Tissue Engineering Requirements for Dermal Wounds

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INTRODUCTION: Wound healing is a complex biological process. In clinical practice patients with wounds have a range of diseases and disorders that can influence this process. The problems of wound healing cost the NHS at least £1 billion a year. Problems associated with diabetic foot disease are the most expensive component of managing diabetics in the health service. Pressure ulcers are often seen as quality indicators of health care provision and at the present time around £250,000 can be awarded to each patient who develops a pressure ulcer that is deemed preventable. Managing leg ulcers in the community consumes up to 75% of nurses time and despite advances in prophylactic antibiotic regimes and surgical techniques around, 10% of clean surgical procedures still become infected. This diverse and challenging clinical problem requires a comprehensive range of therapeutic strategies to both treat and prevent recurrence in as many patients as possible. Tissue engineering has great potential in this area even if in the short term it is only used for hard to heal or complex problems. Close integration between laboratory scientists and clinicians working in this area is required for maximising the potential of tissue engineering and improving the standards of care provided to patients.

Matrix remodelling in Dupuytren's Disease – the cause for progressive contracture

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INTRODUCTION:

It is uncertain whether Dupuytren's contracture is purely a result of matrix deposition or whether cellular contractility also plays a role. We determined the relative contributions made by cells and matrix remodeling in an in-vitro model.

METHODS:

Fibroblasts explanted from Dupuytren's nodule, cord and control carpal ligament were seeded into three dimensional collagen matrices and the force generated by their contraction was measured using a culture force monitor. At 8 hours, 24 hours and 48 hours cytochalasin-D was added to inactivate the actin cytoskeleton and the residual force exerted by the collagen matrix was measured.

RESULTS:

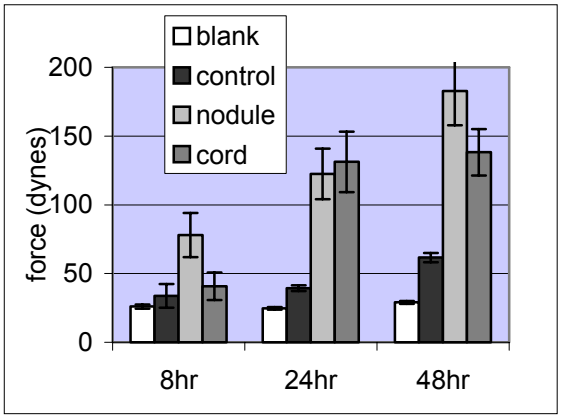

Figure 1. Maximal force generated for n=3 blank gels; n=3 control cell lines; n=5 Dupuytren's nodule cell lines, and n=4 Dupuytren's cord cell lines (error bars = standard error of the mean)

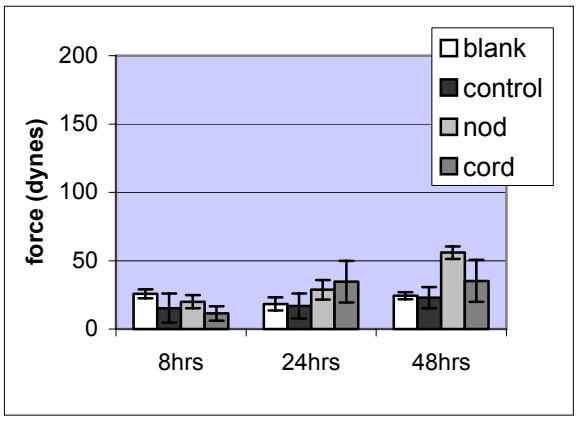

Figure 2. Residual force within collagen matrix after addition of cytochalasin-D at 8,24 and 48hours for n=3 blank gels, n=3 control cell lines, n=5 Dupuytren's nodule cell lines and n=4 Dupuytren's cord cell lines. (error bars = standard error of the mean)

Dupuytren's fibroblasts generate significantly greater contractile force that control fibroblasts ($p=0.008$), and in the case of nodules retain a significantly greater residual force ($p = 0.027$).

DISCUSSION & CONCLUSIONS:

These results indicate that whilst cellular contraction is the main cause for the progression of the contracture in Dupuytren's disease, remodeling of the matrix is the reason the contracture is sustained.

Maggots and Wound Healing: The Effects of *Lucilia sericata* Larval Secretions upon Human Dermal Fibroblasts

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INTRODUCTION: The development of dressings capable of debriding, cleaning and closing recalcitrant wounds, remains as yet, an unmet challenge. Tissue engineering may play a role in meeting this challenge, as demonstrated by recent developments including the use of growth factor delivery systems and skin replacement technology. In conjunction with elucidating how *Lucilia sericata* larvae can often stimulate healing when applied to wounds[1,2], tissue engineering has the potential to provide the total wound care package. Here, the effects of larval secretions upon interactions between human, dermal fibroblasts and extracellular matrix proteins, including the use of 2D and 3D wound assays were investigated.

METHODS: Larval excretion/secretion (ES) collection: One-day-old sterile *L. sericata* larvae were bathed in phosphate buffered saline (PBS). ES/PBS mix was extracted and sterile filtered. Protein concentration and protease activity was estimated using Bio-Rad's protein assay or fluoroscein isothiocyanate (FITC)-Casein assay respectively. Heat-treated ES was heated at 100°C for 30 min, yielding insignificant protease activity.
Cell adhesion: Human, dermal, neonatal fibroblasts were plated into wells pre-coated with fibronectin. An ES blank (PBS) or whole ES was immediately added. Following incubation and aspiration to leave adhered cells for assaying, ATP concentrations were estimated using Packard's ATPLite™-M kit.
Modification of fibronectin by ES: Samples of fibronectin incubated with ES, were tested for evidence of proteolytic degradation products using 12% SDS-PAGE.
Cell spreading: Cells were seeded into fibronectin pre-coated wells, with or without ES. After 4h incubation, images were taken using Leica DMIRB inverted microscope and analysed with QUIPS software.
Cell migration: Cells were seeded upon fibronectin surfaces in 2D *in vitro* wound assays or within 3D gel-based (1.5 mg/ml collagen, 30 μg/ml fibronectin) *in vitro* wound assays, with or without ES. Their migration over 48h was observed and quantitated using still images, time-lapse digital photography and Leica QUIPS software. Additionally, within the 3D assays, cell distribution and morphology was observed using confocal microscopy.

RESULTS: ES reduced fibroblast cell adhesion upon fibronectin[3]. It also modified fibroblast spreading upon the substrate, by reducing cell surface area and increasing roundness[3]. Heat-treatment significantly decreased the modulatory activity of ES, indicating a role for ES proteases[3]. Pre-exposure of fibronectin to ES before addition of fibroblasts, also resulted in modified cell adhesion and the presence of fibronectin proteolytic fragments[3]. The *in vitro* wound assays revealed ES to promote fibroblast migration, both in 2D (*Fig.1*) and within the 3D collagen gel environment. Differences in cell morphology were also observed.

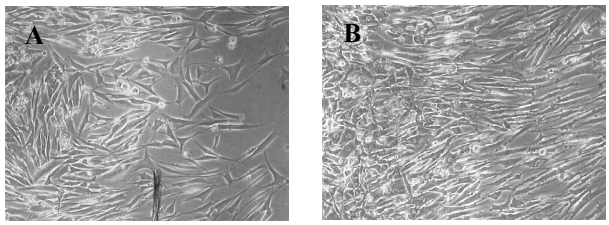

Fig. 1. Migration of fibroblasts across a fibronectin surface, into free space. At 0h, cell boundary positioned along left hand edge of image. Progression seen after 48h in A. ES blank; B. 0.1μg/ml ES.

DISCUSSION & CONCLUSIONS: Interactions between fibroblasts and fibronectin surfaces were shown to be modified by *L. sericata* ES and in particular, by the proteases present within the ES. The observed reduction in cell adhesion and spreading upon fibronectin, may enhance fibroblast migration, as indeed, was seen in the 2D and 3D *in vitro* wound assays. Alteration of fibronectin by the activity of ES may also exert an influence over fibroblast behaviour, as there is evidence that its proteolytic degradation products bind to fibroblasts to elicit different responses[4]. In the clinical setting, ES released by larvae placed onto the wound, may induce fibroblast migration into the wound space, facilitating tissue regeneration.

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Use of peracetic acid to sterilise human donor skin for production of tissue engineered skin matrices for clinical use
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INTRODUCTION: In the use of human tissues for fabrication of tissue engineered products, the concern of disease transmission is a common problem. A terminal sterilisation procedure should be considered and it is practical when the cell viability is not required, for example, in the production of acellular tissue matrices. Currently tissue banks use irradiation and ethylene oxide to sterilise skin grafts. However, there is damage to collagen after irradiation treatment and the use of ethylene oxide is becoming restricted due to its possible mutagenic and genotoxic effects[1]. High concentration of glycerol is used in the preservation of skin and anti-microbial effects have been reported, but it is not a recognised sterilising agent and may not be very effective against bacterial spores. In contrast, peracetic acid is an efficient sterilisation method with natural unharmful reaction products[2]. It has already been used in sterilising several tissues[3]. The aim of this study was to evaluate peracetic acid as an alternative method for the sterilisation of skin grafts.

METHODS: Skin samples were divided into four groups and treated as no sterilisation, sterilisation with peracetic acid (0.1% for three hours), glycerol, and ethylene oxide respectively. Acellular dermal matrices were made after further removal of the epidermis and the cells in the dermis. They were then processed for histological, immunohistochemical, and ultrastructural analysis. The biological function of the ability in supporting the growth of skin cells was evaluated by reconstituting dermal preparations with keratinocytes and fibroblasts *in vitro*. A scoring system was introduced to compare the histology of the reconstructed skin composites.

RESULTS: Dermal matrices sterilised with peracetic acid retained major components of the basement membrane including collagen IV, Lamina Densa and anchoring fibrils. In the dermal area the integrity of structure was maintained with collagen elastin and glycosaminoglycans present and in a normal appearance (Fig1). No significant differences were found in the ability of the sterilised acellular dermal matrices to form reconstructed skin which had a similar structure to native skin (Fig1).

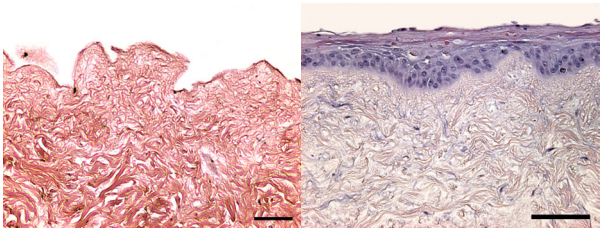

Fig 1: Histology of the peracetic acid treated dermal matrix (left) and the reconstituted skin composite (right)

DISCUSSION & CONCLUSIONS: Peracetic acid offers a convenient alternative protocol to glycerol or ethylene oxide for the sterilisation of human skin. The sterile acellular dermal matrices are suitable for clinical use.

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ACKNOWLEDGEMENTS: We thank the National Blood Service and the National Health Service for the funding of this project. We also thank Meg Stark and Christ Layton for help with TEM and histological study.

DEVELOPMENT OF PHYSICALLY INTERACTIVE BIOREACTORS FOR STUDY OF CELL & TISSUE RESPONSES TO BIOMECHANICAL STIMULATION *IN VITRO*

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INTRODUCTION: Physically interactive bioreactor systems are necessary for investigations of biomechanical regulation of cell function under controlled physiologically relevant conditions and for the development and regeneration of cell-matrix constructs for tissue engineering. It is now recognised that cells respond to physical forces by converting the physical stimulus into biochemical signals that ultimately lead to a change in gene expression and a final cellular response, a process known as mechanotransduction.

Cellular responses to mechanical stimulation. Various systems for studying the mechanotransduction response of cells have been developed. These include dynamic monolayer cultures (eg cell flexing on membranes), systems for delivering fluid shear stress to cells in monolayer, compressive loading and tensile loading of 3D constructs. These systems have been used to show that a variety of cell types respond to mechanical cues by changes in proliferation, apoptosis, the production of matrix proteins and , intercellular signalling molecules. Quantitiative analysis of cell responses remains in its infancy and there are difficulties in comparing the results of different studies due to different cells, animals and physical stimulation systems used.

We have developed two physically interactive systems for the study of the macrophage response to cyclic compressive (COMCELL) and cyclic tensile strain (TENCELL). COMCELL is a servo-assisted displacement controlled device for applying uniaxial cyclic compressive strain to 3-dimensional cell seeded constructs in 12 wells of a 24-well plate. The other 12 wells act as static controls. Cyclic compressive strain of 0-20% can be applied with a sinusoidal waveform. TENCELL delivers uniaxial tensile strain to 3-dimensional cell-seeded constructs housed in eight individual tissue culture wells. There are eight static control wells. A servo-controlled linear actuator delivers linear displacement of 1-10mm. Both systems allow variations in the levels and frequencies of cyclic strain. Using these systems, we have shown that 20% cyclic compressive or tensile strain alone does not stimulate TNF-□ production by macrophages. When UHMWPE wear particles are present at 30□m^3 per cell (not sufficient to activate the cells) there is a synergistic effect of strain plus particles and a significant increase in the levels of TNF-□ secreted.

Physically interactive tissue bioreactors Physically interactive tissue engineering bioreactor systems can be classified as tissue bioreactor systems for the culture of three dimensional constructs, with or without some form of simple physical stimulation and near physiological simulation systems which are tissue specific systems that replicate the functional physical conditions found *in vivo*. Tissue bioreactors include spinner flasks, rotating wall vessels, and perfusion systems. These have been used extensively in cartilage tissue engineering applications and the low shear rotating wall vessels have generally been shown to give optimal results, although no system has yet recapitulated the biochemical and biomechanical properties of native cartilage. Tissue specific systems have been developed for ligaments and blood vessels. We have developed a tissue specific system for heart valve tissue engineering *(Figure1)*

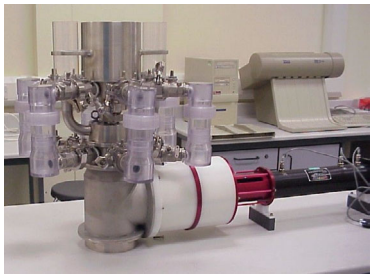

Fig. 1: Leeds heart valve bioreactor..

The bioreactor has a modular design with six stations simulating the aortic position, to allow for replication. The system is designed to stimulate heart dynamics by delivering physiological pulsatile flow and an aortic pressure within physiological levels (80-120mm Hg). The culture medium is conditioned via a conventional chemostat control unit and continuously exchanged. Work is ongoing to determine the optimal conditions in the bioreactor to maintain the viability and biomechanical function of fresh porcine aortic roots.

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Dynamic Measurements of Osteoblast Mechanosensing

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INTRODUCTION:, The biophysical mechanism and the initial down stream signalling of mechanoreception in osteoblasts is at present unknown. Present hypothesis include activation by calcium channels in the membrane, and /or the activation of phospholipase C (PLC) plus other mechanisms. We have used a number of different methods to stimulate primary and tumour derived osteoblast-like cells and have analysed mechanoreption where possible using direct dynamic imaging cell traction force measurements and inducible antisense to specific PLC isoforms.

We find no evidence for any biochemical pathway than activation of PLC β2. The biophysical sensor appears to be located in the actin cytoskeleton and associated with the cell attachment machinery rather than the membrane.

METHODS: Osteoblasts were mechanically stimulated by a) defined uniaxial stretching b) by fluid shear flow and c) by atomic force microscopy.. Intracellular free calcium, membrane potential and nitric oxide were measured by fluorescent techniques. Cell tension was measured dynamically by optical flow. Activated ERK (extracellular receptor linked kinases) were measured by western blot. PLC beta 1 and 2 were inhibited by an inducible vector expressing a specific antisense mRNA.

RESULTS: Intracellular calcium rises between 90 and 120 seconds after a stretch or onset of a stimulatory fluid shear flow with no contribution from extracellular sources of calcium. Stimulating the cells with the AFM shows an influx of calcium through voltage operated calcium channels with a release of IFC 9 to 30 seconds after this. Using a PLC β2 inducible antisense, but not a β1 AS, mechanically induced IFC could be significantly suppressed. PKC inhibitors, PLC β2 AS and an intracellular calcium chelator (BAPTA) could completely suppress ERK 2 activation by mechanical stimulation, indicating that the activation of ERK 2 is through the PLC-PKC pathway in the presence of IFC. Nitric oxide was also suppressed by BAPTA showing that this second messenger pathway was also dependant on IFC. Stimulation of cells using AFM showed an initial Ca++ entry into the cells plus a fast transient increase in traction force. However other experiments showed that an immediate response of traction force is not necessarily linked to Ca++ entry or release. Osteoblasts can also sense substrate stiffness in the order of 10kPa.

DISCUSSION & CONCLUSIONS: Osteoblasts respond to various mechanical forces, such as stretch and fluid shear flow in essentially similar ways. This is most likely because both forms of stimulation distort the mechanosensing apparatus. Although direct stimulation of the membrane can result in the opening of a calcium channel, the most biological significant event is the activation of PLCβ2, which then releases IFC and activates PKC isoforms. No channel is involved before or at the time of cell stretching. Downstream biochemical transduction includes cGMP &nκB, through NO: PKA, through camp and prostaglandins, ERK, through PKC. The nature of the mechano-sensor itself appears to be located somewhere in the actin-myosin-integrin system. Partly this was analysed through the amount of force required to activate the PLC which is very high, at least 10% of the cell tension of 2μN. It has strange properties as the higher the frequency of stimulation required, the less amplitude is needed.

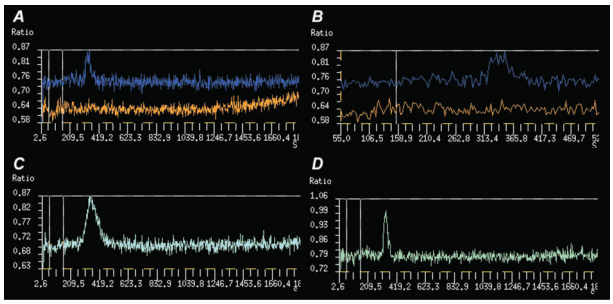

Fig.1. Uniaxial stretch of osteoblasts showing ratio image of FURA. The 2 lines at left of each image show the application of 30 cycles of 1Hz 0.4$% uniaxial strains

REFEERENCES: Jones et al. Proc. Roy. Microscop. Soc. (2003) 38, 67-73

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Effects Of Mechanical Force Application On 3D Bone Tissue Engineered Constructs Using Magnetic Microparticles

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INTRODUCTION: Mechanical forces have been shown to influence bone cell behaviour. Used within the correct parameters, it is possible to increase osteoblast bone related gene expression and ultimately increase the quantity of mineralised matrix production by applying mechanical stimulation. We are studying a novel method of applying mechanical forces, in the piconewton range, directly to the cell over a long-term incubation period. The novel technology employs magnetic nano- and microparticles which have been attached to primary human bone cells via specific membrane receptors which are then exposed to an oscillating magnetic field either in 2D monolayer culture [1] or seeded onto 3D constructs. As this cyclical magnetic field is applied, the magnetic particles respond with the cell experiencing a force as a result of this movement. By applying forces directly to the cell, a variety of non-load-bearing matrices can be used. In this presentation, we describe the effects of this cellular conditioning on human bone cells using PLLA based scaffolds resulting in elevated gene expression and matrix production.

METHODS: Magnetic particles of 4.5 micron and 0.87 micron in diameter have been coated with either an RGD protein or a specific calcium ion channel receptor antibody and adhered to primary human osteoblasts. Approximately four particles per cell were attached (figure 1).

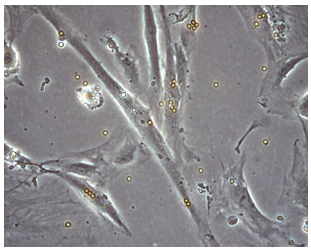

Fig. 1. Light microscopy image of adhered magnetic particles on primary human osteoblasts

These cells/particles were then seeded onto 88±0.02% porous PLLA scaffolds (4mm height x 10mm diameter cylinders). The cell/particle/scaffold was then incubated with 5mls culture media containing osteogenic supplements at 0.1ml/min for one week (media changed at 4 day time point). Six of these constructs (for each of the experimental groups) had a 1Hz oscillating magnetic field of up to 600 Gauss applied. The adhered magnetic particles were attracted to the magnetic field, but were still adhered to the cell. This resulted in a direct mechanical force applied to the cells in the order of 10 piconewtons. Controls of another six constructs had no magnetic field applied. A further control was performed of cells seeded onto PLLA only – with no magnetic particles attached and no magnetic field.

RESULTS: Real-time quantitative RT-PCR showed that the sample group of cells with adhered RGD coated 4.5micron particles and a magnetic field applied showed upregulation of bone related gene expression – Osteocalcin (figure 2), Osteopontin and Collagen Type I.

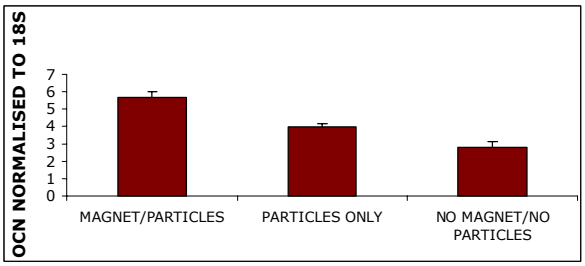

Fig. 2. Osteocalcin gene expression of osteoblasts seeded onto PLLA scaffolds after 1 week. (p = 0.05.

DISCUSSION & CONCLUSIONS: This technique has the potential to be used for the application of mechanical forces to osteoblasts, thus improving the mechanical strength and shortening the in vitro preparation time of bone tissue engineered constructs.

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TISSUE ENGINEERING A LIVING HEART VALVE: BIOLOGICAL AND BIOMECHANICAL ASSESSMENT OF AN ACELLULAR, PORCINE VALVE MATRIX

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INTRODUCTION: Over 225,000 heart valve replacement operations are performed worldwide each year however, there is no heart valve substitute that can survive life-long implantation. Mechanical valves carry a risk of thrombo-embolism, whilst tissue valves have limited durability and none have the capacity for growth and repair. A number of research groups are now focusing on developing tissue engineered heart valves, either from artificial polymer scaffolds or natural tissue sources[2].

The use of natural tissue matrices was chosen as the experimental approach for this project. A detergent-based cell extraction method for generating an acellular valve matrix was developed[3, 4]. This method has since been modified to treat the whole aortic root and the aims of this study were to establish the suitability of this matrix for use in tissue engineering in terms of its biochemical and biomechanical characteristics, biocompatibility and capacity for re-cellularisation.

METHODS:

Acellular Matrices: Excised porcine aortic valve leaflets or intact aortic roots were treated with hypotonic Tris buffer followed by 0.1% SDS in hypotonic Tris buffer, (with an additional trypsin step prior to the SDS treatment for the roots) before being washed in PBS.

Biomechanics: Strips of aorta were subjected to uniaxial tensile loading to failure. Whole aortic roots were tested under simulated physiological pulsatile flow to assess valve competence, kinematics, dilation, and pressure gradients.

