 0.1*	0.9± 0.1	1.09± 0.09*
Strain %	11.6 ±1.3	8.4± 0.6*	11.4± 1.1	11.78± 0.88
Stress (kPa)	13.9 ±4.6	19.7± 3.3*	25.2± 1.7*	22.11± 3.5*
Mod (MPa)	0.1± 0.04	0.2± 0.02*	0.2± 0.03*	0.15± 0.03*

B. Gels with 3T3 cells

P	Coll	GAG/ coll	Coll + CDI + Put	GAG / Coll + CDI+Put
Load (N)	0.7± 0.1	0.6± 0.1	0.7± 0.2	0.5± 0.1*
Strain %	13.8± 2.6	13.3± 1.8	10.8± 1.1*	10.9± 2.2
Stress (kPa)	7.8±0. 9	7.1± 1.1	8.0± 0.7	5.8± 0.4*
Mod (MPa)	0.05± 0.01	0.04± 0.01	0.05± 0.01	0.04± 0.004

REFERENCES:[1]C.S. Osborne, W.H. Reid, M.H. Grant (1999) *Biomaterials* **20**: 283-290.

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Filapodial Sensing of Nanotopography in Osteoprogenitor Cells

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INTRODUCTION: Micro- and nanotopography can have been shown to be able to change cell adhesion, morphology, cytoskeleton, proliferation and differentiation. It is proposed that filopodia presented at the cells leading edge are able to gather special information and thus 'sense' nanotopography around cells. Here, 120 nm diameter pits in both hexagonal and orthogonal arrangement were used to allow observation of filopodia production and interaction from primary human mesenchymal bone marrow stromal cells (HMSC).

METHODS: Electron beam lithography was used to fabricate arrays of pits with diameters of 120 nm and centre to centre spacing of 300 nm in either orthogonal or hexagonal arrangement in Si. Using sputter coating and electroplating, Ni intermediaries were made from which replicas of the Si masters were produced in polymethylmethacrylate (PMMA).

HMSC's at passage 1 were cultured on the structures for 4 days at an intial density of 1 x 10^4/ml in alpha-MEM media with 10% FCS. Half the samples were then fixed with 1.5% gluteraldehyde and prepared for SEM analysis using standard methods. A Hitachi 800 scanning electron microscope running at 5kV was used to observe filopodia.

The other half of the samples were fixed in 4% formaldehyde and Coomassie blue stained. Image J was then used to calculate the perimeters of cells in pixels. Students t-test (* = $p<0.05$) was used to test significance.

RESULTS: Filopodia were observed from HBMCs on each material (fig 1). Following normalizion of the results with cell perimeter, it was observed that cells on nanotopography produce significantly more filopodia compared to those on planar control (fig 2). Filopodial interaction with pits (filopodia deemed to be located at the edge of a pit or in a pit) on the orthogonal and hexagonal structures was detected at 67 and 66 % respectively. This incidence is highly significant given the pits cover 14% of the material surface area when calculating for just the pits or 27% if also including the lips produced by imprinting.

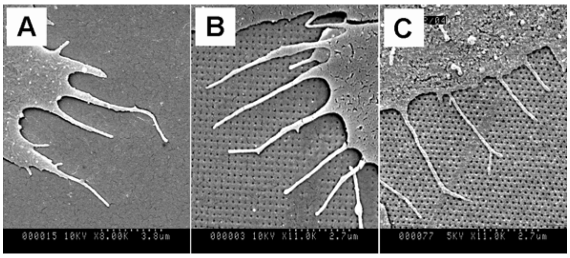

Fig 1: HMSC filopodia on (A) flat control PMMA, (B) orthogonal nanopits and (C) hexagonal nanopits.