Cytotoxicity Assays: Samples of decellularised valve leaflets or aorta were incubated intact or as soluble extracts with confluent monolayers of porcine valve interstitial cells (VIC), smooth muscle cells (SMC), or fibroblasts (Fb). The cultures were then assessed for changes in cell morphology or viability.

Recellularisation: Discs of decellularised leaflets were seeded with VIC, SMC or Fb for 24 hours then processed for SEM analysis of cell attachment. Whole decellularised leaflets were seeded with VIC, SMC or Fb and incubated for 1-4 weeks before histological assessment of cell migration into the matrix.

RESULTS: Specimens of decellularised aorta showed stress-strain and failure characteristics similar to the fresh tissue. Whole decellularised roots under physiological pressures presented complete leaflet competence, physiological kinematics, but reduced transvalvular pressure gradients and increased dilation tendency. No adverse reaction was seen by the cells in contact with the acellular matrices, and there was minimal decrease in cell viability following incubation with soluble tissue extracts. Cell densities of $2.5x10^4$-$7.5x10^4$ cells/cm^2 produced confluent cell monolayers with all porcine cell types. Following cell seeding of whole leaflets, the SMC and VIC migrated throughout the matrix by 4 weeks. In contrast there was no fibroblast migration into the leaflet matrix.

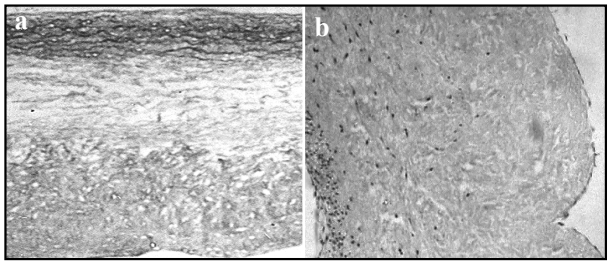

Fig. 1: (a) Acellular 0.1% SDS treated porcine valve leaflet; (b) H&E section showing porcine SMC migration into acellular valve leaflet matrix.

CONCLUSIONS: Decellularised heart valve tissues showed excellent biomechanical characteristics, kinematics and hydrodynamic performance, good biocompatibility and reseeding potential. This decellularisation treatment for heart valves forms a promising platform for the creation of a tissue engineered heart valve replacement.

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Cellular Attachment and Protein Adsorption onto Chemically Patterned Polymer Surfaces.

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Introduction. It is well established that the interaction between biomaterial surfaces and the biological environment is critically important in determining the acceptance or rejection of biomedical devices and in optimizing tissue culture techniques. Modification of biomaterial surface properties, in particular chemistry, is one method for controlling the behaviour of this bio-interface in terms of protein adsorptivity and cellular attachment. Chemically functionalised (polar) surfaces are known to be generally superior to unfunctionalised (non-polar) surfaces in terms of *in vitro* cell attachment, rates of attachment and subsequent growth [1,2] whilst surface chemical patterning can be used to control the spatial position of cell attachment [3,4].

Methods. In the work reported, ultraviolet/ozone oxidation and plasma polymerisation technique have been used to produce polystyrene surfaces with either increasing levels of oxygen functionalities or micropatterned oxygen functionality. These materials have been characterized using XPS, AFM and water contact angle measurement and then used to study the effects of varying surface oxygen chemistry (polarity) on the adsorption of albumin and fibronectin and on the attachment behaviour of a wide range of cells in the presence of serum proteins.

Results. For all of the cells studied, surface oxidation promotes attachment relative to attachment to unoxidised PS. Where oxidised (polar) and unoxidised (non-polar) domains are present adjacently, initial cell attachment is observed to occur at the polar domains and the cell attachment pattern corresponds to that of the chemical pattern. In some instances the chemical pattern dimensions are observed to influence the attached cell geometry and orientation. Over longer culture periods attachment also occurs at non-polar domains and the previously observed cell pattern becomes random. The results clearly show that the presence of polar domains can facilitate subsequent attachment at adjacent non-polar areas which would not normally support attachment. Some insight into the attachment mechanisms at the two surface types is given by the results from albumin adsorption experiments which indicate that relatively high concentrations of strongly bound protein are adsorbed at non-polar hydrophobic PS surfaces where attachment would be expected to occur primarily by dispersion interaction. For oxidised surfaces, albumin adsorption appears to occur by polar interactions resulting in a weaker attachment (possibly by formation of a hydrated interface) and a different conformation, the latter being nearer to that expected in aqueous solution. The substrate chemistry driven differences in protein adsorption, and particularly the differing energies of attachment will be discussed in terms of possible cell adhesion mechanisms.

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GENE EXPRESSION AND MATRIX PROTEASE ACTIVITY IN OSTEOCHONDROSIS DESSICANS

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INTRODUCTION: Osteochondrosis dessicans (OCD) is a disorder of unknown etiology where often a fragment of cartilage and subchondral bone separates from the articular surface. Current treatments for the disease include tissue engineering strategies such as autologous chondrocyte implantation or mosaicplasty. In order to develop successful therapeutic strategies for the disease it is important to understand the anatomy, physiology and biochemistry involved in the etiology of the disorder. The present study was undertaken to examine the gene and protein expression in human OCD cartilage compared to its normal autologous articular cartilage and human osteoarthritic (OA) cartilage.

METHODS Cartilage from five OCD patients (18-34 years) was obtained at the time of surgery. Pieces of cartilage were either snap frozen (in preparation for RNA isolation) or the proteoglycans extracted with 4M GuHCl. Total RNA was isolated from the cartilage using RNeasy mini-columns and reagents (Qiagen) according to the manufacturers protocol. RT-PCR was performed using an RNA PCR kit (Perkin-Elmer) using a number of oligonucleotide primers. GuHCl extracted proteoglycan fragments were analysed using Western blotting with a number of antibodies to aggrecan metabolites, collagen metabolites and the small leucine rich proteoglycans.

RESULTS: When OCD cartilage was compared to normal and human OA cartilage, there was an increase in aggrecan, collagen type II and collagen type X RNA expression. There was no change in RNA expression of link protein or type I collagen. The RNA expression of the aggrecanases (ADAMTS enzymes) was also different in the three different cartilage samples. Neither ADAMTS-1, -4 or –5 was present in the normal cartilage. In contrast, in the OCD cartilage there was expression of both ADAMTS-1 and –4 whereas in the OA cartilage there was expression of ADAMTS-4 and –5. In the case of MMP RNA expression, MMP-3 was decreased and MMP-13 increased in OCD cartilage compared to both normal and OA samples. In addition, the expression of all three TIMP isoforms was increased in the OCD cartilage. Although inflammatory components are not expected in OCD pathology, expression of inflammatory mediators such as COX-2, IL-1α and TNFα were all increased in the OCD cartilage when compared to normal, but expression of these mRNAs in the OA cartilage was higher. Analysis of proteoglycan fragments in the OCD cartilage by Western blotting (Figure 1) showed the presence of aggrecan fragments containing the G1 domain, interglobular domain and the C-terminal neoepitope generated by aggrecanase cleavage. There was also immunoreactivity for biglycan and link protein. This was in contrast to the proteoglycan fragments observed in OA cartilage.

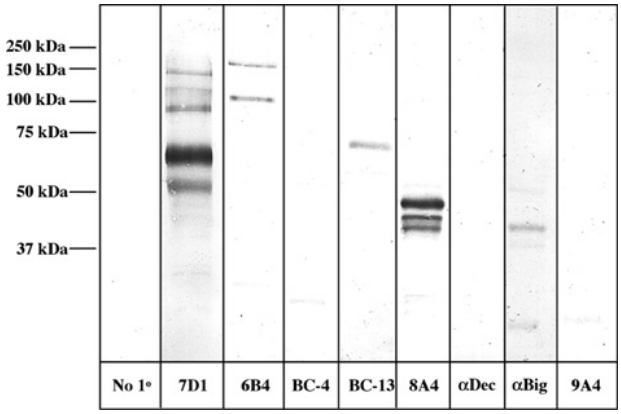

FIGURE 1. Western blot analysis of aggrecan fragments (7D1, 6B4, BC-4 and BC-13), link protein (8A4), small PG's (αDec and αBig) and collagen fragments (9A4). OCD data shown is from 18 year-old female but is representative of all OCD patients.

DISCUSSION & CONCLUSIONS: These results demonstrate marked differences in gene expression patterns and matrix proteinase activity in OCD cartilage compared to normal and OA cartilage. Using this data it may be possible to better understand the etiology of OCD and so develop better therapeutic strategies for the treatment of the disease.

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NON-DESTRUCTIVE OPTICAL MONITORING OF EXTRACELLULAR MATRIX ORGANISATION IN TENDON

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INTRODUCTION: Tendons are highly orientated (anisotropic) fibrous structures adapted for a high load bearing mechanical function.[2] As a tissue engineered tendon construct matures *in vitro*, it is vital to be able to non-invasively monitor its structural development. Elastic Scattering Spectroscopy (ESS) is a novel, low cost, non-invasive technique which delivers white light through an optical fibre to the surface of a material.[1] The pattern of absorption and back scattering of light detected gives real time, quantitative information of structure allowing *in situ* monitoring. However, interpretation depends on understanding responses in normal tendon.

METHODS: Hind paw flexor tendons from New Zealand white rabbits were examined *in situ* using ESS (with a source/detector separation of 300μm). Spectra were obtained in the 350 – 750nm range with tendons in resting position and during passive stretch, i.e. full range of limb motion. Probe was orientated 1) longitudinally (in line with tendon fibre orientation) and 2) perpendicular to the tendon long axis. 12 tendons from 3 animals were tested to identify inter- and intra-tendon differences.

RESULTS: All the spectra obtained had a similar and characteristic appearance with minimal variability between tendons (Fig 1).

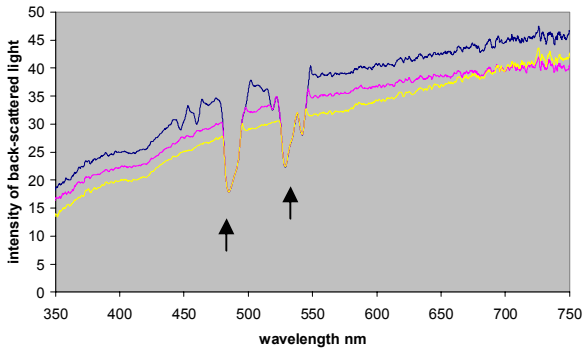

Fig.1: Characteristic spectra from different tendons in relaxed state (arrows show light absorption by haemoglobin)

Spectral signatures at 600nm were analysed with the probe positioned longitudinal & perpendicular to the tendon long axis, hence collagen fibre alignment (Fig. 2). In all specimens there was an alignment of collagen fibrils under load, which could be detected by an increase in optical anisotropy. This anisotropic effect became enhanced with passive stretch of the tendons as the limb position was moved.

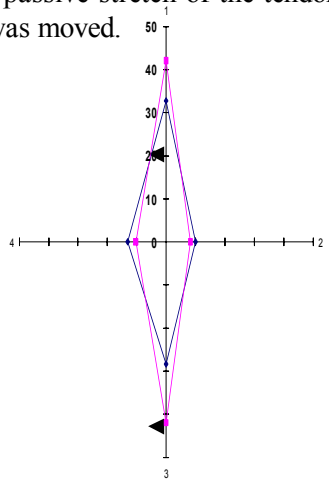

Fig. 2: (— resting position — passive stretch). Axes show arbitrary scale for intensity of back scattered light detected. Probe angle from 0°-90-180-270° (points 1-4) along tendon long axis. As tendon is stretched and collagen fibres align, increased back-scattered light is detected in the longitudinal direction than the transverse. Note: Significant change in shape of graph (arrows).

DISCUSSION & CONCLUSIONS: During mechanical loading, tendon matrix undergoes structural change, with loss of crimp and compaction of collagen fibres.[3] We have analysed and characterized these changes in situ using ESS to identify increased anisotropy (Fig 2). We conclude that ESS is a useful method for monitoring changes in collagen orientation during loading. As such, this technique has major potential for non-invasive monitoring (real time) of structural changes in tissue-engineered constructs.

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NOVEL BIOMATERIALS FOR TISSUE REPAIR

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INTRODUCTION: Cartilage defects present a complicated treatment problem due to the limited capacity for spontaneous repair. Recently developed strategies employ cell transplantation (chondrocytes, chondroprogenitor cells), but are hampered by the difficulty to anchor cells within cartilage lesions. Materials for spatially and temporally controlled delivery of bioactive agents such as growth factors, cytokines, drugs and/or cells are key to facilitating tissue repair. We describe the modification of high MW hyaluronic acid to an ethylene diamine derivative (HAED), which can then be crosslinked under physiological conditions into biocompatible and biodegradable hydrogels. The mechanical properties of the hydrogels have been investigated, as well as cellular toxicity, cell infiltration and inflammatory responses *in vivo*. We demonstrate that the hydrogels are a good delivery system of factors such as BMP-2.

METHODS: Chemical modification of hyaluronic acid and crosslinking into hydrogels has been described previously.[1] Hydrogels contained 9-12mgml^{-1} HAED, collagen type I (pepsinised or fibrilised telocollagen, 1-12mgml^{-1}) and crosslinker (size 3.4-20kDa, ratio 0.1:1 to 2:1 crosslinker:target groups on HA). Rheology: setting hydrogels were subjected to single frequency oscillation (frequency 5Hz, oscillation amplitude 10Pa) to ascertain the hydrogels' elasticity and setting time. *In vitro* cell viability: HCA2 fibroblasts were incorporated into setting hydrogels to create a 3D suspension and the cell viability after 24 hours was assessed by fluorescent live/dead staining (molecular probes). *In vivo* test of hydrogel: The hydrogels (500µl, containing 250µgml^{-1} BMP-2) were subcutaneously implanted into rats and explanted after 10 days and processed for histology.

RESULTS: Rheology: Increasing the quantity of crosslinker produced more elastic hydrogels, with satisfactory hydrogels forming between 0.5:1 and 2:1. Collagen added more elasticity at higher concentrations. Most of the crosslinking reaction took place within the first 15 minutes, with little change in elasticity after 30 minutes. The crosslinking reaction proceeded more rapidly at 37^{o}C. Cell viability: Good cell survival within the hydrogels was observed with 9 mgml^{-1} HAED, 0.6:1 crosslinker ratio with HAED, and with 1mgml^{-1} fibrilised telocollagen. Large molecular weight (20kDa) crosslinkers enabled a sufficiently large pore size within the hydrogel to permit cell survival and infiltration. An increase in crosslinker led to higher cell mortality, as it appears that the resultant smaller pore size is detrimental to cell survival. *In vivo* testing: The rat subcutaneous implantation assay demonstrated cartilage and bone formation resulting from the introduction of BMP-2 into the hydrogel. Cell infiltration from the surrounding tissue was evident, with the optimal hydrogel conditions being 9 mgml^{-1} HAED, 1.2:1 crosslinker:HAED target groups and 1 mgml^{-1} fibrilised telocollagen. Little or no inflammation was observed.

CONCLUSIONS: We have generated hyaluronic acid based biomaterials that are non-toxic to cells, non-inflammatory, support cell infiltration and promote tissue remodelling in the presence of bioactive factors. A biodegradable material will provide a scaffold for cell infiltration and tissue growth, eventually being entirely replaced by the tissue. Here we demonstrate suitability in regards to cartilage and bone formation, but these hydrogels can be more widely applied in other tissue repair strategies.

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Innovative biomineralised polysaccharide templates for human bone cell and gene delivery

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INTRODUCTION: The clinical need for biodegradable materials, with broad applicability, is evidenced by the fact that tissue loss as a result of injury or disease provides reduced quality of life for many at significant socio-economic cost. We describe the development of innovative microcapsule scaffolds based on chitosan and alginate tailored to musculo-skeletal regeneration with potential application to a number of human cell types for a variety of tissues.

METHODS: Semi-permeable polysaccharide microcapsules were produced by a one-step method, in which the deposition of a semi-permeable alginate/chitosan membrane around droplets of sodium alginate was coupled with in-situ precipitation of amorphous calcium phosphate as described by Leveque et al [1]. Nucleation of calcium phosphate could be controlled by the phosphate concentration in the alginate droplets to produce capsules of varying mechanical strength and permeability. Hybrid spheres (750-10,000mm) were generated encapsulating primary human bone marrow cells; STRO-1 selected osteoprogenitors (using magnetic activated cell sorting) and isolated articular chondrocyte populations. The potential for gene delivery and growth factor delivery was assessed using adenoviral green fluorescent protein AdGFP transfected osteoprogenitors and rhBMP-2.

RESULTS: Encapsulated cells remained viable within polysaccharide microcapsules for over 2 weeks as shown by positive alkaline phosphatase staining of encapsulated cells. Thin-walled capsules split and degraded in vitro within 4 days releasing viable osteoprogenitor cells as assessed by alkaline phosphatase activity. Cells expressing GFP, within microspheres, were observed using photomicroscopy indicating the ability to deliver cells, factors and selected genes. Encapsulation and delivery of active BMP-2 was confirmed using the promyoblast cell line C2C12 known to be exquisitely sensitive to BMP-2. Finally aggregation of the polysaccharide microspheres into extended frameworks was achieved using a designed droplet/vapour aerosol system resulting in foams of aggregated beads.

DISCUSSION & CONCLUSIONS: These composite scaffolds offer stable mechanical and chemical biomimetic environments conducive to bone cell function. These natural polysaccharides are also highly amenable to complexation with a range of bioactive molecules to allow molecular level biomimicry and consequently offer tremendous potential in tissue engineering and regeneration of not only hard tissues but soft tissues as well.

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Stem cells: patterning and differentiation.

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INTRODUCTION: In an appropriate environment, adult somatic stem cells can be induced to participate in the formation of a range of tissues. An aim of tissue engineering is to be able to direct new patterns of differentiation and tissue morphology. With this in mind, we have been working to determine the extent to which particular stem cell behaviours are intrinsic, i.e. arise from properties of the stem cells themselves, or are dependent on interactions with their environment.

Characteristic properties of epithelial stem cells are their high capacity for self renewal and their generation of cascades of amplifying cells that augment the differentiating population. In vitro growth and clonal analysis indicates that the generation of a stem and amplifying pattern is an intrinsic stem cell property. In vivo, individual stem cells give rise to clonal clusters of cells associated with small units of structure. Lineage marking shows that these patterns are readily re-formed by cells transplanted to in vivo sites after amplification in vitro. However, the normal regional distribution of stem cells in larger epithelial structures, such as rete, is not re-established after reconstruction. Other work shows that adult epithelial stem cells are normally stably committed to produce cells differentiating in particular regionally determined lineages but that alternative lineages can be generated by appropriate mesenchymal signals. It is concluded that the ability of epithelial stem cells to set up clonal cell hierarchies is an intrinsic property but that re-differentiation and development of complex structure occurs only in response to environmental signals.

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ARTICULAR CARTILAGE PROGENITOR CELLS: CHONDROGENIC POTENTIAL DURING EXPANSION IN MONOLAYER CULTURE

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INTRODUCTION: Current strategies in cartilage repair are based on the transplantation of cells from a variety of sources into the defect in question in order to generate a functional repair tissue. Difficulties arise in the use of chondrocytes due to a limited mitotic ability of mature chondrocytes *in vitro* and rapid loss of phenotype, known as dedifferentiation, in monolayer culture [1]. Previous research has identified a population of articular cartilage progenitor cells in the superficial zone of articular cartilage with an extended cell cycle time, a high affinity for fibronectin in cell adhesion assays, ability to form colonies *in vitro* from a low seeding density [2]. Here we report on the isolation and extensive subculture of these cells together with an evaluation of their chondrogenic ability at various passages by transfer into pellet culture.

METHODS: *Cell isolation:* Superficial zone cells were isolated by sequential digestion in pronase and collagenase of superficial zone cartilage from the articular cartilage of 2-3 week old bovine metatarsophalangeal joints. After isolation, 4000 superficial zone cells in serum free DMEM were seeded into wells of fibronectin coated 6-well plates and incubated at 37°C for 20 minutes. After 20 minutes, the media was removed and discarded. Fresh DMEM containing 10% FCS was added to each well. *Expansion in Culture:* Cells were subsequently transferred into 75cm^2 and 175cm^2 culture flasks. Growth was maintained by continual passaging at a ratio of 1:3. At various passages aliquots of cells were removed for pellet culture and Real-time PCR (Taqman). Controls consisted of normal chondrocytes isolated from the full thickness of bovine articular cartilage. *Pellet Culture*: Aliquots of 250,000 cells were resuspended in a serum free chondrogenic media containing TGFβ-1 and centrifuged to form a pellet. Pellets were incubated for 14 days and then paraffin embedded and sections stained with Safranin O/haematoxylin and immunolabelled with antibodies to collagen I and II. Taqman was used to quantify expression of collagen I, II, aggrecan and versican mRNA in the pellets.

RESULTS: *Growth:* Articular cartilage progenitor cells underwent approximately 61 population doublings over 162 days in culture. At P0 the cells had a doubling time of approximately 24 hours where after the cells maintained a moderately constant rate of growth with cells requiring subculture every 3-4 days. This was maintained until P28 when the rate of growth declined with subculture being required every 7-8 days. After P35 cell growth had virtually stopped. *Pellet Culture:* Articular cartilage progenitor cells expanded up to P22 and grown in pelleted micromasses synthesised a hyaline-like cartilage matrix that stained strongly with Safranin O. P1, P3, P6, P9 and P12 (25 pop doublings) pellets were rich in collagen II protein and mRNA whereas pellets derived from P22 (42 population doublings) cells contained low levels of collagen II. Pellet cultures of freshly isolated normal chondrocytes also stained strongly with Safranin O and contained abundant collagen II. Dedifferentiated normal chondrocytes at P8 contained negligible collagen II.

DISCUSSION & CONCLUSIONS: Although this study has illustrated the high expansion potential of articular cartilage progenitor cells, more importantly, the cells retain the ability to synthesise a cartilage-like hyaline matrix rich in collagen II even after 12 passages (25 pop doublings). In contrast, normal bovine articular chondrocytes rapidly dedifferentiated in monolayer and completely lost the ability to redifferentiate in pellet culture at P8 (13 pop. doublings). This important property to retain the ability to form cartilage after extensive expansion in culture may enable the generation of large cell banks for use in allogeneic tissue engineering applications. Investigations are currently focusing on developing human articular cartilage progenitor cells isolation and expansion protocols and employing these cells in cartilage repair strategies.

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EXPANSION IN FGF2 PROMOTES MATRIX SYNTHESIS DURING CHONDROGENIC DIFFERENTIATION OF HUMAN BONE MARROW MESENCHYMAL CELLS WITHOUT UPREGULATING SOX9

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INTRODUCTION: Bone marrow mesenchymal cells can be manipulated in vitro to differentiate towards a number of different cellular fates and offer a potential source of cells in a variety of tissue engineering applications. Differentiation of chondrocytes *in vivo* is mediated through interactions with growth factors, neighbouring cells and the extracellular matrix, though the detailed mechanisms are unclear. Here we have induced chondrogenesis in human bone marrow mesenchymal cells in a pelleted cell system [1], and show that upregulation of matrix synthesis does not correlate with expression of the cartilage regulating transcription factor SOX9.

METHODS: Human bone marrow mononuclear cells that were adherent after 24 hours on tissue culture plastic were expanded in monolayer culture with or without FGF2 at 1 or 5 ng/ml [2]. Confluent flasks were split 1:3 and cells were used at either passage 2 or 3. For pellet cultures, aliquots of 500,000 cells were pelleted in serum-free defined chondrogenic medium [3] without FGF2 in 15 ml Falcon tubes. The medium was changed on the resulting contracted pellets every 2-3 days for up to 14 days. Pellet chondrogenesis was assessed by changes in wet weight, histology and total GAG and DNA content of pellet extracts. Gene expression in RNA extracted from monolayers and pellets was examined by quantitative real time PCR.

RESULTS: Isolation of human bone marrow mesenchymal cells in medium containing FGF2 gave a cell population with a robust chondrogenic response when tested in the pellet culture system. This method gave a similar result in all different donor samples tested to date (n=9), whereas cells isolated from the same samples without added FGF2 were not overtly chondrogenic. At 14 days there were much higher pellet wet weights in the treated samples, probably due to greater extracellular matrix (GAG) production. Preliminary results suggested that there was also a net gain in cell number in the pellets derived from FGF2 treated monolayers that would also contribute to the weight difference. Sections from pellets derived from FGF-treated cells stained strongly with Safranin O, whereas those from non FGF-treated cells did not. There was a massive upregulation of collagen II mRNA expression (one hundred thousand fold) in pellets from FGF pre-treated cells, but this was not coupled to an increase in SOX9 expression in the same pellets.

DISCUSSION & CONCLUSIONS: Expansion of human bone marrow mesenchymal cells in the presence of FGF2 gave a cell population with enhanced chondrogenic potential, as recently described [2]. This was most clearly shown in the upregulation of GAG synthesis and deposition, and in a massive upregulation of collagen II expression. However this was not matched by an increase in SOX9 expression, which has been proposed to enhance transcription of the COL2A1 gene. This suggested that there were alternative pathways of transcriptional control, or that the SOX9 level already present in the monolayer mesenchymal cells was permissive, but not alone sufficient for collagen II expression. Modest increases in L-SOX5 and SOX6 expression in the pellets may have provided the necessary levels of promoter complex for the massive induction of collagen II expression observed. It has not been established if the isolation procedure with FGF2 alters the overall gene expression of a non-homogenous mesenchymal cell population, priming them for differentiation, or selectively enriches the cultures with cells retaining differentiation potential, although there is some evidence for the latter [4]. This system shows that changes in the level of SOX9 mRNA expression are not controlling matrix production in these differentiating mesenchymal cells.

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PRODUCTION OF HUMAN OSTEOCLASTS IN A THREE-DIMENSIONAL BONE MARROW CULTURE SYSTEM

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INTRODUCTION: Osteoclasts are hemopoietic cells that play important roles in bone remodelling and resorption. Osteoclast development is critically dependent on the bone microenvironment. We have developed a novel human *ex vivo* bone marrow model that mimics bone marrow both structurally and functionally by providing artificial scaffolding. We have utilized the 3-D system to produce multinucleated cells from human bone marrow cultured in the presence of vitamin D_3 and the absence of hydrocortisone and any exogenous growth factors. These multinucleated cells have the phenotypic, and functional characteristics of osteoclasts as determined by their morphology, expression of tartarate resistant acid phosphatase (TRAP), and the ability to resorb bone[1].

METHODS: The 3-D cultures were inoculated with human bone marrow mononuclear cells (3 x 10^6 cells/well) in a packed-bed bioreactors configuration using macroporous microspheres for support. The medium (changed daily) consisted of McCoy's 5A medium supplemented with 1 x 10^{-6} M hydrocortisone and horse serum and fetal calf serum. At week 2, hydrocortisone was lifted and the 3-D cultures were supplemented with 1,25-Dihydroxyvitamin D_3 (1 x 10^{-7} – 1 x 10^{-9} M). At week 4, the 3-D cultures were terminated and cytopsins were prepared along with histological staining on paraffin thin-sections. TRAP staining was performed on the cytospin slides. In addition, a bone resorbing assay was performed by seeding bone wafers with cells harvested from the 3-D cultures at week 4 and culturing them for an additional 10 days in the presence or absence of parathyroid hormone (PTH). Bone resorption was quantified by staining the resorption pits with toluene blue and scoring them using a Reflected Light Microscope. Scanning electron microscopy was utilized to visualize the resorption pits.

RESULTS: Figure 1 shows the presence of morphologically distinguishable "osteoclast-like" cells was observed in the cell output at week 4 from the vitamin D_3-supplemented 3-D human bone marrow cultures. The osteoclast-like cells displayed a polarity defined by a ruffled border that appeared to be still in direct contact with the surface of the matrix, and were TRAP-positive. Figure 2 demonstrates the functionality of the osteoclast-like cells from the 3-D cultures by their ability to resorb bone on bone wafers. Scanning electron micrographs of the bone wafers revealed the resorption lacunae excavated by the osteoclasts. Dissolution of the mineral occurred before the degradation of the organic matrix, i.e., the collagen fibers. The size and shape of the resorption pits varied widely.

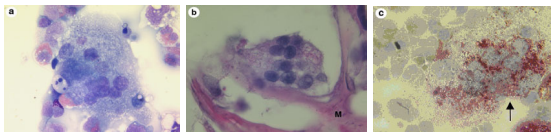

Fig. 1. Results from the 3-D cultures at week 4. (a) an "osteoclast-like" cell in Wright's stained cytospin (160x magnification); (b) "osteoclast-like" cells in paraffin thin-sections (100x magnification); (c) TRAP-positive multinucleated (3 or more nuclei) "osteoclast-like" cells (100x magnification).

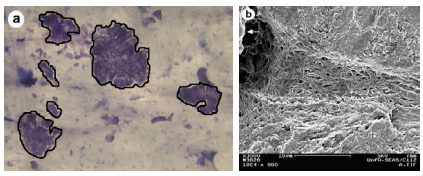

Fig. 2. Bone resorption results. (a) Light photomicrograph of the resorptions pits (10x magnification); (b) low power scanning electron micrograph of the resorption pit with sharp margins.

DISCUSSION & CONCLUSIONS: The results of this study provide qualitative and quantitative evidence that human osteoclastic cells can be generated in a 3-D human bone marrow culture system. Furthermore, the 3-D model with the ability to support extensive stroma development and a 3-D growth configuration represents a suitable, physiologically-relevant model to study *in vitro* osteoclastogenesis in order to elucidate the molecular and cellular signals of these processes.

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Interaction of hard and soft tissues with implant surfaces used for fracture repair

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INTRODUCTION: Studying cell morphology and adhesion on potential implant surfaces gives an indication as to the suitability for fracture repair applications [1-3]. With long term or permanent implants osseointegration is vital to their success. Early soft tissue integration without liquid filled capsule formation is also important for internal fixation plates. Good vascularisation is imperative at the implant / tissue interface, especially for prevention of infection [4]. In certain cases such as distal radius fractures where tendons have to glide over internal fixation plates blood or tissue adhesion may be undesirable. Bacterial adhesion to internal fixation implants is always undesirable. Microtopography is a fundamental factor in controlling cell adhesion. A method to quantify cell morphology and adhesion on implant surfaces is presented to help determine their cytocompatibility and predict the outcome with *in vivo* use.

METHODS: All surfaces should be morphologically characterised using backscattered electron (BSE) imaging with a Scanning Electron Microscope (SEM) [5] and their roughnesses characterised by non-contact optical profilometry. Mouse and human fibroblasts, or rat calvarial primary osteoblasts have been cultured on various surfaces and fixed for morphology [2,3] or undergo immunolabelling of the integral focal adhesion protein, vinculin [3,6,7]. Morphological images show cell shape and are used for quantitative measurements of cell size. Immunogold labelled cells are embedded in resin to enable implant removal and imaging of their adhesion sites directly from the undersurface using low voltage BSE imaging [2]. Only vinculin labelled within the adhesion sites are imaged [6]. A combination of labelling, together with S-phase identification may also be used [8].

RESULTS: There are two types of focal adhesions 'dot', or small immature contacts (0.2-0.5µm), not associated with actin stress fibres and elongated large mature 'dash' contacts (2-10µm) associated with actin stress fibres [9]. Large fibroblast area, as well as a high density of dash adhesions is considered to be a good indication for soft tissue cytocompatibility. In the case of osteoblasts a high amount of dash adhesions only is considered since osteoblast spreading and proliferation has been shown detrimental to expression of bone matrix markers necessary for mineralisation [10]. For both cell types the occurrence of only 'dot' adhesions is considered to show an unfavourable surface. Up to date, results show osteoblasts and fibroblasts display differences in cell spreading, total cell area, amount of adhesion and type of adhesion (dash or dot) to various surfaces.

DISCUSSION & CONCLUSIONS: To reduce intra-sample variation fibroblast monolayers are being used, which also reduces analysis time. A percentage adhesion per unit area of monolayer, rather than per cell, which is a closer representation of the *in vivo* situation for fibroblasts will be produced. For evaluation of surfaces with osteoblasts a three dimensional *ex vivo* cancellous bone culture system [11] will be used, where surfaces can be evaluated in a closer to *in vivo* situation (several cell types in their natural 3D relationship, in realistic numbers, not overpopulated as in standard cell culturing on planar surfaces and where realistic load can be applied).

Quantifying cell-substrate adhesion is a good step towards cytocompatibility evaluation of implant surfaces and should be part of a standard repertoire of cytocompatibility tests before *in vivo* evaluation.

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Human Bone Marrow Mesenchymal Stromal Cells as a Source of Chondrocytes for Treatment of Intervertebral Disc Degeneration.

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INTRODUCTION: Intervertebral disc (IVD) degeneration occurs in over 40% of the adult population and involves loss of discal extracellular matrix leading to instability and pain. Autologous cells are the ideal choice for bioengineering a new disc, but removal of cells from the IVD is not trivial. The purpose of this study was to direct mesenchymal stromal cells (MSCs) down a chondrocytic lineage to mimic disc chondrocyte phenotype.

METHODS: MSCs were either maintained in monolayer culture, pelleted into micromass aggregates or transferred to alginate beads ($1x10^6$ cells per ml). Pellet cultures were used in immunohistochemistry for type II collagen and aggrecan and *in situ* hybridisation for SOX-9 mRNA. Monolayer and alginate cells were cultured in the presence or absence of chondrogenic medium for 4 and 11 days. Monolayer cultured MSCs were also transfected with a SOX-9 adenovirus (pAdloxFLSOX9; MOI=200) and cultured in the presence or absence of TGF-β1. Real-time quantitative PCR was used to analyse expression of chondrocyte markers.

RESULTS: IHC showed increased expression of type II collagen and aggrecan in pellet cultures, while ISH showed that SOX-9 was not expressed by monolayer MSCs, but increased in a time-dependant manner after pelleting (figures 1 and 2). Real-time PCR using alginate-cultured MSCs showed down regulation of type I collagen mRNA expression and up-regulation of SOX-9 that was increased by chondrocgenic medium. SOX-9 transduced monolayer MSCs showed increased type II collagen, aggrecan, SOX-6 and SOX-9 mRNA over controls, while type I collagen levels showed no significant change. Stimulation of transfected MSCs with TGF-β1 showed similar increases in chondrocyte genes.

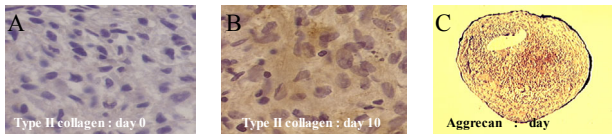

Figure 1: Type II collagen expression in pellet culture at day 0 (A) and day 10 (B) and aggrecan expression in pellet culture at day 10 (C) identified by IHC.

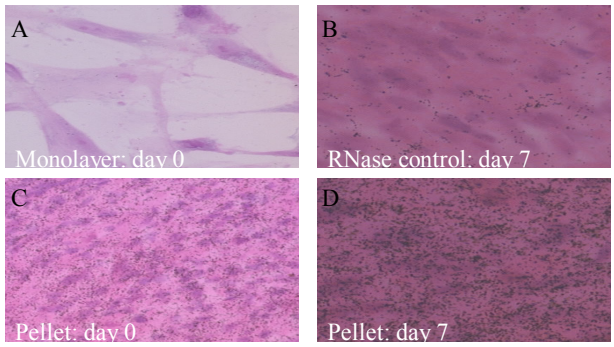

Figure 2: SOX-9 mRNA expression in day 0 monolayer (A) culture and pellet cultures at day 0 (B) and day 7 (C) identified by ISH. The background level is shown by RNase control (D).

DISCUSSION & CONCLUSIONS: Adult human MSCs were induced to differentiate along a chondrocytic phenotype, which was mediated by culture conditions. Alginate and pellet culture both appear to produce a cell that has more chondrogenic characteristics than corresponding monolayer cells. SOX-9 transduced monolayer MSCs appeared to produce a more chondrocytic phenotype and this phenotype was modulated by addition of TGF-β1. Results suggest SOX-9 transfected monolayer MSCs may be used as a source of chondrocytes for repair of degenerate IVD.

ACKNOWLEDGEMENTS:
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Stem Cells in the Treatment of Osteoporosis

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INTRODUCTION: Osteoporosis may be caused in part by an age-related decline in the number of osteoblast-progenitors (mesenchymal stem cells, MSCs) residing in the bone marrow[1]. MSCs can be isolated from marrow, cultured and expanded in vitro[2,3]. It has been hypothesized that such cells from *in vitro* culture might be infused back to osteopenic subjects in order to replenish their stem cell pool. It is hoped that this procedure would result in a positive bone balance and ultimately the regeneration of the osteopenic skeleton.

At present it is unknown how MSCs distribute in a mammalian organism after intravenous infusion and especially whether they have indeed the capacity to preferably 'home' to bone marrow from the blood stream and form colonies as proposed by D.J. Prockop[4] and collaborators.

METHODS: MSCs were labelled with radioactive [^{3}H]thymidine (> 10 dpm/cell) and their organ distribution measured at various time-points for up to 7 days after i.v.-infusion. In order to study seeding, survival and multiplication of cells in various organs including bone, infusion of cells stably expressing green fluorescent protein (GFP) were performed. The therapeutic potential of MSCs was tested under various conditions in rats models of postmenopausal osteoporosis. Syngeneic rat MSCs were harvested and culture expanded from donor rats. Cells were infused one week before or after ovariectomy at a dose of 10 Mio/kg into the tail vein of skeletally mature (8 month old), inbred Fisher F344 rats to prevent bone loss associated with oestrogen deficiency. Injections of parathyroid hormone (PTH), a known bone anabolic principle served as positive control. Non–invasive bone measurements were carried out by pQCT on an XCT-960 (Stratec-Norland, Pforzheim, Germany) in the proximal tibia metaphysis at baseline, 4 and 8 weeks. In separate experiments, GFP-labelled MSCs were injected directly into the metaphysis of anaesthetised oestrogen deficient (ovariectomized, OVX) rats.

RESULTS: Organ distribution: From the data obtained we calculated organ loads relative to organ wet weight. The values suggest that the lung accumulates the majority of cells at 4h and 24h following infusion. Higher than average values were also seen in duodenum, spleen, thymus. Approximately 800-1900 cells were found in the tibia at 24 hours. Organ loads peaked at 24 hours before declining rapidly in all tissues as the cells were removed almost completely by macrophages within 7 days following their administration.

Bone effects: In contrast to PTH, i.v.-administration of MSCs was unable to prevent OVX-induced bone-loss or regenerate bone tissue in osteopenic rats to any significant degree after systemic infusion. However administration of GFP-tagged MSCs directly into the proximal tibia metaphysis conclusively showed that, injected cells were involved in the injury-related bone formation response triggered by the local injection. Locally administered MSCs also failed to prevent the OVX induced bone loss.

DISCUSSION & CONCLUSIONS: Single or multiple i.v.-infusion of syngeneic MSCs at cell-doses of $1x10^7$ is well tolerated in Fisher F344 rats (no anaphylaxis, no ectopic bone or cartilage formation). MSCs did not home preferentially to bone tissue but were found to be enriched in lymphoid organs. The cells did not show long-term seeding. In bone, GFP-labelled osteocytes were detected in the mineralised matrix indicating that injected MSCs did differentiate down the osteogenic pathway and participate in local bone formation processes. However, MSCs did not prevent oestrogen-deficiency induced bone loss in rats after local or systemic administration to any significant degree.

The failure of MSCs in osteoporosis models suggests that, in a situation of oestrogen deficiency, the local 'catabolic' environment does not provide the required stimulus to the MSCs for their differentiation down the osteoblast lineage. It may be necessary to either combine MSCs with an osteoinductive carrier material, or provide an autologous stimulus by expressing a cell differentiation-inducing agent such as BMP or TGFβ into the cells (MSC-based gene therapy) in order to achieve therapeutically significant bone regenerative effects. The local environment may be more favourable and chances for success of stem cell therapy higher in situations such as fracture repair and bony ingrowth of implants, where the local injury provides a strong signal for bone repair.

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Culture of Meniscal Chondrocytes on Alginate Hydrogel Matrices

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INTRODUCTION: Injury to the fibrocartilaginous tissue of the knee meniscus represents a major challenge clinically and failure to repair serious defects can result in joint degeneration. Tissue engineering of the meniscus represents a potential alternative to the use of artificial materials, autogenous or allograft tissue. In this study we have used cells from ovine meniscal tissue which have demonstrated good similarity, at least mechanically, to human tissue[1]. We have investigated the proliferation of ovine meniscal chondrocytes (OMC) to a range of growth factors, and also their attachment to selected matrix proteins, in order to ascertain optimal conditions for *ex vivo* expansion of these cells as a pre-requisite for future tissue engineering studies. Alginate hydrogels, crosslinked ionically (with calcium ions) and covalently (with 1-ethyl-3-dimethylaminopropyl-*N*-hydroxy succinimide carbodiiamide) have also been investigated for their suitability as scaffolds for OMC cell culture.

METHODS: OMC were isolated using the method of Kuettner et al[2] and cultured in DMEM supplemented with 10% FCS. Confluent cultures (passage 3) were used for these studies. To assess the effect of growth factor treatment, OMC were seeded into 48-well plates at a density of $2\times10^4/cm^2$. Recombinant human platelet-derived growth factors AB (PDGF-AB) and BB (PDGF-BB), transforming growth factor β1 (TGF-β1), bone morphogenetic protein 2 (BMP-2), insulin-like growth factors I (IGF-I) and II (IGF-II) and basic fibroblast growth factor (bFGF) were added at final concentrations of 0-100 ng/ml and cells incubated for 72h at 37°C. Proliferation was measured using the MTT assay. Cell attachment to matrix proteins was assessed as follows: Briefly, non cell culture grade 96 well plates were coated with 10 μg/ml fibronectin, laminin, vitronectin, type I collagen or BSA as control, overnight at 4°C. Plates were then washed once with PBS and blocked with 1% heat-inactivated (Δ)BSA for 2h at 37°C. OMC were suspended in adhesion buffer (HBSS supplemented with 20 mM HEPES, 0.1% ΔBSA, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.2 mM $MnCl_2$) and plated at 7.2×10^4 cells/well. Attachment at 0-120 min was measured using the MTT assay. For studies using alginate hydrogels, OMC were encapsulated at $2.6\pm0.6\times10^6$ cells/ml in (2-4% w/v) alginate beads and also seeded on to the surface of flat alginate hydrogels. Scanning electron microscopy (SEM) was used to provide high resolution surface images of alginate beads. The number and viability of adherent cells was assessed using trypan blue, and histological analysis was carried out using sections through beads embedded in O.C.T. fluid, stained using haematoxylin and eosin.

RESULTS: Treatment of OMC with IGF-I, TGF-β1, bFGF, PDGF-AB or PDGF-BB resulted in an increase in proliferation compared to untreated cells. PDGF-AB and –BB had the greatest effect, with maximal proliferation occurring at concentrations of 50 ng/ml. In contrast, BMP-2 and IGF-II had no significant effect over the concentration range tested. The results from matrix protein studies revealed that the greatest cell attachment occurred with fibronectin. Significant increases in attachment were also seen with both vitronectin and type I collagen, compared to BSA controls, with maximal attachment recorded at 60 min. In comparison, OMC adhesion to laminin was minimal. The alginate beads supported cell growth for over 50 days, however, cell numbers and viability in the alginate beads were found to decrease over time. Covalently crosslinked gels were found to disintegrate over a 3 day period, and were generally unsuitable for supporting meniscal chondrocyte growth.

DISCUSSION & CONCLUSIONS: These results demonstrate that short-term (72h) treatment with a range of growth factors is able to increase ovine meniscal fibrochondrocyte proliferation, with the two isomeric forms of PDGF, AB and BB, having significantly greater effects than any other factor tested. We have also shown that these cells attach preferentially to the matrix proteins fibronectin, vitronectin and type I collagen, with the greatest attachment seen consistently with fibronectin. We also show that ionically crosslinked alginate gels will support OMC growth.

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The Effect of Bone Graft Bed Depth on the Short Term Stability of Revision Hip Arthroplasty: a Finite Element Investigation

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INTRODUCTION: Prosthetic components used in hip arthroplasty are subject to wear at prosthesis interfaces. The biological response to the generation of wear particles is degradation of bone around prosthetic components. Thus skeletal structures that were adequate at the time of primary hip arthroplasty, deteriorate to such an extent that revision surgery is required. The 'Slooff-Ling' impaction grafting technique has found favour with many orthopaedic surgeons. A common cause of failure in revision hip arthroplasties is the loss of structural stability in the bone graft in the acetabular region. In order to investigate the short term stability of the acetabular construct following revision hip arthroplasty an idealised plane strain finite element model was developed.

METHODS: Quadrilateral elements were used to generate a structured mesh representative of a transverse section through the acetabular region following revision hip arthroplasty using impacted morselised bone graft. The mesh was comprised of layers of plastic, cement, bone graft, cancellous bone and cortical bone. Load was applied to the acetabular construct through a metal femoral head using smooth sliding surfaces. With the exception of the bone graft, all materials were modelled as isotropic linear elastic. The bone graft was modelled using isotropic non-linear elasticity and non-associated Drucker Prager plasticity. The elastic behaviour of the bone graft was defined using an exponential stiffening curve:

$$p = \exp\left(\frac{\varepsilon_{ve}(1+e_o)}{-\kappa}\right) - 1 \qquad (1)$$

with an initial void ratio e_o of 0.3 and a logarithmic bulk modulus κ of 0.125. The plastic behaviour of the bone graft was defined using Drucker Prager plasticity with a friction angle β of 40°, a dilation angle ψ of 20°, and an interlocking constant d close to zero [1]. Two idealised cyclic load cases were developed to examine the effects of changing load magnitude and changing load direction in isolation [2]. By varying the size of the acetabular cup for a reference acetabular defect four different meshes were produced with varying bone graft bed depths.

RESULTS: Both load cases were applied to each of the four meshes. It was found that cyclic changing of the load magnitude caused the acetabular cup to rotate away from the direction of load application. The increase in the extent of rotation decreased as the number of cycles increased. The extent of rotation increased as the depth of the bone graft increased. For all depths of bone graft irrecoverable plastic strains were observed under normal physiological loading and bone graft was squeezed out to either side of the acetabular cup as shown in Figure 1.