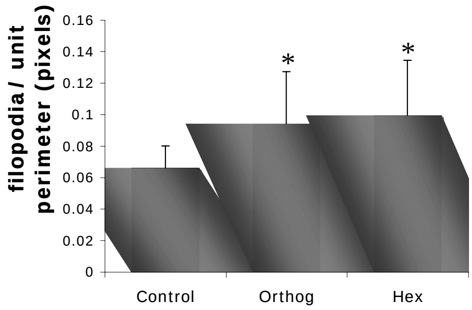

Fig 2: Quantification of number of filopodia / unit perimeter on control and test materials.

DISCUSSION & CONCLUSIONS: Human bone marrow stromal cells are able to detect differences in topography at the nano level. This result adds further evidence that filopodia are important structures when considering cell sensing of their environment. Intriguingly, the results show a higher incidence of interaction than has been previously observed with human fibroblasts on electron beam fabricated anno structures (54 % with the orthogonal arrangement)[1]. The 10% increase in filopodial sensing observed with the human bone marrow stromal cells implicate a possible role for sensing in cell differentiation. This area remains to be fully explored in the design of tissue scaffolds.

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ACKNOWLEDGEMENTS: Matthew Dalby is a BBSRC David Phillips Fellow and Nikolaj Gadegaard is a Royal Society of Edinburgh Fellow. Their research is supported through these routes.

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Surface Engineering of Alginate Scaffolds for the Attachment and Differentiation of Embryonic Stem Cells

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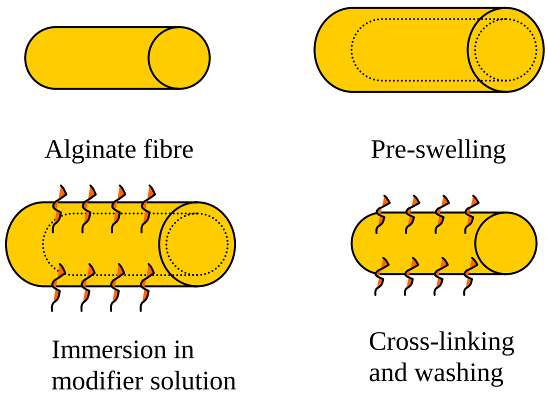
INTRODUCTION: Embryonic stem (ES) cells are a potential cell source for tissue engineering and regenerative medicine due to their capability of unlimited self-renewal and multilineage differentiation. One approach to generate a three-dimensional functional tissue is by use of an ES cell/scaffold composite. To achieve this, an important issue is to encourage ES cell attachment, growth, and selective differentiation on the scaffold.

Alginate, a naturally occurring polysaccharide, has been used as a hydrogel scaffold for tissue regeneration, due to its non-toxicity, biodegradability, and availability. However, the lack of specific cellular interactions limits its potential wider applications, as the initial cell adhesion and growth on the substrate is critical to the regenerative process. The aim of this work was to introduce bioactive molecules within alginate fibres or scaffolds by a physical entrapment process to promote ES cell attachment and differentiation.

METHODS: The entrapment process of bioactive molecules into alginate fibres is illustrated in Figure 1.[1] Briefly, alginate fibres were pre-swollen in a sodium rich $NaCl/CaCl_2$ solution ($NaCl/CaCl_2$ ratio (w/w): 20/1, NaCl concentration: 10 % w/v) for 5 min before exposure to 0.1 % w/v poly(L-lysine) coupled GRGDS (PLL-GRGDS), a well-established cell adhesion peptide sequence, for 10 min. The fibres were then immersed in barium chloride solution (5 % w/v) for 15 min, followed by washing extensively with distilled water and drying in air at ambient temperature to a constant weight. The attachment of undifferentiated murine ES cells (CEE line) fluorescently labeled with Cell Tracker™ green CMFDA on the alginate fibres before and after surface entrapment process was studied. The osteogenic differentiation of ES cells within alginate scaffolds entrapped with PLL-GRGDS was also investigated.