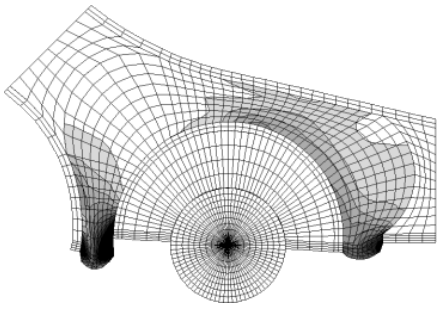

Fig 1: Plastic strain magnitude (increasing with darker shading) in the bone graft following cyclic changing of the load magnitude.

It was found that cyclic changing of the load direction also caused the acetabular cup to rotate away from the direction of load application. The extent of rotation in either direction decreased as the number of cycles increased.

DISCUSSION & CONCLUSIONS: The study suggests that clinical benefits may be gained by minimising the bone graft bed depth, and that stabilisation of the acetabular construct may occur after initial rotation of the acetabular cup.

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IN VIVO HUMAN BONE AND CARTILAGE FORMATION USING POROUS POLYMER SCAFFOLDS ENCAPSULATED WITH BMP-2

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INTRODUCTION: The ability to deliver, over time, biologically active osteogenic growth factors using designed scaffolds to sites of tissue regeneration offers tremendous therapeutic opportunities in a variety of musculo-skeletal diseases. The aims of this study were to generate porous biodegradable scaffolds encapsulating an osteogenic protein, bone morphogenetic protein-2 (BMP-2) and to examine the ability of BMP-2 released from encapsulated constructs to promote human osteoprogenitor adhesion, migration, expansion and differentiation on 3-D scaffolds *in vitro* and within an innovative *ex vivo* chick chorioallantoic membrane bone formation model as well as by in vivo bone formation assays.

METHODS: Recombinant human BMP-2 encapsulated Poly(DL-lactic acid) (PLA) scaffolds (100ng rhBMP-2/mg PLA) were generated using an innovative supercritical fluid process developed for solvent sensitive and thermolabile growth factors. The bioactivity of rhBMP-2 encapsulated PLA scaffolds were confirmed by induction of the C2C12 promyoblast cell line into the osteogenic lineage as detected by alkaline phosphatase expression. Human bone marrow stromal cells and scaffold constructs were implanted subcutaneously or sealed in diffusion chamber and peritoneally implanted in nude mice up to 10 weeks. Samples were assayed for x-ray and histochemistry. Chick chorioallantoic membrane model was used for angiogenesis assay.

RESULTS: No induction of alkaline phosphatase-positive cells was observed using blank scaffolds. BMP-2 released from encapsulated constructs promoted adhesion, migration, expansion and differentiation of human osteoprogenitor cells on 3-D scaffolds. Angiogenesis and enhanced matrix synthesis (evidenced by alcian blue, sirius red and collagen birefringence) on growth factor encapsulated scaffolds was observed following culture of human osteoprogenitors on explants of chick femoral bone wedge defects in an ex vivo model of bone formation developed using the chick chorioallantoic membrane model. In vivo studies using diffusion chamber implantation (10 weeks) and subcutaneous implantation (6 weeks) of human osteoprogenitors on rhBMP-2 encapsulated scaffolds showed morphologic evidence of new bone matrix and cartilage formation in athymic mice as assessed by x-ray analysis, immunocytochemistry and birefringence.

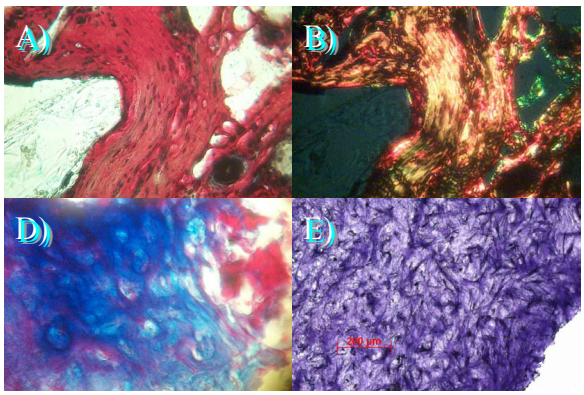

Fig. 1. In vivo bone (A-C) and cartilage (C) formation by human bone marrow stromal cells on rhBMP-2 encapsulated PLA scaffold. A) & D) Alcian blue and Sirius red staining; B) birefringence; D) Only fibre tissue formation on PLA scaffold alone in diffusion chamber model.

DISCUSSION & CONCLUSIONS: These studies provide evidence of controlled release of bioactive rhBMP-2 from biodegradable polymer scaffolds initiating new bone and cartilage formation in vivo. The successful generation of 3-D porous biomimetic structures incorporating slow release osteoinductive factors indicates the potential for de novo bone formation and the potential for more complex tissue regeneration strategies that will incorporate temporal and sequential release strategies for the augmentation of tissue regeneration.

Improvement of bone cell adhesion on PLLA substrate by a biological matrix

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INTRODUCTION: One of the major challenges for tissue engineering is to create a favourable environment for the proliferation and differentiation of cells into functioning tissues. Since the first interaction between cells and substrate (scaffold) is cell adhesion, the surface properties of the scaffold become a key factor in governing the success of the engineered tissue. It has been demonstrated that coating the scaffold surface with single extracellular matrix (ECM) molecules such as collagen, fibronectin, laminin, or pre-soaking scaffolds in media improves cell seeding efficiency and spreading. In this study, we take a further step by coating substrates with whole extracellular matrix extracted from autologous cells. The aim of this study is two fold: 1) to compare the effect of coating with whole extracellular matrix to coating by a single ECM molecule, collagen type I on Poly(lactic acid) (PLLA) substrate, and 2) to establish a methodology which enables observation of cell morphology on PLLA or other opaque polymer substrates.

EXPERIMENTAL: PLLA films with a thickness of about 10 μm were produced by solvent-casting. The film was then adhered to coverslips by a silicon adhesive. The coverslips thus covered by the PLLA film were still transparent. MG63 bone cell line and primary human bone cells from patients were used in the study. For production of a homogenised cell coating, 0.4 x 10^6 cell pellet was ground with a mortar and the ground solution in PBS was filtered through a 10 μm sieve. The coverslips were coated by the filtrate at room temperature for 2 hours. For lysed cell coating, $0.2x10^6$ cells were seeded on a coverslip and cultured until confluent. The cells were lysed by water and exposed to 20 mM NH_4OH solution for 3 min., then rinsed with distilled water and PBS. Some coverslips were coated with calf collagen type I at 7.5 μg/cm^2 density. The same patient cells were seeded on such treated coverslips and the cell morphology was observed at 1 hour and 16 hour culture periods by light microscopy. To test the effect of the coating on the cell adhesion in response to mechanical loading, the seeded cells on differently coated PLLA substrates were subjected to 4-point bending cyclically for 1 hour. After 24 hour post-culture, the coverslips were fixed and stained by H & E.

RESULTS: Due to the transparent feature, the cell morphology on the PLLA substrates could be observed and recorded directly in a light microscope with transmission mode. Both MG63 cell line and primary bone cells showed dramatically different attachment ability to PLLA with various coatings. Using the cell shape as an indicator of adhesion, e.g. rounded cells indicating less adhesion and elongated cells indicating more adhesion, the relative degree of cell attachment after 1 hour incubation was as follows: PLLA control < collagen coating < homogenised cell coating ≤ lysed cell coating.

Overnight culture of the cells on the PLLA substrates showed that cells on cell extract coated substrates had a more elongated cell morphology and denser network of confluent cells. The collagen coated substrate showed improved cell adhesion compared to the non-coated substrate. However, after mechanical loading, the coating with whole cell extracts exhibited a more robust attachment with fewer cells detaching from the surface of the PLLA substrate.

DISCUSSION AND CONCLUSION: Adhering a thin film on coverslips allows us to establish a convenient and reliable method to study cell morphology on opaque polymers in response to different surface treatments and mechanical loading. Lysed cell solution or homogenised cell mixture contains almost all of ECM molecules produced by the same type cell, potentially including some growth factors which promote cell adhesion in the first attachment and proliferation stage. Furthermore, the stronger adhesion between cells and the substrate reduces cell loss when the cells are subjected to mechanical force, which is valuable for bone tissue engineering to improve the quality of engineered tissues.

Osteoblast adhesion and morphology and *Staphylococcus aureus* adhesion on various coated orthopaedic implant surfaces.

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Introduction: Determination of surface cytocompatibility using cells and bacteria helps determine the suitability for *in vivo* use. The ability of *Staphylococcus aureus* to adhere to biomaterials is a significant factor in the pathogenesis of medical-device related infections[1, 2]. Osteoblast adhesion and morphology along with *S. aureus* adhesion was evaluated for various surfaces, to indicate which are likely to promote osseointegration or infection.

Materials and Methods: A Laser profilometer (LP) and Scanning Electron Microscope (SEM) were used to characterise the surface topographies (Table 1). Primary rat calvarial osteoblasts were cultured[3], 20,000 cells on each surface for 48hrs, (sub-confluent). For the morphological analysis cells were fixed then imaged with an SEM using the high emission backscattered electron (BSE) technique[4] at low voltage (5kV). The area of 30 cells from random positions was calculated. The focal adhesion protein vinculin was immunogold-labelled[5] and enlarged with gold enhancer to allow imaging of labelled whole cells with the SEM at 6kV[6]. The percentage area of labelling on 20 random cells was measured.

Label	Description	Ra (µm)
NS	Standard anodised TAN (Ti AL Nb)	0.99
NT	Tiodised TAN	0.79
TS	Standard anodised Ti tanium	1.15
TT	Tiodised TS	0.51
TSS	Titanium-101 gold anodized control	0.83
THY	TSS with grafted sodium hyaluronate (THY)	1.09
TAST	TSS with polymer cell promotion (TAST)	1.09
TIG	Nitrogen ion implanted TSS (TIG)	1.05
TLF	Low friction grey anodized TSS (TLF)	1.14

Table 1- Substrates investigated.

S. aureus were cultured on the surfaces in brain heart infusion broth for 1h at 37°C, stained with fluorescent redox dye, 5-cyano,2-ditolyl tetrazolium chloride (CTC)[7] for 1h, and visualised with a Zeiss Axioplan 2 Epifluorescence microscope fitted with a digital camera. The density of live bacteria adhering to the surface was quantitatively measured using image analysis software.

Results: Cells were generally well spread on all surfaces except THY, to which no cells adhered (Fig. 1). The degree of cell spreading varied slightly between surfaces. Dash adhesion labelling was visible on the TAST, TIG, TS and NT surfaces, suggesting these surfaces may be more cytocompatible. TT, TLF and TSS surfaces showed no mature dash adhesion labelling (Figure 2). Bright surface artefacts interfered with image analysis of NS samples (✦).

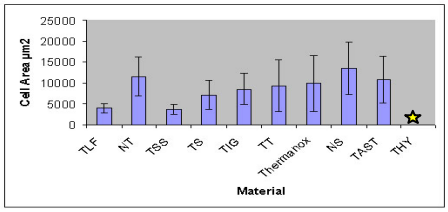

Fig. 1 *Average cell area on surfaces.*

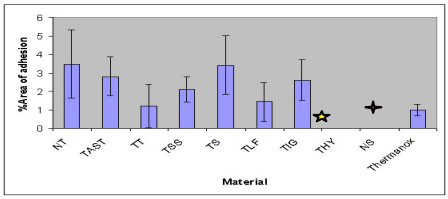

Fig. 2 *Average % labelled adhesion area.*

Fluorescence microscopy showed *S. aureus* adhered to all the surfaces (Fig. 3), with the exception of THY. The amount of adhesion was low with TIG, and TLF.

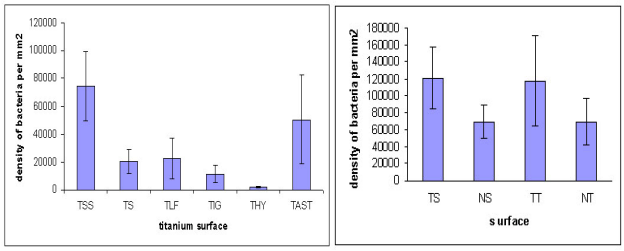

Fig. 3 *Density of S. aureus adhesion to the surfaces.*

Conclusions: The surfaces showed different degrees of cytocompatibility with regards to osteoblast morphology and adhesion. These surfaces (exception sodium hyaluronate coated surface) did not have a significant effect on the adhesion of *S. aureus*. The least cytocompatible surface to osteoblasts and *S. aureus* was THY.

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Fixation of Mouse Articular Cartilage.

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INTRODUCTION: Articular cartilage (AC) has been studied extensively during the past century but despite this there is still no consensus about its ultrastructure as viewed with the electron microscope. Fixation is an extremely important aspect of biological electron microscopy; it can have a significant affect on the final tissue appearance and is probably one of the reasons for discrepancies in the literature regarding AC structure. Traditionally chemical fixation was exclusively used but cryofixation methods have become more popular in recent years[1] and have led to novel structures being revealed[2, 3]. The work described here shows the structure of mouse AC following preparation with both chemical and cryo-fixation techniques.

METHODS: Mouse tibias were freshly obtained and either cryofixed using rapid freezing and freeze-substitution techniques or chemically fixed in either 4% formaldehyde or 1% acrolein in 0.1M PIPES buffer (pH 8.0) and dehydrated in an acetone series. The samples were then dried using a critical point drier. The tibias were fractured either at room temperature or cooled under liquid nitrogen (in a special container that released the liquid nitrogen gas and prevented condensation onto the samples while warming up to room temperature). The fractured tibias were mounted onto stubs and coated with platinum/palladium before being viewed and imaged in a Hitachi S-4700 field emission SEM.

RESULTS & DISCUSSION: All fixation regimes applied resulted in the AC general appearance being consistent with that described in other studies[4]. The collagen fibrils and chondrocytes were orientated perpendicular to the articular surface in the deep zone, arched over in the intermediate zone and were parallel to the surface in the superficial zone. Only closer examination of the AC revealed differences between the types of fixation.

Both the chemical fixation protocols resulted in separation of the collagen fibrils, evident only at higher magnifications. Some regions of the acrolein fixed AC, immediately surrounding the chondrocytes, appeared to be less disrupted and more closely resembled that of the cryofixed AC. Cryofixation techniques have been used for AC in recent years in several different animal species and are thought to be superior to chemical fixation methods, producing fewer artefacts[1]. The most

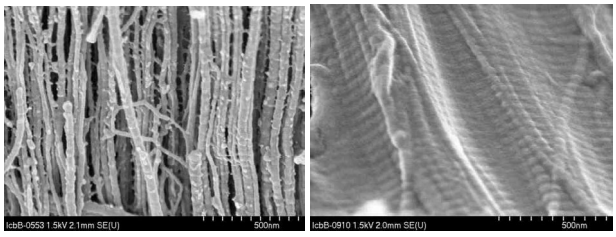

Fig. 1: Formaldehyde fixed (right) and cryofixed (left) collagen fibrils in mouse articular cartilage. The formaldehyde fixation resulted in separation of the collagen fibrils, which appeared as closely bound sheets following cryofixation.

obvious artefact is ice crystal formation. AC has very little free-water molecules available, most being associated with the proteoglycan and collagen molecules [5]. Thus there is relatively little water to form ice crystals and although some of the samples showed ice-crystal disruption, evident from fibril separation, there were specimens that had no apparent damage. In these samples the collagen fibrils in the AC were closely and continuously bound together to form sheet-like structures. Occasionally, tubules were also seen. Small orthogonally arranged fibril-like structures were observed to be raised above the surface of the main collagen fibrils, in register with the D-periodic banding of the collagen, possibly acting to bind the collagen together. There appeared to be much less tissue disruption and artefact formation using the cryofixation methods. The close association of the collagen fibrils observed would act as a barrier to lateral fluid flow in AC, correlating with the known biomechanical properties of the tissue in acting as a load-bearing structure.

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CELL-CELL INTERACTIONS IN A 2-D ULTRASOUND TRAP

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INTRODUCTION: A novel ultrasound trap is described as a technique that can characterise and control the progression of cell-to-cell adhesion in suspension away from any deformable substratum. Mechanically strong and manipulable 2-D arrays of interacting cells can be produced in suspension. The technique allows continuous monitoring of the progression of 2-D cell aggregate formation from initial cell-cell interaction, offering the prospect to address fundamental biological questions with reference to tissue construction, intercellular communication and differentiation.

METHODS: The main features of the ultrasonic trap are: a transducer, a sample region and a quartz glass reflector that provides optical access to the sample region. Interchamber temperatures can be held from 10-40^{0}C. A thermocouple located at the centre of the sample area allowed accurate temperature measurements without disturbing the sound field. Human erythrocytes, calf cartilage cells and rat neural cells were used as model mammalian cell systems. The aggregation process was epi-microscopically observed and video recorded [1].

RESULTS: Human erythrocytes formed closely packed but weak aggregates due to the low affinity interactions of the sialic acids of glycophorin A present in the plasma membrane. A strong dendritic aggregate morphology was formed in the presence of wheat germ agglutinin (WGA). Surface zone articular cartilage cells (fully differentiated) formed packed weak aggregates due to the presence of integrin receptors, an observation expected as these cells are supported by cell to matrix rather than cell to cell adhesion. The calcium and/or temperature dependencies of membrane spreading occurring in neighbouring neural cells [Fig.1] were also investigated. At low temperatures (12^{0}C) membrane spreading was repressed, whereas at temperatures as high as 33^{0}C, neighbouring cells progressively spread over each other resulting in the loss of some of the individual cell boundaries. Increased concentrations of EDTA gradually diminished membrane spreading. Membrane spreading of erythrocytes in the presence of WGA was also observed.

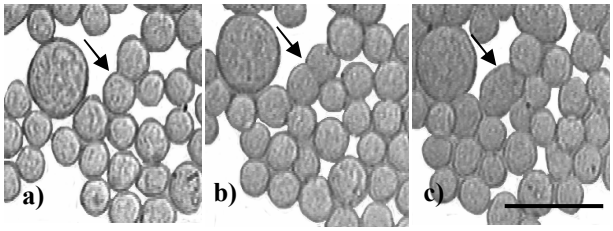

Fig.1: Development of membrane spreading (arrows) in a neural cell aggregate suspended in neuronal supplemented medium (33^{0}C) a) 0 min, b) 10 min and c) 20 min after ultrasound exposure (scale bar 25μm)

DISCUSSION & CONCLUSIONS: The 2–D aggregate morphologies observed in the trap correlate well with the cells' surface properties. The non-invasive nature of the ultrasound trap allows cells to adhere on a strictly biological basis, forming aggregates whose morphology (intimately linked to the cell surface receptors) can be extensively studied, therefore offering valuable information on the expression of different receptor molecules during the aggregation process and the dynamics that complement it (i.e. membrane spreading). There was no measurable temperature increase (i.e. $\Delta T < 0.5^0$C) in the trap. The cell to cell interactions occur in suspension away from the constraints imposed by surface specific or elasticity effects of solid inorganic substrata, hence arguably more closely represent biological interactions *in vivo*. The integrity and robustness of the 2-D dendritic aggregates (as shown for erythrocytes) suggest applications for the trap of producing single layer lamellae of, for instance, epithelial cells that could be deposited on a substratum or investigated in suspension.

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POTENTIAL DRUG - CELL DELIVERY ROUTES USING MAGNETIC NANOPARTICLES

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INTRODUCTION: The main problems associated with systemic drug delivery include an uneven biodistribution throughout the body, a lack of drug target-specificity, the necessity of a large dose to achieve high local concentrations and adverse side effects due to such high doses. Drug targeting aims resolve many of these problems (1). Amongst the current principles schemes of drug targeting is magnetic targeting, i.e. the targeting of a drug immobilised on magnetic nanoparticles held in position by the action of an external magnetic field. Magnetic nanoparticles have been used for bio-medical purposes including drug delivery, cell destruction and as MRI contrast agents (2). A wide variety of iron oxide nanoparticles have been synthesised. They differ in type of coating material used (eg. dextran, starch, albumin and silicones), and size, resulting in hydrodynamic particle size varying between 10 and 3500 nm (3). However, prior to use *in vivo*, the particles must initially be characterised *in vitro*. To this end, magnetic nanoparticles (8-15 nm diameter) were synthesised and derivitised with various coatings by Liquids Research Ltd., producing stable fluids of neutral pH.

METHODS: In this study, dextran and transferrin derivatised magnetic nanoparticles were synthesised (8-20 nm diameter), alongside inert iron oxide particles bearing no specific coating (figure 1a). Human dermal fibroblasts were cultured on glass coverslips for 24 hours and challenged with the nanoparticles (0.05 $mg.ml^{-1}$) for up to 72 hours. Biological analysis included cell viability, cell morphology, cytoskeletal changes, and possible particle internalization (figure 1b).

RESULTS: Uncoated particles were rapidly uptaken into the control cell cultures, to the point of initiating cell death, probably through apoptotic means (results not included here). The dextran Derivatisation induced cytoskeletal changes linked to areas of particle uptake, while the transferrin Derivatisation particles appeared to specifically localise on the cell surface receptors with no adverse effects in culture.

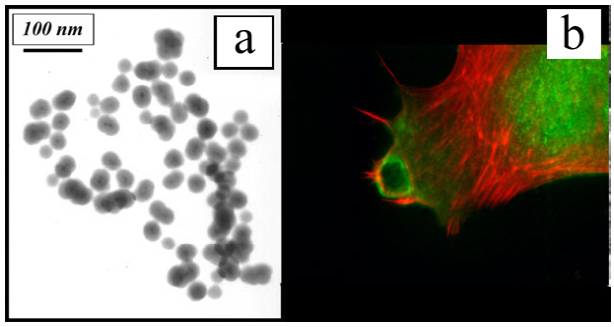

Fig. 1. TEM image of underivatised magnetic nanoparticles (left) F-actin(red) and clathrin (green)staining of fibroblasts incubated with dextran derivatised particles (right).

DISCUSSION & CONCLUSIONS: The cell response to the dextran derivatised particles was surprising considering that several dextran preparations are currently being used in clinical trials (4). However many *in vitro* studies detailed in the literature concerning dextran are short term, usually several hours of incubation, and therefore may not identify longer term problems. Transferin derivatised magnetic nanoparticles are also commonly used in research due to the high affinity of transferrin with its receptor (CD71) followed by receptor mediated endocytosis (5, 6). In this case, however, endocytosis of the particles did not occur. It may be that although the biological effects and chemistry of the protein are retained, the resultant size or shape due to derivatisation with the nanoparticles presents a stoichiometric hurdle for the receptor.

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DIFFERENTIATION AND PROLIFERATIVE CAPACITY OF ARTICULAR CARTILAGE PROGENITOR CELLS

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INTRODUCTION: In recent years, autologous chondrocyte implantation (ACI) has been increasingly used to repair articular cartilage defects. The number of chondrocytes that can be harvested from a patient for this procedure is limited by biopsy size, and the age of the patient. Previous studies demonstrate that primary chondrocytes as well as having a limited growth potential in culture lose their cartilage-specific phenotype. (1). The dedifferentiated phenotype can be recovered by culturing cells in an environment supporting a spherical morphology. We have identified a population of chondroprogenitor cells in the superficial zone of articular cartilage (2). Here we report on the isolation and extensive expansion of these cells and their ability to redifferentiate when transferred into a 3D environment. The effect of different growth factors on expansion of cloned chondroprogenitor cells was also studied within a 2d monolayer culture to evaluate a procedure to quickly expand a small cell number.

METHODS: *Chondrocyte Isolation*: Surface zones (SZ) chondrocytes were isolated from the metacarpal-phalangeal joints of 7-day-old calves as previously described (2) *Differential Adhesion*: 35mm dishes were coated with 10μg/ml fibronectin in PBS containing 1mM $CaCl_2$ and 1mM $Mgcl_2$ (PBS+) overnight at 4°C. Fibronectin was removed and SZ chondrocytes plated onto the dishes at 4000 cells/ml in serum free DMEM/F12 and allowed to adhere to the dish for 20 minutes at 37°C. Media and non-adherent cells were removed and the remaining cells cultured for 6 days in DMEM/F12 +10% FCS (DMEM+). *Cloning Chondrocyte Progenitor Cells*: 8 colonies of 32+ cells were identified and isolated using the cloning ring method and cultured in 35mm dishes and cultured in DMEM+. *Expansion in culture:* When cells in the 35mm dish approached confluency (P0), cells were trypsinised and transferred to 25cm^2 culture flasks (P1). Subsequent growth in 125cm^2 culture flasks was carried out by continual passage at a ratio of approx 1:6. In addition 56 clones were cultured whereby throughout the expansion phase (P1 and P2) cells were cultured in DMEM+ (1% or 10% FCS) or DMEM+ supplemented with growth factors involved in cartilage metabolism. Factors tested included 5ng/ml FGF-2, 10ng/ml EGF, 10ng/ml PDGFbb, 1ng/ml of TGF-β, and a combination of 5ng/ml FGF-2 and 10ng/ml TGF-β or 10ng/ml IL-1β. *Pellet Culture:* Aliquots of 250,000 cells were resuspended in chondrogenesis media (DMEM supplemented with gentamycin; ITS premix, ascorbate-2-phosphate (100μm) and TGFβ-1 (10ng/ml)). *Histology and immunolabelling:* Pellets were snap frozen in liquid nitrogen and sections of 10μm thickness were cut and stained with Safranin O/haematoxylin. For immunolabelling, sections were labelled with antibodies to collagen I and II and visualised using appropriate secondary Alexa Fluor 488 conjugated antibodies.

RESULTS: The growth kinetics of the 8 clones were investigated from the primary culture through 17 passages, corresponding to about 45 population doublings. P1 cultures reached confluence in about 2 weeks and an average 18 doublings, a doubling time of less than 24 hours. On replating the clones had slowed their proliferation rate, with a growth rate of about 3-4 days per population doubling. Cultures of cloned progenitor cells comprised a polygonal flattened morphological appearance.

Pellets cultures derived from early passaged chondroprogenitors and redifferentiated in chondrogenic media showed focal areas of glycosaminoglycans (GAG) deposition (Safranin O), were positive for type II collagen labelling and contained small rounded chondrocytes. Interestingly, although pellets derived from P17 stained for GAGs, the labelling for collagen type II was relatively weak. Control pellets derived from full depth isolated chondrocytes contained larger, vacuolated cells, separated by intensely stained matrix. However, pellets derived from full depth cultures at P3 although stained positively with Safranin O had very little collagen type II labelling. Not all the growth factors tested during chondroprogenitor expansion in monolayer induced a significant decrease in the doubling times of exponential growth. However, some growth factors were more effective at reducing doubling times than others. The growth factors, which promoted the highest cell proliferation rates, were FGF-2 and TGFβ1 in combination by significantly reducing the doubling time by 15%.

DISCUSSION & CONCLUSIONS: The data presented in this study indicate that, the population of expanded clones (32+ cells) maintained their ability to proliferate and redifferentiate into a hyaline cartilage like tissue. Expansion of cloned chondroprogenitor cells using FGF-2 & TGFβ1 would also allow faster amplification and to reduce the amount of donor tissue required during repair procedures. It is hoped that the development of a two-phase culture system (monolayer culture followed by pellet culture) using growth factors and chondrogenic media would exploit the chondroprogenitor's already enhanced ability to form cartilage after extensive expansion in culture

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Nano-Topography Induces Mechanotransduction In Human Fibroblasts

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INTRODUCTION: Macro- and micro-topography can present strong cues for cell adhesion, alignment and differentiation. More recently, developments in electronics research have allowed initial observations into cell response to nano-topography, and it appears that cells do react to such small surface features. New, rapid, methods of nano-production are becoming available for biologists; these include polymer demixing and colloidal lithography. This report forms a start point in observing fibroblast growth on nano-features produced using colloidal lithography. Colloidal lithography uses etching around adsorbed colloids to produce nano- columns. Here, microscopical (fluorescent, optical, SEM and TEM) and molecular (microarray) techniques were used to study human fibroblast adhesion, cytoskeleton, growth and signalling on nano-columns. Also studied was nucleus morphology and the position of post S-phase interphase chromosome 3 centromeres to see if the relative position of interphase chromosomes was altered on nano-topography

METHODS: The features used were cylindrical in shape roughly 100nm in diameter and 160 nm high (fig 1) fabricated on sheets of PMMA. Flat controls with around 2-4 nm roughnesses were also studied. H-Tert BJ1 human fibroblasts were used as a cell model. Optical, SEM and fluorescence microscopy were used to observed morphology and cytoskeleton in response to the islands. TEM was used to view ultrastructure. 1718 gene microarrays were then used to measure gene regulations in response to the nano-columns. Mean gene responses were calculated from 4 replicate arrays, and large up- and down- regulations will be reported. FISH for the centromeres of chromosome 3 was also performed. Fluorescence observation of post S-phase (when 2 centromeres were apparent) was then used and the mean centromere spacings measured.

RESULTS & DISCUSSION: The nanocolumns reduced cell spreading and the fibroblasts had less organised microtubules, microfilaments and intermediate filaments. As a result of these factors, the nuclear morphology was altered in to a more spherical shape, and the positioning of chromosome 3 was altered during interphase. This result may indicate that the nucleus being mechanically integrated within the cell is under less tension in the more rounded cells compared to the more spread cells on the planar surface. 1718 gene microarray was also used to observe down-stream changes within the cell genome, of which most were down-regulations. It has been hypothesised that interphase choromosomes have a consistence of position within the nucleus. Our results indicate that by altering the positions of the chromosomes, changes in gene regulation are observed. These changes directly relate to proliferative and phenotypical responses, and thus may be significant in producing nano-materials for tissue engineering.

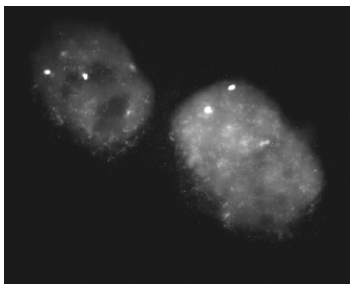

Fig. 1. Fibroblast nuclei with chromosome 3 painted. Measurements were made between the centromeres for post S-phase interphase nuclei. Significant differences were observed for cells cultured on the nanocolumns.

	Flat Control	Nano-Columns
Nucleus area (μm^2)	1057 ± 359	822 ± 303*
Intensity (arb units)	65261 ± 40710	88237 ± 40987*
Chromosome distance (µm)	3.76 ± 0.97	2.34 ± 1.1*

Table 1. Quantification of nucleus area, thickness (intensity) and distance between post S-phase chromosome 3 centromeres .

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NOVEL STRATEGIES FOR ENHANCING TISSUE INTEGRATION IN CARTILAGE REPAIR

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INTRODUCTION: Articular cartilage is unable to initiate a spontaneous repair response when injured due to its avascular and aneural properties. Within adult cartilage, chondrocytes are entrapped within an extensive extracellular matrix and are unable to migrate to sites of injury to regulate tissue repair. Injury to this tissue therefore inevitably leads to degeneration of the cartilage and the development of diseases such as osteoarthritis.

The surgical technique of autologous chondrocyte transplantation (ACT) was developed for the treatment of full thickness cartilage defects[1]. Implantation of chondrocytes into the defect site repairs the injury with a mixture of fibrocartilaginous and hyaline-like tissue that poorly integrates with the existing cartilage and frequently degenerates with time. In this current study we have developed an in vitro model to investigate methods for enhancing this integration and the development of a more biomechanically stable repair tissue.

METHODS: Bovine articular cartilage explants from the *metacarpalphalangeal* joint were experimentally injured using a stainless steel trephine and cultured for a period of 28 days. Autologous chondrocytes in an agarose suspension were injected into the interface region at the injury site. Culture media was collected and analysed for proteoglycan and collagen content using the DMMB and hydroxyproline assays respectively. Matrix metalloproteinase (MMP) expression was also analysed using zymography and an adapted collagen fibril assay.

RESULTS: Morphological analyses indicate attempts at repair and integration within both control and experimental treatment groups although the presence of autologous chondrocytes appeared to amplify this repair response. Considerable differences in proteoglycan release between injured explants and the intact control group were seen over the 28 day culture period although these differences were not statistically significant. Collagen release into the media was only seen at day 28 within the experimental culture group. Western Blotting will ascertain the nature of this collagen and whether it is present in media as a consequence of degradation or synthesis will be investigated using radiolabelling techniques. An up-regulation of MMPs 2 and 9 was seen within the experimental cultures compared to the controls using zymography and preliminary data also suggests up-regulation of collagenases in the experimental group.

DISCUSSION & CONCLUSIONS: As seen with clinical ACT treatment the presence of autologous chondrocytes appears to enhance repair and integration attempts however morphologically this repair tissue appears to be fibrocartilaginous. This in vitro model will enable the temporal and spatial expression of the chondrocytes to be monitored during the "repair process". Establishment of this in vitro model will allow investigation into the effects of different growth factors on enhancing this repair and integration process.

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Motoneuron Growth on Protein Surfaces Modified with a "Stop" Signal

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INTRODUCTION: To successfully engineer functional tissues, some degree of manipulation of cellular behaviour is necessary. For example, for the site-specific *in vitro* innervation of engineered skeletal muscle, we must be able to direct motoneurons towards the target muscle fiber and arrest neurite extension at a defined point. The tripeptide sequence LRE, found in s-laminin at the neuromuscular junction, has previously been shown to bind to and act as a "stop" signal for a motoneuron-like cell line and chick ciliary ganglion cells *in vitro*.[1] To investigate this molecule in primary mammalian cells, we seeded a motoneuron-enriched rat embryonic spinal cord population on to protein coated surfaces that had been chemically altered with synthetic LRE and examined the effect on neurite outgrowth.

METHODS: We have recently described the chemical engineering of cell monolayers using selective periodate oxidation of surface sialic acid residues, enabling their decoration with molecules of our choosing.[2] This was extended to laminin-coated tissue culture plastic, utilizing the glycosylation of the peptide to generate aldehydes as "chemical handles". Culture plates were coated with laminin using standard protocols. Wells were then treated with PBS or sodium periodate solution and subsequently incubated with PBS, biotin hydrazide or LRE hydrazide.

Motoneuron-enriched embryonic spinal cord cells were isolated using a variation of the methods described by Schnaar & Schaffner[3] and Camu & Henderson[4]. Cells were plated at a density of 10 000 cm^{-2} in defined medium and fixed after 24 hours growth. The cells were then imaged by phase contrast microscopy and neurite lengths measured using ImageJ software.

RESULTS: Figure 1 demonstrates the successful ligation of biotin to laminin-coated surfaces, detected by subsequent staining with FITC-conjugated avidin. The effect of different surface modifications on neurite outgrowth from cultured motoneurons is shown in figure 2, in comparison to a control surface of unmodified laminin. Mean neurite length on LRE-modified surfaces was 31.5 ± 3.4 µm in comparison to 52.1 ± 8.2 µm for control cells, a reduction of 39 %. Cells on surfaces expressing aldehyde- or biotin-modified surfaces were unaffected.

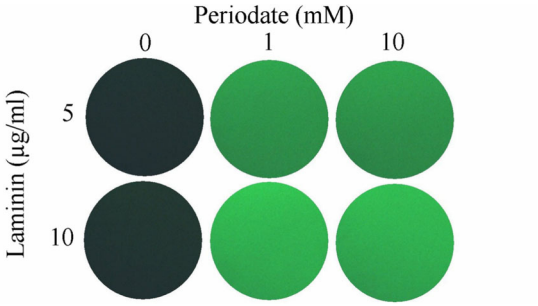

Fig. 1. Chemical engineering of laminin surfaces with biotin.

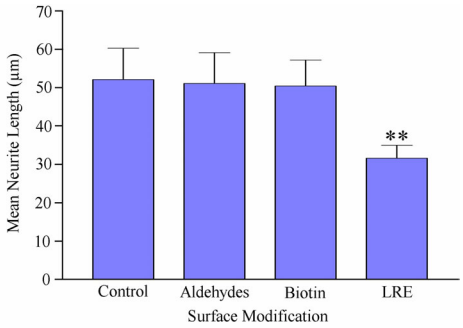

Fig. 2. The effect of laminin surface modifications on neurite length in cultured motoneurons.

DISCUSSION & CONCLUSIONS: We have demonstrated that a simple chemical technique can be employed to modify cell culture surfaces with bioactive molecules or our choosing. Using this technique, the tripeptide LRE sequence exhibited an ability to significantly reduce the outgrowth of neurites from cultured motoneurons. Combining this with surface patterning methods will potentially provide us with greater control over cell behaviour *in vitro*.

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NOTCH MODULATION IN ARTICULAR CARTILAGE.

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INTRODUCTION: We have previously described the isolation of a population of progenitor cells from the surface of articular cartilage. These cells express members of the Notch family of cell surface signalling molecules and several reports highlight the importance of Notch signalling in both articular cartilage and growth plate development. Mature Notch is presented at the cell membrane after S1 cleavage by a furin-like convertase. A second cleavage at the cell surface by members of the ADAM family (ADAM 10 and ADAM 17) allows Notch to interact with its ligands (Delta and Jagged). After activation, S3 cleavage by the gamma secretase complex releases the Notch Intracellular domain (NICD) which mediates transcription of various genes.

Here we report on the effect of interfering with Notch signalling at various steps in the Notch cleavage pathway using inhibitors of gamma secretase mediated S3 cleavage and ADAM-mediated S2 cleavage *in vitro* and discuss possible implications for clinical use

METHODS: Full depth articular cartilage explants were excised from 7 day-old bovine metacarpal-phalangeal joints and incubated in DMEM/F12 containing the gamma secretase inhibitor DAPT (50nM in DMSO) or rh TIMP 1 and 3 (0.1 and 0.01 uM) for up to 7 days. In some experiments explants were incubated with both rhIL-1 (1ng ml^{-1}) and 50 nM DAPT for up to 7 days. Media samples were assayed for GAG content and explants were either fixed and processed for histology or digested with papain for GAG analysis using the DMMB assay.

Chondroprogenitor cells, were isolated using differential adhesion to fibronectinfor 20 minutes and incubated in DMEM/F12 containing gamma secretase inhibitors as described for explants. Both initial adhesion and the colony forming efficiency of these cells was calculated.

Human osteoarthritic cartilage samples were obtained from patients undergoing knee arthroplasty. Full depth samples were fixed, wax embedded and labelled with antibodies to Notch family members.

Whole knee joints were obtained from rats/mice up to 14 days after the onset of antigen induced arthritis and wax embedded and labelled with antibodies to Notch family members.

RESULTS: In samples treated with DAPT, a hypocellular area was present in the transitional zone of the articular cartilage and this zone was very weakly stained with toluidine blue suggesting GAG loss from this region. DMMB assays revealed that GAG was not lost to the media with time and that the area of hypocellularity was due to inhibition of proliferation in the surface zone of the explants.

We also showed that the gamma secretase inhibitor DAPT did not prevent chondroprogenitor adhesion but did abolish colony forming efficiency. The use of S2 cleavage blockers (TIMP 1 and TIMP 3) similarly abolished the clonality of chondroprogenitors.

The addition of DAPT to IL-1 treated cartilage explants abolished the GAG loss associated with IL-1 treatment, suggesting that DAPT may be of possible use in the treatment of osteoarthritis.

Finally, immunohistochemical studies reveal the recapitulation of Notch signalling molecules in both human OA joint tissues and in a collagen induced model of arthritis in the mouse, particularly the expression of Notch 1.

DISCUSSION: These results highlight the importance of Notch signalling mechanisms during articular cartilage growth and development and the re-expression of Notch signalling molecules during both OA and RA highlight these molecules as possible targets for both pharmaceutical intervention and manipulation of the chondrocyte phenotype during *ex vivo* growth for tissue engineering.

ACKNOWLEDGEMENTS: This work was supported by the BBSRC and Smith and Nephew Research Centre.

A Novel Biodegradable Scaffold To Minimise Mass Transfer Limitations In Tissue Culture To Aid Healing of Non-Union Bone Injuries

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INTRODUCTION: Bone tissue engineering is well recognised as a potential treatment for trauma injuries, osteoporosis, arthritis and bone cancers [1,2]. In comparison with traditional treatments such as fixation and joint replacement, bone tissue engineering could offer an enhanced treatment and a reduction in healing time. A key problem is fracture non-union and wounds that result in bone loss too large for natural healing. Treatment of this type of bone condition requires the replacement of tissue to bridge the gap and to provide cues for healing as well as to provide mechanical support. Current approaches have involved autologous bone grafts of which there is a finite amount of tissue available[3]. Bone tissue engineering seeks to rectify this shortage by manufacturing a bone construct by culturing a scaffold seeded with cells in a dynamic bioreactor environment. The resulting tissue construct is then placed in vivo to aid healing. However, the production of functional bone tissue on a clinically relevant scale is limited by the problem of necrosis due to poor nutrient supply and the accumulation of toxic wastes[4]. Our work seeks to address the limitations of nutrient and waste product transport to and from the centre of nascent tissue constructs.

METHODS: 25w/w% 50:50PLGA was dissolved in 1-methyl-2-pyrrolidinone (NMP) or dioxane. The membranes were cast by pouring polymer solution onto a flat glass sheet and spreading with a glass rod, kept at a height of 200µm from the glass sheet. The glass sheet was submerged in distilled water and left until the polymer sheet lifted from the glass. The membranes were soaked for three days in water, which was changed twice a day. The membrane was then soaked for 30min in 70% ethanol, rinsed with DMEM, and left in 'complete media' (see below) for 20min until required.

The cell line $560P_{zip}U19$ was maintained in 'complete media', containing DMEM Glutamax, supplemented with 10% (v/v) heat inactivated foetal calf serum (FCS), 1mM sodium pyruvate, 100µM Ascorbate-2-phosphate, 1% Non Essential Amino Acid (NEAA) and 1% pen/strep antibiotic. 10^{-8}M Dx, and 50µg/ml G418 was also added. Cell solutions were made up to give a seeding density of 100,000 cell/cm^2. The cell solutions were pipetted onto the centre of the substrates and left in the incubator for 24h.

RESULTS: Highly porous scaffolds were formed using the phase inversion technique. The microarchitecture differs with process conditions (Figure 1). The membranes have anisotropic porosity as show in Figure 1. The membranes were shown to successfully support the osteoblast cell line $560P_{zip}U19$.

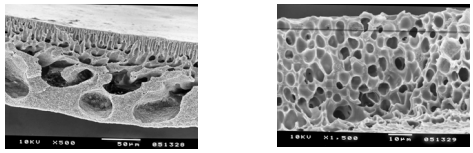

Fig. 1. SEMs showing structure of PLGA flat sheet membrane prepared with NMP solvent (left) and Dioxane solvent (right)

DISCUSSION & CONCLUSIONS: The scaffold microarchitecutre can be controlled by altering the process conditions such as the solvent used,polymer concentration, casting thickness and temperature. The scaffolds formed using this phase inversion technique were shown to support osteoblast-like cell attachment, proliferation and function. The varying porosity in the membranes will allow controlled mass transfer of nutrients and removal of toxins. Future work will combine the use of a bioreactor module with this scaffold to further improve the culture environment.

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MATRIX DEPOSITION BY TENDON CELLS IN SUSPENSION CULTURE

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INTRODUCTION: Tendons connect muscles to bone, and so must transmit high tensile loads. They consist of longitudinally running parallel bundles of collagen with rows of tendon cells between them. The tendon cells have an intimate relationship with one another and with the collagen bundles [1]. Recent *in vitro* studies indicate that the two gap junctions that are important in regulation of tendon cells response to mechanical loading; communication via connexin 32 containing gap junctions upregulates collagen synthesis, whereas via connexin 43 gap junctions does the opposite [2].

An important aim of tissue engineering of tendons is to replicate such environments *in vitro*. Here we describe the behaviour of tendon cells in a novel 3-dimensional culture system designed to allow cells to establish cell-cell contacts and deposit matrix in the absence of scaffold components and without disturbance by medium changes.

METHODS: Tendon fibroblasts were isolated by sequential protease and collagenase digestion from 50-day-old chicken feet [3]. Grown to passage 3 in Dulbecco`s modified eagle medium (DMEM) with 5% foetal calf serum, 1% antibiotic, 1% L-glutamine at 37^0C, 5% CO^2. Cells were suspended at $3x10^7$ cells/ml and 1 ml placed in a DispoDialyzer tube and sealed. This was placed into a 50 ml centrifuge tube with 40 ml DMEM and ascorbate (1mg/ml). After 24 hours, 7, 14 and 21 days the cell aggregates were fixed in 90% methanol (4^0C), washed in PBS and rapidly frozen on dry ice in Cry-M-Bed embedding medium (Cryo-M-Bed Ltd.)

Sections were cut at 10-15 μm, collected on histobond slides and immunolabelled. Sections labelled with polyclonal type I collagen were pretreated with hyaluronidase/chondroitinase. Sections were labelled with monoclonal type II, III and V collagen, actin, vimentin and decorin, connexins 32 and 43, vinculin, Pan cadherin and N-cadherin. Polyclonal antibody binding was detected using FITC conjugated secondary antibody goat anti-rabbit. Monoclonal antibody binding was detected using Alexa488 conjugated goat anti-mouse immunoglobulins. Labelled sections were mounted using Vectashield with propidium iodide. Sections were examined on a Leica Labrolux 12 epifluorescence microscope and photographs taken on a digital camera.

RESULTS: Cells in suspension culture formed elongated aggregates up to 3cm long. Immunolabels showed that at 7 days type I and III collagens were present, predominantly in the periphery. At 14 days the collagens were uniformly distributed throughout the aggregates and showed a degree of organisation. It is also clear from propidium iodide label that the cell nuclei have distinct areas of alignment. The aggregates labelled positively for actin stress fibres, N-cadherin, decorin and connexin 32. Type II collagen and connexin 43 showed no conclusive label.