RESULTS: As shown in Figure 2, there was minimal ES cell attachment on the original alginate fibres. However, the surface entrapment of PLL-GRGDS into the alginate fibres improved the ES cell adhesion. It has also been demonstrated that this surface modification technique provided an effective approach to promoting ES cell osteogenic differentiation within alginate scaffolds.

Fig. 1: Modification of pre-formed alginate fibres by a physical entrapment process.



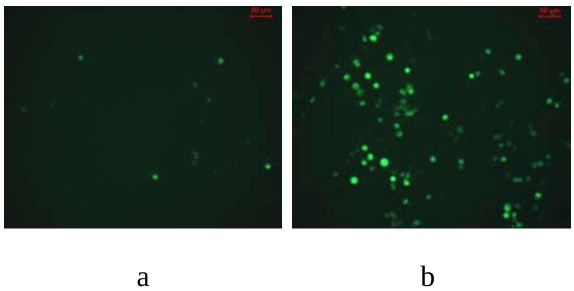


Fig. 2: Murine ES cell attachment behaviour on the different alginate fibres. a) non-treated alginate fibres; b) PLL-GRGDS treated alginate fibres, PLL-GRGDS concentration: 0.1% w/v.

DISCUSSION & CONCLUSIONS: These results indicate the feasibility of this physical entrapment process in introducing bioactive molecules within alginate scaffolds for improved ES cell attachment/recruitment.

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ACKNOWLEDGEMENTS: We thank the EPSRC for funding.

Growth Characteristics of Chondroprogenitor Cells *In Vitro*

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INTRODUCTION: Cell transplantation therapy has shown promise in augmenting the inadequate repair mechanisms within damaged articular cartilage. It has been reported that the proliferative potential of autologous chondrocytes decreases with increasing age (1) posing a major problem in providing adequate cell numbers for repairing osteoarthritic lesions. Hence, alternative sources of donor cells have been sought in order to make cell based therapies more readily available for patients. The isolation of a putative chondroprogenitor (CP) cell from the surface zone of articular cartilage by differential adhesion to fibronectin (2) broadens the horizon for articular cartilage repair strategies. Some of the problems associated with the use of MSCs such as the lack of integration and unstable phenotypes and reduced proliferation capacity may potentially be overcome using CP cells.

METHODS: Chondroprogenitor cells isolated by differential adhesion and expanded approximately 20 population doublings were resuspended in the experimental culture medium at a concentration of 2 x 10^5 cells per 10 μL medium. Aliquots of cells were placed at the centre of either 12 well culture dishes or 8 well chamber slides (Labtek, Nunc International, IL). Micromass cultures were incubated in a humidified 37°C, 5% CO_2 air incubator for 5 hours prior to being flooded very carefully with matching medium. The base medium used in micromass culture studies was DMEM (containing 4500 mg/L D-glucose, 4 mM L-glutamine), 1 mM sodium pyruvate, 0.1% Gentamycin, 50 μg $mL^{-}1$ ascorbate-2-phosphate, 10 mM HEPES. To the latter medium was added either insulin-transferrin-selenium (ITS; 10 mg $mL^{-}1$, 5.5 μg $mL^{-}1$, 6.6 ng $mL^{-}1$, respectively; Gibco) and/or TGF β1 (5 ng $mL^{-}1$; Peprotech).

RESULTS: Chondroprogenitor clones were isolated and expanded for a minimum of 20 population doublings. Clones were grown as micromasses and formed spheroid aggregates, a typical stem cell behavioural characteristic. All spheroids were alcian blue positive. When grown in the presence of TGFβ1 and ITS we observed that a homogenous cell mass was formed (Figure 1), whereas individual growth factor addition enhanced spheroid formation. RT-PCR analysis showed that TGFβ1 immediately activates Sox9 expression on day 0 following micromass formation but that expression falls below detectable levels after 3 days. In the presence of ITS, Sox9 transcripts appear after day 1 and are maintained over 7 days of culture.