DISCUSSION & CONCLUSIONS: The suspension cultures clearly show that tendon cells are capable of assembling an extracellular matrix in culture, rather than just releasing their collagen to the media. The structures formed were remarkably similar to tendons in their cell and matrix organisation, with parallel longitudinal rows of tendon cells interspersed with longitudinal collagen fibres. The amount of collagen produced by the cells is affected by time, though quantification must be obtained.

The differences in connexin label may relate to the growth and deposition of matrix. From antisense knockout studies we know that connexin 32 is stimulatory to collagen synthesis and connexin 43 inhibitory [2].It is important to note the orientation of the cell nuclei corresponding to that of the matrix orientation.

These results suggest that this system is influenced by growth factor combinations and that matrix deposition could be further enhanced and controlled.

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Mineral biomimicry - Generation of biomimetic microporous calcium carbonate spheres for skeletal regeneration

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INTRODUCTION: The development of self-assembling biomineral complexes for cell growth, growth factor and gene delivery offers tremendous opportunities for skeletal repair. Natural biological ceramic structures possess arrangements of structural elements that govern and optimise tissue function, nutrition and organisation may provide an innovative strategy to address this clinical need. The aim of this study was to fabricate biomineral microporous shells with highly complex forms and to examine their ability to interact with human osteoprogenitor cells as cell and growth factor delivery vehicles.

METHODS: Microporous vaterite shells were generated using a synthetic in-solution mineralisation technique in which mineral is spontaneously deposited around vesicular templates[1]. Porous and textured self-organising hollow microspheres (5-20 μm) were generated expressing controlled and uniform shapes. These micropores puncture the surface at high densities and are interconnected throughout the sphere. Primary human bone marrow cells labelled with Cell Tracker Green (CTG) and ethidium homodimer-1 fluorescent labels and osteoprogenitors transfected with an adenoviral vector expressing Green Fluorescent Protein (AdGFP) were cultured with vaterite shells over three weeks.

RESULTS: Cell biocompatibility of these biomimetic spheres was confirmed by confocal fluorescence and light microscopy in primary human bone marrow cultures labelled with CTG and bone marrow cultures transfected with AdGFP. At three weeks microspheres were encapsulated and integrated with osteoprogenitor cells. Histological analysis confirmed expression of alkaline phosphatase, extracellular matrix synthesis and the capacity for extensive mineralisation. Examination by fluorescent, SEM and light microscopy showed that the growth of osteoprogenitors transfected with AdGFP encapsulated and integrated with vaterite sphere in pellet culture and integration of vaterite spheres within the osteoprogenitor cell matrix indicating the potential of growth factor delivery. To determine the potential of the spheres to encapsulate selected proteins, microporous spheres were incubated with bovine haemoglobin. FITC microscopic examination showed haemoglobin could be entrapped inside the spheres and between the biomineral crystal plates during self-assembly.

DISCUSSION & CONCLUSIONS: In conclusion, these studies demonstrate the development of facile techniques for the generation of porous microsphere scaffolds that are biocompatible, aid mineralisation with potential for cell and growth factor delivery. These biomineral complexes present an innovative material for skeletal regeneration and for tissue engineering.

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ACKNOWLEDGEMENTS: We thank Professor Walter Sebald (University of Wurzburg, Germany) for generous provision of rhBMP-2. This work was supported by grants from the BBSRC and EPSRC. DWG is supported by the BBSRC. DG and DW are funded by the BBSRC and EPSRC respectively.

Mathematical Modelling in Tissue Engineering

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We believe that mathematical modelling has an important role to play in understanding and interpreting experimental data. We aim to outline below the process of mathematical modelling and illustrate the potential insights it may afford in medicine and in tissue engineering in particular.

Biological systems are inherently complex. The art of mathematical modelling is to unravel this complexity by identifying the key features of a given system. We do this by deriving equations based on fundamental principles such as conservation of mass and momentum, which govern its behaviour. Using various mathematical techniques and computer simulations, we can solve these equations over a wide range of physiological parameter values, thereby generating predictions which can be tested in the laboratory. The resulting insights can accelerate progress by suggesting the most fruitful lines of inquiry and thus reduce costs of experimental efforts. Furthermore, model predictions may also suggest new avenues for investigation.

A particularly productive field of mathematical medicine has been the study of solid tumour growth, [2] being a notable example. The aim of this study was to help resolve the debate in the literature on the mechanism of tumour encapsulation, there being two leading hypotheses: *active* (surrounding cells secrete collagen in an attempt to confine the tumour) and *passive* (tumour expansion compresses the surrounding extra-cellular matrix into a capsule). Using mathematical models, the authors found that the passive hypothesis provides the more natural explanation of encapsulation.

The field of tissue engineering presents numerous opportunities for mathematical modelling, in areas such as cell signalling and cell-substrate interactions. For example, a recent study on cell signalling in engineered urothelium tissue showed that a proposed reaction pathway for cellular differentiation (in response to the drug Troglidazone) was inadequate to explain the experimental observations [3]. A new feedback mechanism, in which activation of the signalling pathway stimulated active transport of the drug across the cell membrane, was postulated. The new model appears to show better agreement with the behaviour observed in the laboratory; it now remains for experimental investigations to determine the mechanism that operates in practice.

Our particular focus is on optimising the formation, functionality and viability of liver-cell spheroids *in vitro,* by modelling the interplay of cell-cell and cell-matrix interactions. Preliminary results from a mathematical model of the early stages of spheroid formation appear to indicate that compliant substrates (those with small Young's modulus and viscosity) are preferential for aggregation, as they offer least resistance to the motion of the cells.

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Preparation and in vitro Evaluation of Pullulan Nanoparticles as Gene Transfection System

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INTRODUCTION: Over the past few decades, there has been considerable interest in developing biodegradable nanoparticles as effective delivery vehicle of drugs, proteins, peptides and nucleotides [1]. Pullulan is a water soluble, neutral linear polysaccharide consisting of α-1,4 and α-1,6 glycosidic linkages [2]. The aim of this study was to prepare hydrogel nanoparticles of pullulan that can encapsulate water-soluble materials for intracellular delivery and targeting.

METHODS : In this study, pullulan nanoparticles in narrow size range encapsulating DNA (pBUDLacZ) have been prepared inside the aqueous core of the reverse micelles formed by AOT/n-hexane (w/o microemulsion). The effect of pullulan-DNA nanoparticles on cell viability has been illustrated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) on HEK293 and COS-7 cells with transfection potential of these nanoparticles evaluated using LipofectAMINE2000 (Invitrogen Ltd.) as a positive control.

RESULTS: Pullulan nanoparticles encapsulating plasmid pBUDLACZ were prepared using the highly monodispersed aqueous core of AOT/n-hexane reverse micellar droplets with the size in the range of 35-40nm in diameter with narrow size distribution. The integrity of the plasmid encapsulated inside the pullulan nanoparticles was illustrated by agarose gel electrophoresis. The nanoparticles encapsulating plasmid treated with DNase showed band of similar intensity as the nanoparticles-DNA without treatment (figure-1).

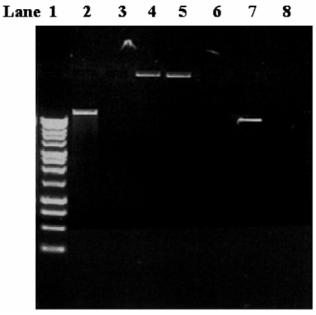

Fig.-1: Agarose Gel Electrphoresis. Lane1: Molecular weight marker; Lane2: Free pBUDLacZ DNA; Lane3: Free pBUDLacZ DNA treated with Dnase; Lane4: Pullulan nanoparticles encapsulating pBUDLacZ DNA; Lane5: Pullulan nanoparticles encapsulating pBUDLacZ DNA treated with Dnase; Lane6: Void pullulan nanoparticles; Lane7: Void pullulan nanoparticles with adsorbed DNA; Lane8: Void pullulan nanoparticles with adsorbed DNA treated with DNase

For eventual applicability in vivo, a required characteristic of gene delivery system is absence of cytotoxicity. The viability of Cos7 and Hek cells after incubation was measured by MTT assay after culturing for 24 hours. As it is evident from figure, the cytotoxicity of the nanoparticles was found to increase in relation to increased concentration of pullulan. The pullulan nanoparticles were found to be more than 100% viable relative to control at the concentration as high as 1mg/ml (fig.2). The transfection efficiency

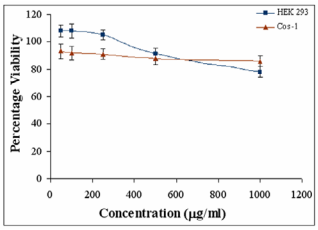

Figure-2: Cytotoxicity Profile of pullulan nanoparticles in HEK293 and COS-7 Cells as Determined by MTT Assay

of the pullulan nanoparticles was evaluated in Cos-7 and Hek 293 cells and compared with commercial reagent-LipofectAMINE2000 (fig.-3). The efficacy of transfection in vitro on HEK293 and COS-7 cells demonstrated cell type dependence with Cos-7 cells found to have a higher gene expression as compared to HEK293. The β-gal expression in Cos-7 cells by pullulan nanoparticle was comparable to commercially available LipofectAMINE2000.

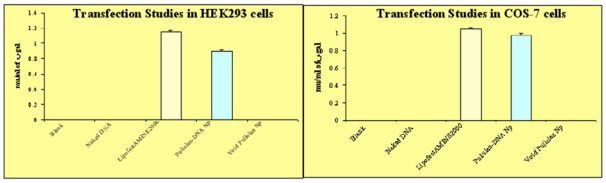

Figure-3: Comparison of Transfection Efficiency of Pullulan Nanoparticles with LipofectAMINE2000 incubated with HEK293 and Cos-7 cells

DISCUSSION & CONCLUSIONS: The nanoparticles of pullulan were prepared in the aqueous core of the reverse micelles formed by dissolving a surfactant AOT in n-Hexane with the size in the range of 35-40nm in diameter with narrow size distribution. Cell viability studies following incubation with the nanoparticles showed the lack of toxicity of pullulan. The efficacy of transfection in vitro on HEK293 and COS-7 cells demonstrated cell type dependence, with COS cells having a higher gene expression. The results of this study are very encouraging for the development of pullulan nanoparticles as an intracellular delivery system for drugs and genes. Further investigations on using these nanoparticles as a receptor-mediated drug carrier can be achieved by the use of cell specific ligands on the surface of these nanoparticles.

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Polymeric Nanoparticles Encapsulating NSAIDs for Ocular Delivery: Corneal Penetration and Polymorphonuclear Leukocyte Migration Studies*

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INTRODUCTION: As the ocular efficacy of drugs is greatly influenced by the corneal contact time, the most common method of improving the ocular availability of drugs is to increase precorneal residence time by using adequate drug delivery systems [1-2]. This paper describes the efficacy of mucoadhesive copolymeric micelle nanoparticles made of N-isopropylacrylamide (NIPAAM), N-vinylpyrrolidone (VP) and acrylic acid (AA) having cross-linkage with N, N' Methylene *bis* acrylamide (MBA) and containing non-steroidal anti-inflammatory drugs (NSAIDs) into the polymeric network as ocular drug carriers for enhanced bioavailability.

METHODS: NSAIDs loaded copolymeric nanoparticles of NIPAAM, VP and AA were synthesized as reported in our recent patent [3]. The drug loaded nanoparticles were characterized by various physicochemical methods like DLS, TEM, SEM, FT-IR, ^{1}H-NMR, XRD, UV/visible and fluorescence spectroscopic studies. The Drug release studies from the nanoparticles were done in different pH buffers at 25 and 37^{o}C.

In this study, an attempt was made to examine the performance of these drug loaded nanoparticle formulations by in vitro corneal permeability through goat cornea as well as in vivo anti-inflammatory activity in rabbit eyes by determining the polymorphonuclear (PMN) leukocyte migration in tears.

RESULTS AND DISCUSSION: Copolymer formed by random copolymerisation of NIPAAM and VP with AA, when dissolved in water shows amphiphilic character and forms micelle with a hydrophobic inner core and hydrophilic outer shell. Water insoluble NSAIDs like indomethacin or nimesulide was dissolved into the polymeric micelles. The size of the drug-loaded particles as measured by dynamic light scattering was found to be around 35-nm diameter at 25^{o}C and temperature dependent. TEM picture showed that the particles are spherical and highly monodispersed. The release of the drug from the nanoparticles was found to be temperature and pH dependent.

In vitro corneal permeation studies (fig-1) with nanoparticulated drug indicated much higher increase in ocular availability with no corneal damage compared to an aqueous suspension containing same amount of drug as in nanoparticles. The formulations showed that the percent inhibition of PMN migration with nanoparticle formulations was much higher and longer lasting than aqueous suspension of drug of equivalent concentration (Fig.2).

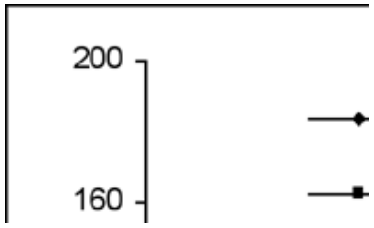

Fig.-1: Comparison of permeation of indomethacin (solid line) and nimesulide (broken lines) from nanoparticle formulation with aqueous suspension of drug of same concentration (2.0 mg/ml) through goat cornea.

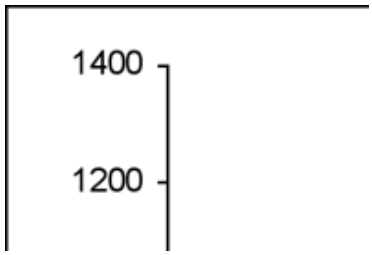

Figure2: Comparison of effect of indomethacin (solid line) and nimesulide (broken lines) loaded micelle formulation with aqueous suspension of drug of same concentration (0.05%) on PMN migration in tears of rabbit eye.

CONCLUSIONS: The above studies indicate that these formulations can be effectively used as ocular drug carriers in ocular delivery for higher therapeutic efficacy at much lower concentrations of drugs.

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The Attachment of Human Fibrochondrocytes to Ultraviolet Ozone Treated Polystyrene Surfaces

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INTRODUCTION: The important knee meniscus is prone to injury and has limited intrinsic healing potential despite surgical repair. *In vitro* culture of meniscal tissue and cells enables the scientist to investigate fibrochondrocytes and to possibly enhance their function and healing capability [1]. Ultraviolet Ozone (UVO) modified polystyrene surfaces have been characterised chemically and topographically [2]. These surfaces have been shown to promote attachment and proliferation of certain cell types [3]. The molecular mechanisms involved in cellular attachment to these surfaces remain unclear. However it has been established that cells attach to extracellular matrices via various transmembrane molecules of which the integrins are considered to be the most important group and some are specific in their ligand binding properties [4]. The integrin α5β1 binds to the adhesive glycoprotein Fibronectin and α2β1 to Collagen type I. This study investigated the attachment of human fibrochondrocytes to UVO modified surfaces and the possible role of the integrin subunits α2 and α5 in this process.

METHODS: Tissue samples from three separate human adult medial menisci were obtained at knee arthroscopy. Meniscal cells were isolated by standard cell culture techniques and cultured to 100% confluence before seeding onto UVO modified surfaces at a density of 10^4 cells /cm^2. The internal, untreated polystyrene surfaces of tissue culture dishes (Nunc, Denmark) were oxidatively modified with an ultraviolet ozone treater (Jelight Company Inc., California). By means of masking with squares of aluminium foil some dishes had both hydrophobic (UVO protected) and hydrophilic (UVO exposed) regions, enabling a visual comparison to be made between the two areas on the same dish. Images of cells in culture were obtained with a Leica Digital Camera (Switzerland) mounted on an optical microscope Leica DMIL (Germany). After 48 and 96 hours of culture standard wet transfer Western Blots were undertaken using primary antibodies to the α2 and α5 integrin subunits (Santa Cruz Biotechnology, California).

RESULTS: Meniscal cells attached preferentially to the UVO treated areas of polystyrene *Figure 1*. Western blot analysis showed the integrin subunit α5 to be present in the cell lysate from UVO exposed polystyrene dishes after being cultured for 48 and 96 hours *Figure 2*. No band was visible at the 126 kDa level suggesting that the α2 subunit was not present in the cell lysate.

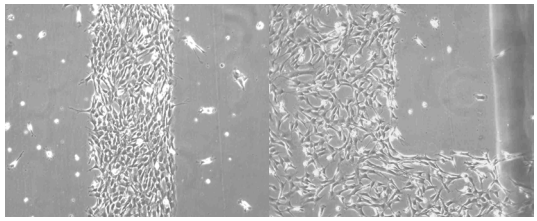

Fig. 1. Fibrochondrocyte attachment to UVO patterned polystyrene surfaces.

Fig. 2. Western Blot for the α5 integrin subunit.

DISCUSSION & CONCLUSIONS: Ultraviolet ozone modified surfaces have been shown to promote the attachment of Chinese Hamster Ovarian cells, skin fibroblasts, osteoblasts and now human meniscal cells. Although the surface oxygen concentration for optimal meniscal cell attachment is still to be confirmed. Collagen Type I is the predominant collagen in the extracellular matrix of meniscal tissue. The α2 subunit was not present in the cell lysate of UVO treated polystyrene surfaces after culture for 48 or 96 hours. Whether this is due to defective or absent collagen I at this stage of the culture remains to be answered. Fibronectin is also an extracellular constituent of the meniscus and is well known to promote cellular attachment to extracellular matrices. The α5 subunit's presence suggests that Fibronectin may facilitate the attachment of fibrochondrocytes to UVO modified polystyrene surfaces. Although whether this Fibronectin is produced by the fibrochondrocytes or from the serum supplemented media is still to be established.

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α-MELANOCYTE STIMULATING HORMONE ANTI-INFLAMMATORY ACTION IN HUMAN DERMAL FIBROBLAST CELLS

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INTRODUCTION: Inflammation resulting from a burn injury to skin tissue can seriously compromise the success of graft acceptance. However, alleviating inflammation with steroid compounds is often associated with side effects, including inhibition of wound healing. We have previously reported on a tridecapeptide α-melanocyte stimulating hormone (α-MSH) as a potent anti-inflammatory molecule in human skin melanocytes and keratinocytes[1, 2] acting via the melanocortin-1 receptor (MC-1R). Signalling arises predominantly via a carboxyl ligand tripeptide sequence (KPV) [1,2]. However, no evidence exists on a role of MSH peptides in human dermal fibroblasts, and hence whether MSH peptides may be of value in dermal inflammation. We therefore investigated cultured fibroblasts for expression of MC-1R and the potential of MSH peptides to: i) activate intracellular calcium responses; ii) inhibit pro-inflammatory cytokine (TNF-α) activation of NF-κB (a key transcription factor controlling inflammation) and iii) inhibit the TNF-α stimulated upregulation of intercellular adhesion molcecule-1 (ICAM-1).

METHODS: Human dermal fibroblasts were cultured as described previously[3]. MC-1R expression was detected by immunofluorescent microscopy (anti-MC-1R, Santa Cruz Inc., USA, [N-19]). Cells were preincubated with MSH peptides in the presence or absence of serum in the culture medium (10^{-12}M to 10^{-6}M; 15 minutes to 24 hours) prior to TNF-α addition (200 units/ml, 60 minutes). NF-κB was immunofluorescently labelled (anti-p65, Santa Cruz [sc-302]) and mean nuclear intensities of fluorescence determined using epifluorescence microscopy. ICAM-1 Western blotting (anti-ICAM-1, Santa Cruz [H-108]) was performed on cells preincubated with MSH peptides as described above prior to TNF–α addition (200 units/ml; 24 hours). Intracellular calcium responses were studied using Fura-2AM loaded cells, as described[4].

RESULTS: Fibroblasts labelled positively for the MC-1 receptor. TNF-□ activated fibroblast NF-□B after 60 minutes stimulation. Activation was inhibited by preincubating cells with KP-D-V (Figure 1). NF-κB inhibition was greatest at 10^{-12}M KP-D-V, with a 4 hour preincubation in the absence of serum proteins (84% ± 8%). Overall, MSH peptides were more potent in the absence of serum proteins.

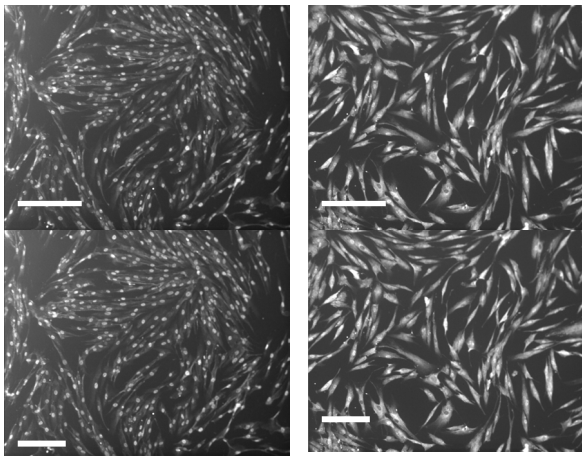

Figure 1:**a)** TNF–α induced nuclear translocation of NF-κB in fibroblasts is inhibited by **b)** KP-D-V (at 10^{-12}M; 4 hour preincubation). Bar=200μm.

Fibroblast ICAM-1 expression was dramatically upregulated by TNF-α after 24 hours. Addition of α-MSH (10^{-12}-10^{-6}M) prior to TNF-α addition inhibited ICAM-1. Fibroblasts also responded to MSH peptides at 10^{-14}M-10^{-6}M with a rapid and acute release of intracellular calcium.

DISCUSSION & CONCLUSIONS: MC-1R expression is fundamental for MSH peptide signalling in dermal fibroblast cells. TNF-α challenge rapidly activated NF-κB and upregulated ICAM-1, typical of inflammatory signalling. MSH peptides were observed to inhibit NF-κB activation and ICAM-1 upregulation in fibroblasts. The present study therefore supports potential value for use of MSH peptides in dermal inflammation, e.g. skin grafting for full thickness burns injuries.

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ACKNOWLEDGEMENTS: We thank the EPSRC (UK) for financial support.

Mechanical Stimulation of Calcium Signaling Pathways in Human Bone Cells Using Ferromagnetic Micro-particles: Implications for Tissue Engineering

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INTRODUCTION: Mechanical forces are known to influence bone cell behaviour *in vivo* and *in vitro*, leading to a range of cellular responses that ultimately influence bone matrix production. Techniques have previously been described that allow mechanical forces to be applied to specific cell surface receptors, such as integrins, via the use of micron-scale magnetic particles coated in specific ligands or antibodies [1, 2]. We are investigating the potential for using magnetic particle based techniques to mechanically condition cells via the activation of specific mechanotransduction pathways. Here we provide evidence that both primary human osteoblasts and bone marrow stromal cells respond to magnetic particle mediated mechanical stimulation via the generation of intracellular calcium transients.

METHODS: 4.5µm ferromagnetic particles were attached to integrin receptors on primary human osteoblasts and bone marrow stromal cells via an RGD peptide coating. Typically 4-6 particles were attached to each cell. Cells were then exposed to static magnetic fields (600G) using permanent rare Earth magnets. The forces acting on the membrane bound particles are estimated to be in the range of 10-30pN. Fluo-3 was used in conjunction with laser scanning confocal microscopy and time-lapse software to monitor intracellular calcium levels of populations of cells in real time. Cells were monitored over a period of 5-15 minutes and the activity of stimulated cells was compared to normal cells without particles, and also to cells with particles but no applied magnetic field. The effect of magnetic field alone was also investigated. The number of cells responding, magnitude, time to onset and duration of calcium responses was evaluated.

RESULTS: Magnet field alone had no effect on either cell type. The presence of magnetic particles in the absence of an applied magnetic field also had little effect on calcium signalling, although there was a slight increase in the percentage of cells exhibiting Ca^{2+} transients compared to normal cells. Both osteoblasts and bone marrow stromal cells exhibited increased levels of calcium activity in response to magnetic loading. There were however significant differences in both the background levels of spontaneous calcium activity and the characteristics of magnetically stimulated calcium responses between the different cell types. Bone marrow stromal cells typically responded with a single Ca^{2+} transient whereas responsive osteoblast cells demonstrated both single and oscillating Ca^{2+} transients. Time to onset of response was also found to vary between cell types.

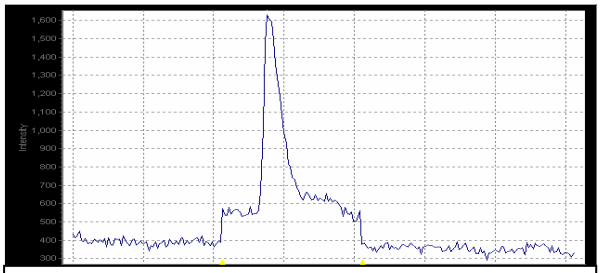

Fig 1. Ca^{2+} trace from hBMSc exposed to magnetic loading. Magnet applied between yellow arrows. Image every 2 sec.

DISCUSSION & CONCLUSIONS: Magnetic particles offer a valuable tool for mechanically stimulating cells in-vitro, and possibly in-vivo. The ability to apply mechanical forces directly to cell surface receptors has several advantages over more traditional approaches where compressive or tensile forces are applied to cell seeded scaffolds. It is clear that the different cell types investigated herein displayed differential responses to similar modes of stimulation. These differences may be of importance when deciding on the pattern of loading and optimal cell source to be used for the generation of tissue engineered bone constructs in-vitro. Future directions include the development of strategies that will allow the magnetic localisation and differentiation of human mesenchymal stem cells *in vivo* for the repair of damaged or diseased tissue.

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ACKNOWLEDGEMENTS: This work is supported by the European Commission Framework V programme BITES, QRLT-1999-00559, BBSRC grant No. 323/REI18446, and a Welcome Trust Showcase Award.

Biocompatibility of Glancing Angle Deposited Thin Nanostructured Silicon Films: Relevance of Angle for Fibroblasts Reaction

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INTRODUCTION: Biological relevant cellular interactions with artificial materials are illustrated by a surface and cell type specific morphological, biochemical and behavioural response. Even slight differences in topography or chemistry can produce a wide variety of cell responses [1]. The underlying physicochemical and biological mechanisms for these effects are still a matter for debate. Here we set out to investigate how hTert human fibroblasts react to the same material (silicium) deposited as columnar thin films where the tilting angle of the columns was varied.

METHODS: The surface modification technique used, Glancing Angle Deposition (GLAD), is a modification of vacuum vapour evaporation. In our case the silicon was electron-beam heated while a substrate (glass) was positioned 50cm directly above the 2.5cm diameter source. The film structures produced were chemically identical slanted columnar thin films. The substrate was rotated continuously for a total of 5 turns for each film, deposited at 0, 70, 80, and 85 degrees. The evaporated mass was identical for each film, yielding films of approximately 500nm thickness [2, 3]. hTert fibroblasts were seeded onto the structures at 30k/ml, 3ml/disk and established overnight. The surface and structures were studied live using phase contrast- and interference reflection microscopy and after fixation by fluorescence, SEM and AFM.

RESULTS: Cells adhered initially (1h) on all surfaces. On 80º films the cells developed blebs and died within hours of seeding leaving a cell free surface. On 0º surfaces the fibroblasts were able to adhere and spread, only then to die within the next 12h. Cells survived and thrived on the 70º surfaces as well as on glass. On the 85º surface cells survived but could be seen to phagocytose the surface. See Table and Figure for details.

Table 1. Cellular reaction to GLAD surfaces.

	Glass	0º	70º	80º	85º
Adhesion	X	X	X	(x)	X
Spreading	X	X	X	—	X
Survival	X	—	X	—	X

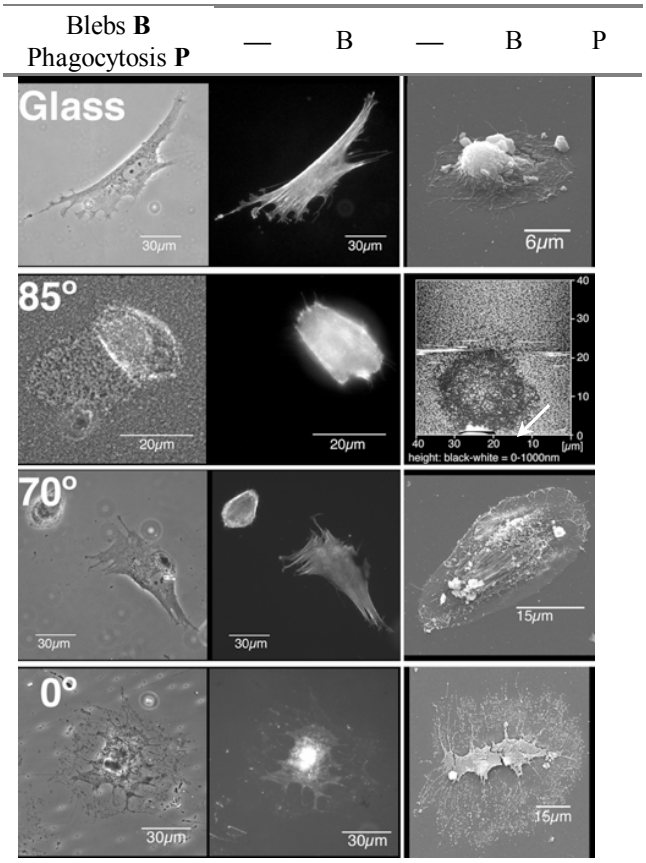

Fig. 1. Cell morphology of hTert fibroblasts after 1d on GAD surfaces. 1st panel phase contrast, 2nd panel F-actin and 3rd panel SEM except 85°, AFM image: surface gouged out by the cell, which has vanished off to the bottom (arrow: cell edge). No cells were found on 80° after 1d.

DISCUSSION & CONCLUSIONS: We have shown that despite being of the same chemical composition the angle of deposition of a nanocolumnar film has a distinct biological effect on cell survival, morphology and behaviour.

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ACKNOWLEDGEMENTS: Funding from EPSRC (UK) and NSERC (Canada) is acknowledged.

ATTACHMENT AND SPATIAL CONTROL OF CELLS IN SERUM ENRICHED MEDIA USING MICRO-PATTERNED UV/OZONE TREATMENT.

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INTRODUCTION: There is increasing interest in the development of quick and cost effective techniques that produce surfaces of well-defined chemical heterogeneity, which are suitable for promoting rapid cellular adhesion and spreading[1]. Surfaces are now being designed at a molecular level to facilitate specific attachment of different types of cells and to spatially direct their growth. The spatial resolution of micro-contact printing[2], self assembling peptides, patterned self assembled monolayers (SAMs)[3] and plasma polymerisation are sufficient to produce patterns of cellular dimensions. This work demonstrates that UV/Ozone treatments may pattern polystyrene (PS) surfaces and highlights some of the physicochemical factors that may be of importance in achieving high spatial resolution using this approach.

METHODS: The internal surfaces of 45mm PS dishes were modified with a UV/Ozone treatment at a variety of times (10-900s). Surface chemical composition of treated and untreated dishes were characterised by XPS. Topographic changes were examined by AFM under ambient conditions. A range of photo masks was used to chemically pattern the internal surfaces of PS dishes[4]. 3T3.L1, CHO, HOS TE85 and primary human osteoblast (isolated from osteoarthritic bone) cells were grown to 70-80% confluence in cell culture media with 10% FCS in 5% CO_2 at 37°C. Spatial control of cells on patterned surfaces was determined by using dishes plated at 10^4cells/cm^2.

RESULTS & DISCUSSION: Exposure to the UV/Ozone oxidation resulted in an increase in surface oxygen from 0 to ~36% unwashed and ~28% washed after 900s treatment. This reduction in surface oxygen after washing is due to the removal of low molecular weight oxidation debris. Contact angle analysis showed there was a marked reduction in contact angle with increasing treatment time and therefore an increase in surface energy due to the introduction of hydrophilic oxygen species to the surface. Roughness analysis of the treated surfaces showed an increase from ~2 to ~7nm which is less than has been reported to effect cell attachment and growth. Figures 1a and b show examples of the cellular attachment to patterned surfaces. Cells have attached to the treated, more hydrophilic, areas on the surfaces.

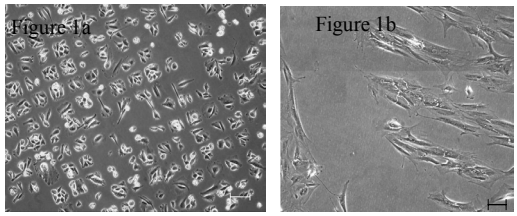

Figure 1. Optical images of cellular patterned surfaces; 1a shows CHO cells after 48h incubation, 20 s UV/O exposure, 250 mesh grid. 1b shows primary human osteoblast cells after 72h incubation, 60 s UV/O exposure, Sjøstrand mesh grid, where the curved edge of the grid is clearly visible. Scale bar = 100μm.

CONCLUSIONS: UV/Ozone treatment can be used as a simple, rapid, and cost effective method for chemically patterning surfaces. The resulting surfaces have both a controllable chemical functionality and a pattern resolution comparable to other more widely used patterning techniques. Surface roughening using this technique is minimal, less than would be expected to effect cellular growth. This work demonstrates that chemical patterns with spatial resolution of less than 50 μm can be achieved using UV/Ozone treatments. Furthermore, this technique may be used to pattern rough surfaces, such as PS and be used to achieve spatial control, cellular attachment and proliferation of 3T3.L1, CHO, HOS TE85 and primary human osteoblast cells.

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ACKNOWLEDGEMENTS: Financial aid from the EPSRC grant no. GR/M86996/01 (S.A.Mitchell), SHEFCRDG1008 & EPSRC (A.H.C.Poulsson & M.R.Davidson). The Orthopaedic Department, Aberdeen University Medical School, Forresterhill, and kindly providing primary human osteoblast and HOS cells.

AFM and XPS Study on Albumin Adsorption onto Graphite Surfaces

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INTRODUCTION: Although many proteins adsorb spontaneously onto solid surfaces, they often denature or adopt undesirable orientations,[1] so the cells in contact with an implanted material will interact with the adsorbed protein layer rather than with the biomaterial surface itself. The aim of this work is to study the behavior of HSA in contact with a carbon surface.

METHODS: HSA adsorption onto freshly cleaved highly oriented pyrolytic graphite (HOPG) surface was studied. Protein solutions (from 0.001 to 2 mg/ml) were prepared using HSA (essentially fatty acid and globulin free lyophilized powder) in Dulbecco's phosphate buffered saline. Droplets (200µl) of HSA solutions were incubated at 37ºC for 1 hour in contact with surfaces then washed in MilliQ water for 4 hours. The AFM analysis was performed immediately after drying of the samples under ambient conditions in tapping mode (TM). The surface chemical composition was analysed using a Kratos X-ray photoelectron spectrometer

RESULTS&DISSCUSION: The AFM images of the HSA solution (concentration higher than 0.005mg/ml) show the formation of a network structure, with peaks and ridges (Fig. 1c and 1d). As the HSA solution concentration increases, the albumin has a tendency to aggregate into a more compact network, with narrower valleys and wider ridges and the RMS roughness values also increase(Fig 2).

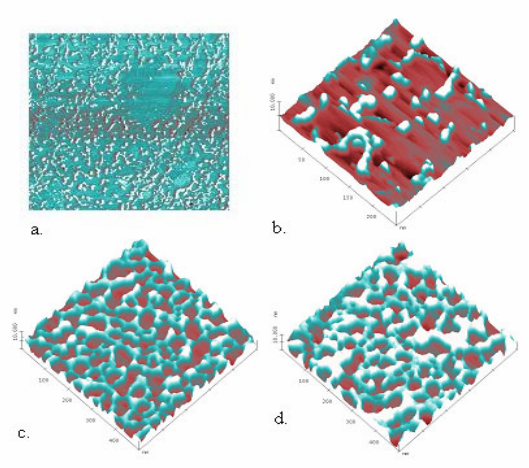

Fig. 1:TM-AFM images (a,c,d:500x500x5nm; b:250x250x5nm) of HSA solution (concentration-a,b:0.001mg/ml; c:0.1mg/ml and d:2mg/ml) adsorbed on HOPG (a. phase; b ,c ,d height images)

The phase contrast in the case of adsorption from the weakest protein solution(0.001mg/ml) showed individual features (Fig. 1a) and chainlike aggregates along the steps. These features have two different shapes: triangular / circular (diameters: 25±3nm) and oblong (35±5nmx24±2nm) with the height in the range 2.5±0.3nm (Fig 1b). Due to the tip radius of curvature (5-10nm), the apparent width of the imaged molecules is extended by 10-20nm. The triangular or circular imaged features, similar to those reported on mica surfaces,[2] may be HSA in normal conformation (8x3nm) and the oblong might be the elongated F form (4x12.9nm)[3] the conformation difference being induced by the surface binding process.[3]

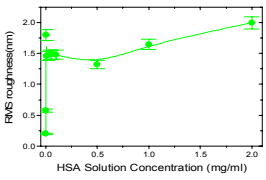

Fig.2. RMS roughness function of HSA solution concentration

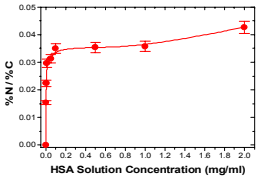

Fig.3. %N/%C of the surface function of HSA solution concentration

From the XPS narrow scan spectra (C1s, N1s and O1s) we calculated surface elemental compositions. The nitrogen atomic concentration of the surface is a function only of the irreversibly bound albumin because the surfaces have been thoroughly washed with analytically pure water. As shown in Fig.3, the nitrogen concentration was found to increase with increasing HSA solution concentration.

CONCLUSIONS: AFM topographic analysis showed the formation of a network structure, suggesting strong interactions between the adsorbed protein molecules. For adsorption from the solution of concentration 0.001mg/ml onto HOPG individual molecules have been imaged. These appear to take one of two forms depending upon the adsorption energy and site.

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Fibroblast adhesion & morphology on titanium & TAN with various surface treatments

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Introduction: Determination of cytocompatibility of a surface gives an indication of it's suitability for *in vivo* use. A cytocompatible surface should allow a cell to behave as it would in its natural *in vivo* surroundings. In this study some determinants of fibroblast cytocompatibility were evaluated for various test surfaces (promotion of cell attachment, adhesion, spreading and proliferation).

Materials and Methods: A Laser profilometer (LP) and Scanning Electron Microscope (SEM) were used to characterise surface topographies (Table 1). Balb/c 3T3 fibroblasts were cultured[1], 20,000 cells on each surface for 48hrs (sub-confluent). For morphological analysis cells were fixed[2] and imaged with an SEM using the high emission backscattered electron (BSE) technique[3] at low voltage (5kV). The area of 30 cells was calculated. The focal adhesion protein vinculin was immunogold-labelled[4] and enlarged with gold enhancer to allow SEM imaging of labelled whole cells at 6kV [5]. The percentage area of labelling on 20 cells was measured.

Results: LP data displayed topography of circular turnings of several of the surfaces which may well have reduced effect upon the cells of the ensuing surface treatment (TSS, TIG, TLF, TAST and THY). Cells on TSS, Therm, TIG, TLF, and TAST were generally well spread with a large proportion of the cellular edges closely associated with the various substrata. Cells on TS and NS surfaces were much smaller and were generally raised away from the substratum. No cells attached to the THY surface. Quantitative analysis of the cell area showed no major difference except on NS that were much smaller than the others (Fig. 1).

Label	Description	Ra (µm)
NS	Standard anodised TAN (Ti AL Nb)	0.99
NT	Tiodised TAN	0.79
TS	Standard anodised Ti tanium	1.15
TT	Tiodised TS	0.51
TSS	Titanium-101 gold anodized control	0.83
THY	TSS with grafted sodium hyaluronate (THY)	1.09
TAST	TSS with polymer cell promotion (TAST)	1.09
TIG	Nitrogen ion implanted TSS (TIG)	1.05
TLF	Low friction grey anodized TSS (TLF)	1.14
Therm	Thermanox plastic	0.06

Table 1- Substrates investigated.

Specific labelling of vinculin in mature 'dash' focal adhesions was observed on all surfaces (except THY, where no cells grew). Quantitative analysis showed the highest area of labelling on the NT surface. A high area of labelling was also observed on TIG and TLF. There was no significant difference between the percentage labelling of the cells on TT, TAST, TS, TSS and Therm. The cells on both negative controls had a very low amount of background label. Cells on the NS surface could not be analysed as the cells were damaged during substrate removal from the resin.

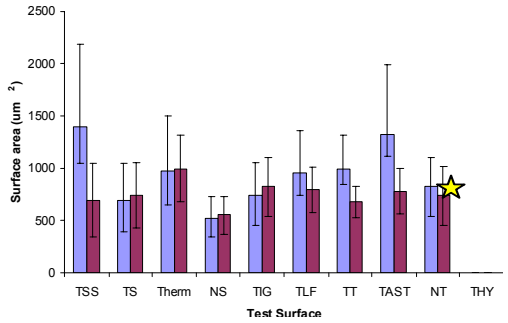

Fig. 1. *Average cell area on the surfaces. Results for both cultures combined. Error bar- twice the standard area.*

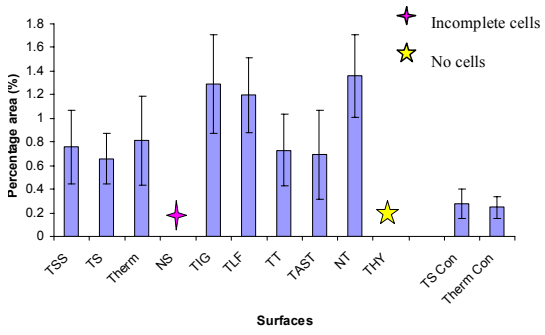

Fig 2. *Average percentage labelled cell area on the surfaces. Cells on TS and Therm also used as negative controls. Error bar - twice the standard area.*

Conclusions: There was a difference in the fibroblast morphological and adhesive response to the surfaces indicating varying degrees of cytocompatibility. THY did not allow any fibroblast growth in 48 hours and was the least cytocompatible.

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SPATIAL CONTROL OF CELLULAR GROWTH ON A PHYSICALLY MODIFIED POLYMERIC SURFACE

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INTRODUCTION: Biodegradable poly α-hydroxy acid polymers have been widely investigated as candidates for tissue engineering biomaterials. poly(DL-lactic acid) ($P_{DL}LA$) is of particular interest due to its biocompatibility and suitability for scaffold formation using supercritical carbon dioxide. It is recognised however that the affinity for cell attachment to PLA is relatively low. We aim, through an increased physicochemical understanding of the $P_{DL}LA$ and its modification by poly(ethylene glycol) (PEG), to modulate and improve cellular interactions with the surface by physical modification of the polymer surface.

METHODS: The modification of a PLA continuous phase with poly(ethylene glycol) (PEG) was achieved using a method of physical entrapment[1]. In order to determine the effect of PEG concentration on cell-surface interactions, this approach was employed to spatially control entrapment of PEG on the PLA surface. Bulk and surface specific thermal analysis techniques have been utilised to analyse the surface properties of the modified PLA, scanning thermal microscopy (SThM) localised at the surface and bulk properties with differential scanning calorimetry (DSC).

RESULTS: The interface generated by spatial control of entrapment was assessed by quantification of cell adhesion thus allowing comparison of differences between modified and unmodified regions. A significant difference in both cellular attachment and morphology was observed. It has been showed that a relatively sharp boundary was generated and that PEG was able to render the PLA surface protein resistant.

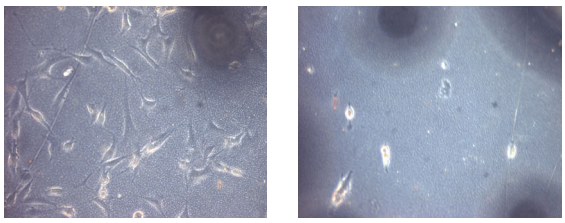

Fig. 1: Differential cell adhesion created by modification of PLA matrix with PEG. Untreated region (left), PEG modified region right.

The results from both thermal techniques suggest that the two discreet regions within the PLA construct can be distinguished, the first of these being the unmodified bulk or core PLA region and the second being the PEG-modified surface layer. The incorporated PEG appears to act as a plasticiser for the PLA and hence suggests that a miscible blend is being produced between the two polymer species.

The chemical nature of the boundary created by physical entrapment of PEG has also been evaluated using Time-of-flight secondary ion mass spectrometry (TOF-SIMS). The images show a boundary between the modified and unmodified regions, highlighted by the different ionic species found in these areas. The brighter regions correspond to an abundance of the ionic species being mapped.

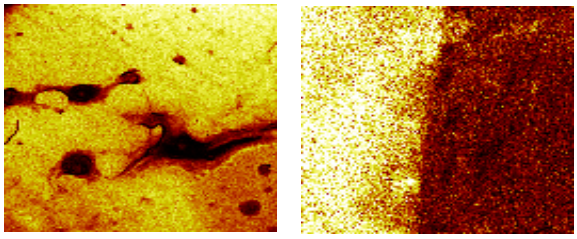

Fig. 2: (left) TOF-SIMS image of phase separated PEG regions,4mm^2 scan size, diagnostic peak m/z 55 (C_3H_3O). (Right) Image of boundary created by physical modification, 500μm^2 scan, diagnostic peak m/z 26$^-$ (CN).

DISCUSSION & CONCLUSIONS: We have demonstrated and characterized a simple and rapid method of physically engineering a polymer surface. This method has been employed to spatially control the surface modification hence allowing direct comparisons to be made between different regions of differential cellular response. Thermal analysis has helped us to gain the fundamental understanding of the entrapment process needed to allow optimization of the system and subsequent control of cellular response.

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IN VITRO ASSESSMENT OF CELL PENETRATION INTO POROUS HYDROXYAPATITE SCAFFOLDS WITH ALIGNED MACROARCHITECTURE

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INTRODUCTION: Bone defects that occur during surgery, as a result of trauma, or from abnormal development, incur a major cost to healthcare systems as well as a reducing the patients' quality of life[1]. The *in vitro* generation of such tissues and/or biocompatible scaffolds for implantation, using tissue engineering strategies[2], would be a major breakthrough for orthopaedic surgery. However, a common problem encountered in tissue engineering is the rapid formation of tissue on the outer edge of the scaffold whilst the centre becomes necrotic. To address this, the scaffold has to incorporate a design to improve nutrient and cell transfer to the scaffold centre. In this study, aligned channels of different diameter were inserted into the centre of a random porous hydroxyapatite scaffold and cell coverage assessed to determine the optimum channel diameter for improved cell proliferation within the centre of a scaffold.