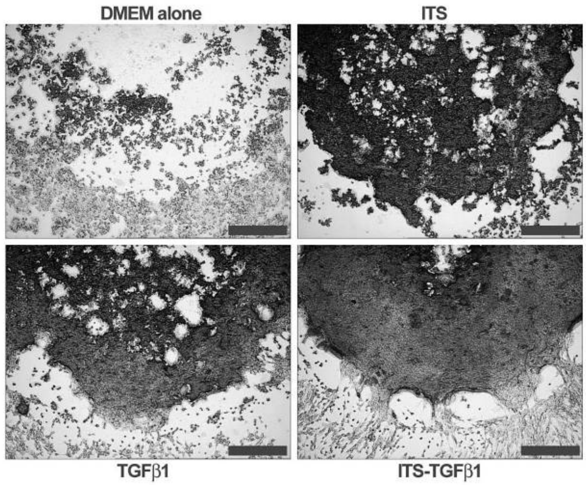

Figure. 1: Alcian Blue (pH 1) staining of chondroprogenitor clones grown as micromass cultures. Bar 500 μm.

DISCUSSION & CONCLUSIONS: Our work shows chondroprogenitors grown in micromass culture recapitulate aspects of early development in that they form condensations or spheroid bodies. Addition of growth factors to the basal medium led to an increase in sulphated glycosaminoglycans (GAG) being deposited in micromasses following the order; DMEM alone<plus TGFβ1<plus ITS<TGFβ1 + ITS. Synergistic activity of TGFβ1 and ITS may account for increases in GAG deposition and increased metabolic activity and this link is being investigated further.

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Regulation of Hypertrophic Gene Expression in Chondrogenic Cultures of Human Bone Marrow Mesenchymal Cells

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INTRODUCTION: Bone marrow mesenchymal cells (MSC) are a potentially useful source of progenitor cells for cartilage tissue engineering. Human MSCs can be directed in culture to assume a cell fate with some characteristics of articular chondrocytes; however the expression profile of these chondrogenic cultures has also been shown to include elements of a hypertrophic chondrocyte phenotype such as collagen type X [1]. In previous studies we have also demonstrated the expression of other molecules characteristic of chondrocyte terminal differentiation such as Indian hedgehog (IHH) and components of the parathyroid hormone-related peptide (PTHrP) signalling pathway in MSC chondrogenic pellet cultures. This study was performed to investigate the effects on MSC chondrogenic differentiation of molecules thought to be important mediators in the interacting pathways underlying the development and control of chondrocyte proliferation and hypertrophy (BMP2, FGF2 and PTHrP) [2].

METHODS: Adherent cultures of human bone marrow mesenchymal cells were derived from bone marrow mononuclear cells (Cambrex) and expanded in medium containing FGF2. Chondrogenic pellet cultures (5 x 10^5 cells) were performed as described [3] and were additionally supplemented with BMP2, FGF2 or PTHrP. Time course experiments were sampled at 1,3,7 and 14 days and pellets were analysed for DNA/Glycosaminoglycan (GAG) content, gene expression by quantitative RT-PCR, and immunohistochemistry.

RESULTS: Time course analysis of gene expression in the chondrogenic pellet cultures revealed an unexpected apparently co-ordinate expression of collagen II and X. Other markers of chondrocyte hypertrophy such as PTHrP receptor and Indian hedgehog (Ihh) were also upregulated over 14 days, though not in the temporal or spatial manner characteristic of the growth plate. Addition of BMP2 to the pellet cultures during the 14 day time course resulted in several key changes compared with controls. BMP2 treatment increased cellular proliferation and accelerated the onset of collagen II expression to a higher steady state level with a more even distribution of protein, whilst having no effect on collagen I mRNA levels. However, collagen X expression was stimulated in a similar fashion to that of collagen II, and other markers of the hypertrophic phenotype (Ihh, PTHrP-R) were upregulated earlier in the time course. In contrast, when FGF2 was present during the time course there was a small degree of inhibition of proliferation and a reduction of about one half in the amount of GAG deposited into the matrix on a per cell basis at day 14. FGF treatment reduced expression of the hypertrophic marker genes, but also lowered the expression of collagen II and aggrecan. PTHrP treated pellet cultures accumulated size and weight at the same rate as controls. Despite this, GAG appeared to be deposited more slowly in the PTHrP treated cultures. The PTHrP treatment completely down regulated expression of Ihh and lowered the expression level of collagen X mRNA by 100 fold at day 14. Up regulation of collagen II expression was also inhibited by PTHrP addition, though to a lesser degree - a 20 fold lower steady state level was seen at day 14.

DISCUSSION & CONCLUSIONS: The 14 day chondrogenic cultures of hMSC appeared to be a composite of articular and hypertrophic chondrocyte phenotypes. This apparent hypertrophy might represent a problem in an implanted articular tissue engineered device, if local influences in the joint environment were unable to reverse or control the differentiation state of the cells. Our results showed that cells in the pellet cultures were able to respond to at least some of the pathways thought to control chondrocyte terminal differentiation and suggest the promise that the differentiation of hMSCs into chondrocytes could be controlled by appropriate factors if they were applied in the correct temporal and spatial manner.

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Fibroblasts Transformed to a Wound Healing Phenotype Accumulate a Hyaluronan-Rich Extracellular Matrix Through Reduced Degradation

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INTRODUCTION: Wound healing involves a complex sequence of interactions leading to tissue repair and resolution of injury. Some injuries, however, fail to resolve, resulting in chronic scarring and fibrosis. A common goal of groups investigating scarring and wound healing is understanding the mechanisms differentiating between scarred and scar-free healing. Fibroblasts are central to both the healing and scarring processes and have distinct phenotypes depending on the site from which they are isolated.

The importance of fibroblasts with a myofibroblastic phenotype to the progression of wound healing has been emphasised by the work of several groups in recent years. We have recently investigated differences in glycosaminoglycan (GAG) synthesis, structure and function associated with the possession of a myofibroblastic phenotype compared to that of a normal fibroblast. A major finding was that the important linear polysaccharide, hyaluronan (HA) synthesis was more than 4 fold increased in myofibroblasts. HA is ubiquitously distributed in the extracellular matrix (ECM) and is implicated in the regulation of diverse biological processes including morphogenesis, angiogenesis, tumourigenesis, inflammation, cellular transformation and tissue repair through a direct effect on cell proliferation, phenotype and migration. The present study investigated the synthesis and turnover of HA by these cells.

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METHODS: Fibroblasts were differentiated to myofibroblasts by treatment with TGFβ1. HA synthesis was measured by incorporation of ^{3}H-glucosamine over 24 hrs. HA size was determined following ion exchange and size exclusion chromatography. mRNA levels for hyaluronan synthases (HAS) or hyaluronidases (HYAL) were assessed by RT-PCR. HYAL activity was visualised by HA zymography and degradation of ^{3}H-HA. HYAL was localized by immunohistochemistry

RESULTS: Upto 4 fold more HA was secreted into the culture medium and pericellular matrix following myofibroblastic differentiation. Inhibition of HAS activity and analysis of mRNA levels, showed that this was not due to induction of HAS enzymes or the increased synthesis of HA. Rather exogenously supplied HA and endogenously synthesised HA were degraded at a reduced rate by myofibroblasts. Immunoblotting of culture supernatants demonstrated more HYAL 1and HYAL 2 in the myofibroblast medium suggesting it had been shed or secreted. Acidification of the medium to the HYAL pH optimum, however, showed that most of this enzyme was inactive. Immunolocalisation of HYALs and the HA receptor CD44 by confocal microscopy showed a much more diffuse pattern of expression in myofibroblasts, while they were very discretely localised in fibroblasts.

DISCUSSION & CONCLUSIONS:

Both the endogenously increased expression and the over-expression of HA synthase 2 with a resulting increase in HA synthesis have previously been linked to cellular transformation. The results of the current study, however, found no causal link between HA synthesis and myofibrblastic differentiation. Rather there is a major reorganisation of the mechanism for degrading HA which leaves the cell surrounded by an extensive HA-rich matrix.

In vivo this matrix is likely to provide a stablising environment in which the myofibroblast exists protected from the modifying effects of other cells. To alter this phenotype it may therefore be possible to reverse the changes in HYAL reorganisation. It may be that this would contribute to the generation of a fibroblastic phenotype with the potential for scarless wound healing.

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Biomimetic Synthesis of Apatite on Polycaprolactone for Bone Tissue Engineering

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INTRODUCTION: Biodegradable polymers such as polycaprolactone (PCL) have been used for bone tissue engineering (TE) scaffolds. The material surface must be modified to support the cell attachment, proliferation and differentiation. It has been reported that nanocrystalline apatite can facilitate the osteo-inductivity and osteo-conductivity of the polymer scaffolds [1]. The objective of the work is to produce an apatite coating on PCL using a biomimetic method. To induce the apatite formation on PCL, a chemical treatment was carried out to functionalise the PCL surface with carboxyl ligands.

METHODS: PCL (CAPA®6500/6800 Solvay) with a MW of 120,000 was used as film substrates. They were surface treated with NaOH solutions of different concentrations (1M, 5M, and 10M) and subsequently dipped in 0.4M of Ca^{2+} and PO_4^{2-} solutions alternatively. After rinsing and drying, the samples were immersed in a stimulated body fluid (SBF) at 37°C. The SBF solution was prepared according to a newly improved procedure[2] to minimize the precipitation. Scanning electron microscopy (SEM) with an EDS attachment was used to characterize the morphology and chemical composition of apatite coatings. Attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR) was used to analyse the surface chemistry of PCL films before and after treatments.

RESULTS: Figure 1 shows the surface treated PCL samples after immersing in SBF for different time. After 14 days, apatite was formed on the PCL. EDS analysis showed that the Ca/P ratio of the apatite was 1.63, close to that of hydroxyapatite (1.67). When the immerse time was increased to 30 days, the Ca/P ratio was reduced to 1.44, which was between that of tricalcium phosphate (1.50) and octacalcium phosphate (1.33). Platelike morphology was observed at high magnification (Fig.2). FT-IR results (Figure 3) showed gradual changes when the PCL was immersed in SBF. P-O peaks around 1000 cm^{-1} were only detectable after 14 days, which is in agreement with the SEM observation.

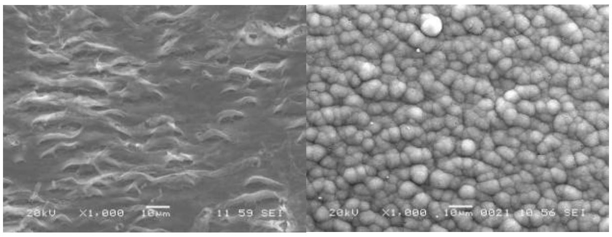

Fig. 1: 5M NaOH surface treated PCL films incubated in 1xSBF solution for different time: left-7days; right-14 days.

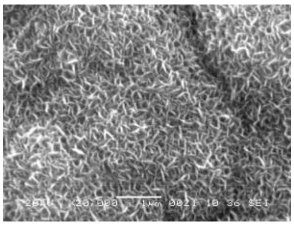

Fig. 2: SEM micrograph of the PCL film surface treated with 1M NaOH, showing platelike apatite formed after 30 days in SBF.

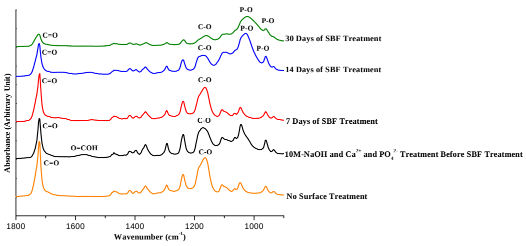

Fig. 3: FT-IR spectra for 10M NaOH treated PCL films at different stages.

DISCUSSION & CONCLUSIONS: NaOH surface treatment of PCL is crucial in inducing the apatite formation. The reason is mainly due to the PCL hydrolysis, resulting in chain scission of the polyester chains in PCL and formation of carboxylic acid ligands on the surface. FT-IR results clearly show a new peak around 1560 cm^{-1} which is representative of -COOH. This peak increases as the NaOH concentration increase. Carboxyl ligands have been reported to induce apatite formation through strong binding to positively charged Ca^{2+} ions to form nuclei and undergo subsequent growth to apatite.

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Effects of Cyclic Loads on Transport of Fluid and Solutes in Cell Seeded Constructs

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INTRODUCTION: Repetitive compression of soft tissues affects tissue mechanical properties and metabolic activities [1]. The effect is attributed, in part, to the movement of water and solutes in extracellular matrix, which alters the mechanical, e.g. fluid shear stress and chemical, e.g. growth factors, cytokines and hormones microenvironments for cells in the tissue [2]. To quantify contributions of external cyclic loads on solute transport, a theoretical model is built that mimics experimental set-ups of mechanical compression of cell seeded constructs with different loading conditions. Effects of loading frequency and amplitude are studied, and transport of different sized molecules is simulated.

METHODS: Poroelastic theory is applied for the deformation of the matrix and transport of fluid and solutes in a simplified two-dimensional model (Figure 1). The solid phase represents the matrix of the cell-culture construct and the liquid phase represents the interstitial fluid. The deformable construct embedded with cells is immersed in a solution inside a well with rigid, impermeable walls. On top of the matrix, solution with known solute concentration exists. Solute moves into the construct and is consumed by cells. Metabolic activity of cells follows a step function of solute concentration. Cyclic loads are applied over a central area on the top surface of the construct, causing its deformation and extracellular fluid movement. Resulting cell density in the construct changes with time and location in the construct.

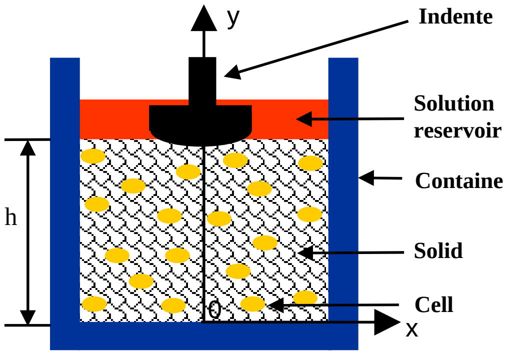

Figure 1. Schematic drawing of the model.

RESULTS: Movement of the extracellular fluid coupled with solute diffusion contributes to the overall solute transport in the construct. Following results are observed:

1. Cyclic loads facilitate solute transport into the soft construct.
2. Higher loading frequency has a greater effect.
3. Increase in loading amplitude improves solute transport, but the effect is less significant than that by increasing loading frequency.
4. At given location in the matrix, increase in solute concentration with time can be approximated by a mono-exponential function. Its time constant is affected by the loading frequency.
5. Increase in solute concentration following cyclic load is most significant for large sized molecules.
6. More cells (in deep regions of the construct) are metabolically active under cyclic load, when solute concentration in deep regions is raised to above the critical level.

DISCUSSION & CONCLUSIONS: Quantitative analysis of solute concentration distribution in the construct makes it possible to predict regions where cells are activated by the improved solute supply. The fact that more cells are metabolically active under cyclic loads exemplified most directly the effect of external dynamic loads on solute transport into soft tissues. The model can be extended to predict the transport of newly synthesized molecules by cells, e.g. proteoglycans, and the removal of waste products in extracellular matrices.

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Novel Chondroitin Sulphation Motifs As Putative Biomarkers of Articular Cartilage Chondroprogenitor Cells

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INTRODUCTION: The cellular mechanisms responsible for the zonal stratification of articular cartilage are still not fully understood. However, accumulating evidence indicates that the tissue is maintained by appositional growth occurring from the surface zone[1] which contains a population of chondroprogenitor cells[2]. In this study, we demonstrate that monoclonal antibodies (mAbs) recognising novel chondroitin sulphation (CS) motifs in CS glycosaminoglycans (GAGs)[3,4] can be used to identify and separate this chondroprogenitor cell sub-population.

METHODS: For immunohistochemistry, cryo-sections of articular cartilage (from the hock joints of 7 day bovines) were labeled by standard immuno-fluorescence procedures using mAbs 3B3(-), 7D4 and 4C3 which recognise novel sulphation motifs in CS GAGs [3,4] before counterstaining with propidium iodide. Sections were photographed on a Leica epi-fluorescent microscope equipped with digital image acquisition. For FACS analysis, thin surface slices of articular cartilage were digested overnight at 37°C in 1.2U/ml dispase II (Roche # 295825) and 100U/ml type II collagenase (Worthington #X4N7639) to enrich for superficial zone chondrons (*i.e.* cell and pericellular matrix). Isolated chondrons were immuno-fluorescently labeled with mAbs 3B3(-), 4C3 and 7D4 as described above. Labeled suspensions of chondrons were analysed in a FACS Calibur instrument. Ten thousand events were registered per sample and analysis of whole chondrons was performed using appropriate scatter gates to avoid cellular debris and aggregates.

RESULTS: Monoclonal antibodies 3B3(-), 7D4 and 4C3 strongly immuno-located novel CS motifs at the cell membrane and pericellular matrix of chondrocytes in the superficial zone of articular cartilage (figure 1). FACs analysis of isolated chondrons demonstrated that the former two mAbs, but not 7D4, could be used to identify and isolate this surface zone population (figure 2).

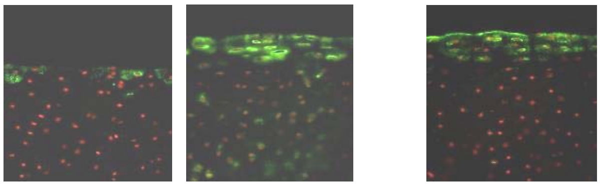

Fig. 1: Distribution of novel CS sulphation motifs in articular surface zone with mAbs 3B3(-), 4C3 & 7D4. (left to right)

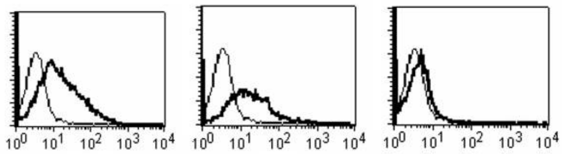

Fig. 2: FACS plots showing 3B3(-), 4C3 & 7D4 positive cells (left to right; bold line) compared to unlabelled controls.

DISCUSSION & CONCLUSIONS: In this study we show that the superficial zone cells of articular cartilage, recognized as a chondroprogenitor cell population[2], can be identified and separated by the presence of membrane-bound/pericellular novel CS glycosaminoglycan sulphation motif epitopes using monoclonal antibodies 3B3(-) and 4C3. Consistent with previous findings[5], the CS epitopes recognised by mAb 7D4 appear to be lost during the enzymatic procedure used to isolate intact chondrons. However it is anticipated that they may be retained following a mechanical isolation procedure (currently being evaluated). The identification, isolation and characterisation of this chondroprogenitor cell sub-population will lead to a greater understanding of the role novel CS sulphation plays in articular cartilage development and will be of benefit to new cell-based techniques for cartilage repair.

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