METHODS: Hydroxyapatite (HA) scaffolds with random porosity were manufactured using a modified slip-casting methodology. Whilst in the green state, a single channel was inserted through the centre, which upon shrinkage (due to sintering) produced a series of scaffolds with channel diameters ranging from approximately 150-500 µm. Human osteoblast sarcoma (HOS) cells were seeded into these scaffolds with agitation (100 rpm on an orbital shaker) and cultured statically for 8 days. Scaffolds were cut to reveal the channel in longitudinal section and prepared for scanning electron microscopy[3]. Cell penetration and average cell coverage within each of the channel diameters was assessed using image analysis software (*Fig 1*).

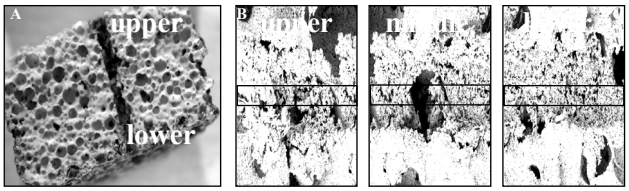

Fig 1: HOS seeded HA scaffold, cut into longitudinal section and prepared for SEM (A). Image analysis to assess cell coverage in aligned channels of varying diameter (B).

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RESULTS: Increased cell coverage was observed with increasing channel diameter (*Fig 2*). Extrapolating this data, the minimum pore or channel diameter for cell penetration in this system was 82 µm (where the line cuts the x axis). Cell coverage within the middle section of the channel (at the core of the scaffold) increased with increasing channel diameter (*Fig 3*).

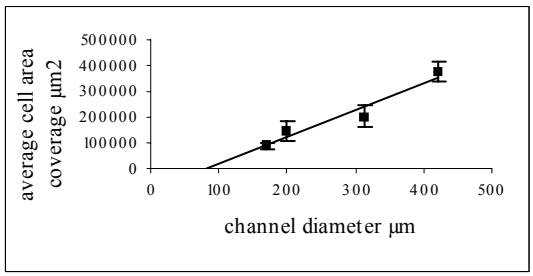

*Fig 2: Average total cell area coverage within channels of varying diameter (µm²). Statistical significance using the Tukey-Kramer multiple comparison post-test * p<0.05, ** p<0.01, ***p<0.001; n=4.*

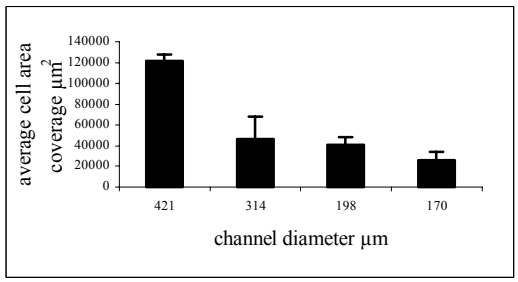

*Fig 3: Average cell area coverage within the middle section of channels of varying diameter (µm²). Statistical significance using the Tukey-Kramer multiple comparison post-test * p<0.05; n=4*

DISCUSSION & CONCLUSIONS: This study demonstrates that aligned channels improve cell and nutrient transfer within a porous scaffold, in particular within the centre of the scaffold. The optimum channel diameter for this application is 421 µm and the minimum pore or channel diameter size for osteoblast penetration into HA scaffolds is 82 µm.

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ION CHANNEL EXPRESION IN INTERVERTEBRAL DISC CELLS

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INTRODUCTION: Musculoskeletal system cells exhibit the property of mechanotransduction. Various groups are currently researching the possibility of inducing cellular upregulation by mechanotransduction of membrane ion channels in bone cells. However, as yet this approach has not been applied to intervertebral disc cells. Furthermore very little has been reported about ion channel expression of intervertebral disc cells. The purpose of this study was to determine the ion channel expression of intervertebral disc cells with particular reference to the Voltage Operated Calcium Channel, and the potassium channel TREK-1.

METHODS: Human intervertebral disc cells, harvested from patients at the time of routine surgery, were used for this study. All samples were taken after informed consent had been obtained from patients.

Intervertebral disc cells from 4 patients were used for this study. Nucleus pulposus and annulus fibrosus cells were isolated from the intervertebral disc samples. Both cells types were cultured in a monloayer under standard conditions. Cell samples were used for RNA extraction, using a commercially available kit. Protein extraction was also performed on cell samples. A reverse transcription reaction was performed using the extracted RNA. After polymerase chain reaction (PCR) was performed to analyse for gene expression of the VOCC and TREK-1. Western blot analysis was performed using the extracted protein for the VOCC and TREK-1.

RESULTS: PCR identified the gene for the VOCC, in particular the $\alpha_2\delta_1$ subunit was identified. PCR was also positive for TREK-1. See Figure 1.

Western blotting also revealed bands corresponding to the VOCC and TREK-1. See Figure 2.

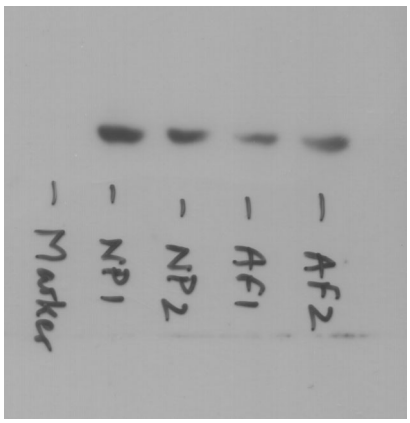

Fig. 1:Bands seen on western blot analysis. NP (Nucleus Pulposus) and AF (Annulus Fibrosus)

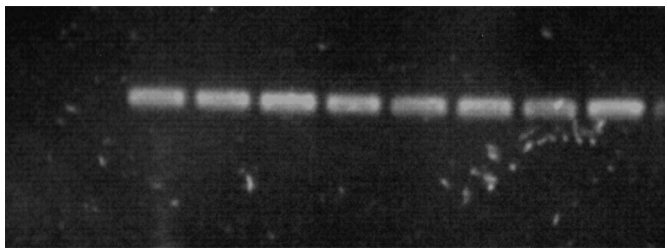

Fig. 2: Bands seen on PCR. 4 bands on the left are from Nucleus Pulposus cells and the 4 on the right are from Annulus Fibrosus cells.

DISCUSSION & CONCLUSIONS: Both nucleus pulposus and annulus fibrosus cells possess the VOCC and TREK-1 ion channels. These findings advance our current knowledge of the cellular biology of intervertebral disc cells. We believe that the cellular function of disc cells can be upregulated by the application of force, and that this may be achieved by applying force directly to the VOCC and TREK-1 ion channels, using biomagnetic manipulation.

Measurement of NF-κB as an indicator of stress in skin cells in 2D culture and a 3D reconstructed skin model

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INTRODUCTION: NF-κB is a DNA-binding transcription factor that plays an essential role in the regulation of many genes, especially those involved in stress, immune and inflammatory responses. Human skin keratinocytes and fibroblasts, in common with many other cell types in the body, respond to common proinflammatory cytokines such as TNF-α with a rapid activation of NF-κB. However, various stimuli including viral infection, lipopolysaccharide, UV irradiation, shear stress and intracellular pH can also activate NF-κB. The aim of the present study was to develop an immunofluorescence method for measuring acute NF-κB activity in keratinocytes and fibroblasts in normal and reconstructed human skin, based on a similar approach for measurement of cells in monolayers. We also investigated the robustness of the technique by exposing cells to TNF-α, pH and a depletion of culture medium buffering potential.

METHODS: Culture of human dermal fibroblasts, preparation of human skin and reconstructed human skin were as previously described [1]. Cells were stimulated with TNF-α, incubated with pH-adjusted medium or incubated in an unbuffered atmosphere to investigate inflammatory, pH and carbon dioxide tension as indices of stress. Immunofluorescent staining of p65/NF-κB was performed as reported previously [1,2]. Fluorescence micrographs of immunolabelled samples were taken using a Leica DM-IRB inverted fluorescent microscope using epifluorescent illumination at λ_{ex}= 495nm, λ_{em}= 515nm (for FITC / NF-κB visualization) and λ_{ex}= 358nm, λ_{em}= 461nm (for DAPI / nuclei visualization). NF-κB activation was calculated based on nuclear versus cytoplasmic localisation using Improvision Openlab v3.0.2 [2]. Normal and reconstructed human skin were treated as above and then processed for cryosectioning (5μm), followed by analysis of NF-κB using the above immunolabelling approach.

RESULTS: Incubation of fibroblast cells with TNF-α rapidly activated NF-κB. This was observed by immunofluorescence microscopy as rapid translocation from the cytoplasm to the nucleus. Incubation of cells for 60 minutes in medium with pH adjusted from 6.3 to 8.5 also activated NF-κB, in comparison to cells maintained at a physiological pH of 7.3 (Figure 1). Fibroblasts were also depleted of buffered culture medium by incubation in 21% O_2 / 79% N_2. Similarly, an increase in NF-κB activation was found with time, correlating with medium acidosis. NF-κB analysis by immunofluorescent microscopy was then used for the analysis of normal and reconstructed human skin containing keratinocytes and fibroblasts. A similar activation of NF-κB to TNF-α, pH and unbuffered conditions for both cell types was observed. However, it was also possible to monitor the relative activation of the two cells types based on cellular position in the tissue.

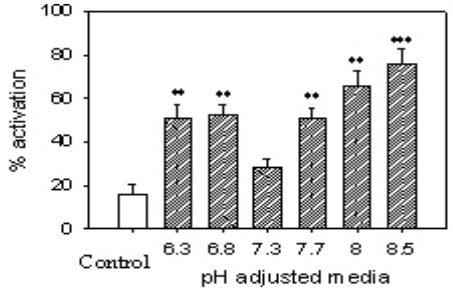

Figure 1. Effect of pH on human fibroblast NF-κB activation

DISCUSSION & CONCLUSIONS: We have developed an immunofluorescent technique for measuring NF-κB activity, initially used for analysis of cells in 2D, for analysis of cells in a 3D skin model. Using this method, inflammatory stimuli, pH and culture medium buffering potential were found to activate NF-κB in cultured fibroblast monolayers, and keratinocytes and fibroblasts in normal human and reconstructed skin. This study highlights the sensitivity of using NF-κB transcription factor activation as an indictor of inflammatory, pH or environmental stress. Application of this technique for measuring NF-κB of cells contained in tissues will have considerable value for the analysis of cellular stress in tissue engineering applications.

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Alpha-MSH inhibits inflammatory signalling in Glial cells

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INTRODUCTION: α-melanocyte stimulating hormone (α-MSH) has a potent anti-inflammatory role in skin, vascular and nervous tissue[1]. It inhibits the production and action of proinflammatory cytokines and specifically can inhibit TNF-α activation of the transcription factor NF-κB via the melanocortin-1 receptor (MC-1R)[2,3]. NF-κB activation synthesizes several inflammatory genes, and its inhibition by α-MSH is a pathway by which inflammation can be controlled. We have investigated the ability of α-MSH to inhibit proinflammatory cytokine activation of NF-κB in primary olfactory ensheathing cells (OECs) and Schwann cells. These two glial cells have both been used with limited success in nerve repair where acute inflammation propagates nerve injury and damages host cells and donor glial cell transplants.

METHODS: α-MSH (10^{-8}M or 10^{-10}M) or forskolin (10^{-4}M) was added to the glial cells 15 minutes prior to TNF-α (1000 units/ml) or γ-IFN (1000 unit/ml). Cells were incubated with cytokines for 60 minutes and then fixed for fluorescent staining using 4% paraformaldehyde. Cells were permeablised for immunolabelling of NF-κB/p65, whereas non-permeablised cells were used for immunolabelling of the MC-1 receptor. NF-κB/p65 in the cells was observed using epifluorescent microscopy. Relative levels of NF-kB activation in cells were assessed according to nuclear verses cytoplasmic localization using image analysis software.

RESULTS: Inhibition of cytokine-stimulated NF-κB was investigated by immunolabelling for the transcriptionally active p65 subunit of NF-κB. OECs and Schwann cells both responded to TNF-α and INF-γ by nuclear localisation of the p65 NF-κB subunit. When the glial cells were co-incubated with α-MSH, the amount of nuclear NF-κB activation was decreased significantly. Co-incubation with forskolin also significantly decreased nuclear translocation of NF-κB. OECs and Schwann cells both immunolabelled positively for the melocortin-1 receptor.

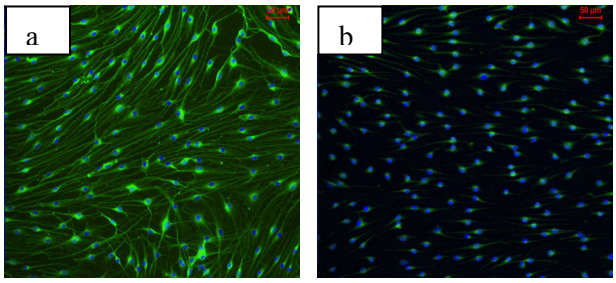

Fig. 1. a) Cytoplasmic localisation of NF-kB/p65 in unstimulated control Schwann cells. b) Nuclear localisation of NF-kB in cytokine activated Schwann cells. NF-κB/p65 (green), DAPI (blue).

DISCUSSION & CONCLUSIONS: This study demonstrates that primary OECs and Schwann cells express the transcription factor NF-κB. Both glial cells responded to the proinflammatory cytokine molecules TNF-α and γ-IFN with a rapid activation of the p65 subunit of NF-κB, observed as a translocation from the cytoplasm to the nucleus. Cells were also found to label positively for the type-1 melanocortin receptor and responded to the receptor ligand (α-MSH) at very low concentrations with a reduction in the extent of cytokine-stimulated NF-κB activation. Co-incubation of cells with forskolin (to elevate cAMP independently of the MC-1 receptors) inhibited the activation of NF-kB. This is supportive of a cAMP mediated action of cytokine inhibition and has been reported to be the intracellular route responsible for α-MSH / MC-1R suppression of inflammation. These results show that different glial cells are able to respond to proinflammatory and anti-inflammatory signals. Therefore, the use of α-MSH and related melanocortin peptides may be of value in augmenting cell-based glial therapy in nerve injury.

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ACKNOWLEDGEMENTS: We would like to thank the RPSGB for funding of Katrina Teare.

SOX9 TRANSDUCTION AND TGFβ-3 TREATMENT OF LATE PASSAGE HUMAN ARTICULAR CHONDROCYTES IN PELLET CULTURE POTENTIATES CARTILAGE MATRIX FORMATION

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INTRODUCTION: The expansion of articular chondrocytes for tissue engineering purposes leads to loss of chondrocytic function that can be difficult to regain. We have transduced SOX9, a transcription factor crucial for the induction and regulation of the chondrocyte phenotype[1], into monolayer expanded human articular chondrocytes using a retroviral vector[2]. The cells were grown as pellet cultures in the presence of TGFβ-3 and IGF-1 and the expression of marker genes and extracellular matrix (ECM) production was compared with that of control cultures.

METHODS: Human articular chondrocytes (HAC), from tissue obtained following total knee arthroplasties, were grown as monolayers and between passages 2-5 cell growth was accelerated by stimulation with growth factors. During this time the cells were transduced with a retrovirus bicistronically expressing human SOX9 and green fluorescent protein. After transduction, the cells were >90% positive. Control cells were transduced with a retrovirus containing GFP only. Subsequently, the cells were further expanded in monolayer to passages 7-10 and then grown as pellet cultures for 14 days in medium containing 10ng/ml TGFβ-3 and/or 100ng/ml IGF-1. Glycosaminoglycan (GAG) accumulation was measured using the dimethylmethylene blue assay on papain-digested pellets. Pellets were fixed in 4% formaldehyde and embedded in paraffin wax for histological analysis. 5μm sections were cut and stained with 0.1% safranin-O. Total RNA was prepared from pellet cultures using Tri Reagent. cDNA was reverse transcribed and then amplified by PCR with an MJ Opticon 2 using a SYBR Green Core Kit (Eurogentec) with collagen I, II and SOX9 specific primers. Relative expression levels were normalised using GAPDH. Protein extracts were prepared by grinding the pellets in RIPA buffer. They were run on 4-12% Nu-PAGE gels and western blots were carried out using antibodies to collagen I and II and GAPDH.

RESULTS: SOX9 transduction led to larger, heavier pellets after 14 days in culture. The size of the pellets was further increased by the addition of TGFβ-3 and IGF-1 to the medium during culture. These growth factor treated SOX9 transduced cultures displayed elevated levels of GAG retention in the pellets as assayed using DMB. Histologically, SOX9 transduced chondrocytes treated with TGFβ-3 and IGF-1 displayed strong safranin-O stained ECM populated by rounded cells. Control pellets under all conditions did not display cartilage matrix morphology and neither did SOX9 transduced cells cultured without growth factors or with IGF-1 alone. Treatment of transduced cells with TGFβ-3 and IGF-1 led to maximal collagen II gene expression 2 times higher than in growth factor free SOX9 transduced cultures and nearly 1700 times higher than growth factor free control cultures. Collagen I was upregulated in all pellet cultures when TGFβ-3 was present and did not seem to be affected by SOX9 expression levels. Western blotting of protein extracts of growth factor treated pellets showed a large increase in collagen II accumulation in the pellets formed from SOX9 transduced cells when compared to control cell pellets. Collagen I was found at similar levels in both transduced and non-transduced cell pellets.

DISCUSSION: Passaged HAC show major loss of expression of matrix genes. We have shown that continuous expression of SOX9 via a retrovirus improves collagen II expression in monolayer culture of late passage HAC and also enhances their ability to respond to 3-dimensional cultures[2]. We have used these cells to produce a maximal chondrogenic system by stimulating them in pellet cultures with TGFβ-3 and IGF-1. In these cultures, synthesis of ECM genes and the retention of ECM proteins around the cells is significantly upregulated. Furthermore, under these conditions, the cells show a typically rounded chondrocyte-like morphology not seen in any of our other pellet cultures. It would appear that the synthesis of appropriate cartilage matrix components and their localized retention is dependant on SOX9 expression and is an important factor in further influencing the morphology and phenotype of monolayer expanded articular chondrocytes.

Extracellular Matrix Macromolecules Enhance Schwann Cell Growth and Peripheral Nerve Regeneration Through Bio-engineered Conduits

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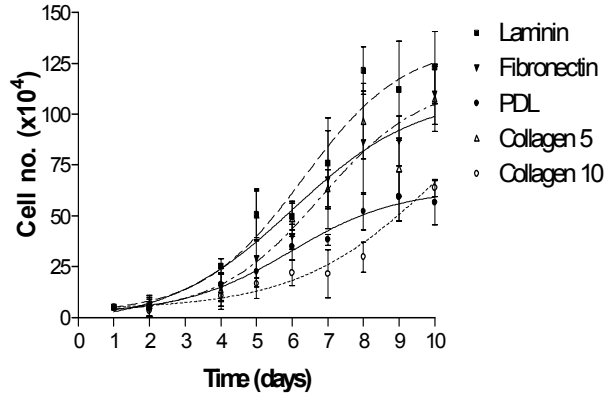
INTRODUCTION: Extracellular matrix macromolecules (ECMMs) play an important role in the ultra-structure and homeostasis of all tissues. Molecules such as laminin, fibronectin and collagen closely interact with and support cellular elements *in-vivo*.[1] The development of bio-engineered peripheral nerve conduits eludes to closely mimicking the structure of a peripheral nerve. This study aimed to assess the effect of the combination of ECMMs and Schwann cells (SCs) following transplantation within a biodegradable peripheral nerve conduit.

METHODS: *In-vitro* investigations on the effect of Schwann cell growth in the presence of laminin, fibronectin, collagen and poly-D-lysine (control) substrata were performed prior to an *in-vivo* study. For the *in-vivo* study polyhydroxybutyrate (PHB) conduits were used to span a 1cm gap in the rat sciatic nerve (n=5).[2] Dissociated fibres of PHB were pre-coated in either laminin, fibronectin or collagen prior to insertion into the lumen of the conduits. Following grafting, SCs retrovirally labelled with green fluorescent protein were seeded in the conduits[3]. Conduits were harvested at 14 days and analysed with immunofluorescent histochemical techniques to measure SC and axonal regeneration distances and to assess transplanted SC survival.

RESULTS: *In-vitro* studies showed that ECMM substrata significantly improved Schwann cell growth rate and confluence density, with laminin showing the greatest benefit, followed by fibronectin and low density collagen (*Figure 1.*). *In-vivo* studies showed that conduits coated in ECMMs had increased SC and axonal regeneration distances and a greater number of surviving transplanted SCs in comparison to uncoated controls.

Figure 1. Schwann cell growth curves on ECMM substrata. Collagen 5 and 10 represent 5 and 10 μm/cm² respectively. PDL (poly-D-lysine) represents the control.



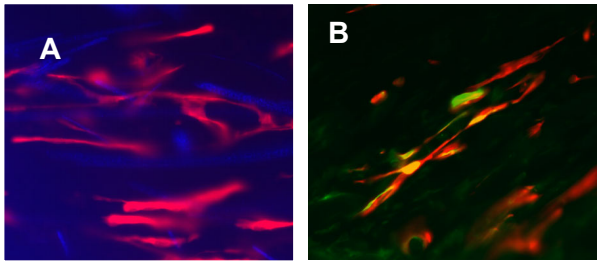


Figure 2. (A) Schwann cells (red) adherent to PHB fibres (blue) within conduit. (B) GFP co-localisation with S100-Cy3 confirms integration of transplanted Schwann cells. (X40).

DISCUSSION & CONCLUSIONS: A priori, mimicking the internal milieu of any tissue is fundamental to building bio-engineered systems. The beneficial effect of ECMMs on Schwann cell proliferation and axonal regeneration within a bio-engineered nerve supports the need for an engineered extracellular matrix. ECMMs are known to promote cellular adhesion and proliferation and to influence the expression of cell adhesion molecules. Further characterisation of the effect of these molecules at the cellular and molecular level will lead to important information relating to and advances in, the behaviour of cellular transplants within bio-engineered systems.

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Chondroprogenitor transplantation into partial depth articular cartilage lesions.

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INTRODUCTION: Arthroscopic lavage and debridement gives pain relief and increased joint function to patients with minor articular cartilage lesions[1], which may last from 6 months to 5 years, but averages 1 to 3 years[2,3,4]. However, this removes the surface layer of chondrocytes within the damaged area, which has recently been identified as containing a progenitor cell population[5]. If chondroprogenitors were returned to the damaged cartilage surface during the initial arthroscopy would the subsequent outcome be improved? If so, the time to repeat surgery would be increased, or even removed. Thus, the aims of this initial study were to discover whether chondroprogenitors transplanted into a partial depth lesion would adhere and secrete a cartilaginous matrix.

METHODS: Surface and full depth chondrocytes from the articular cartilage of 7-day-old bovine metacarpophalangeal (MCP) joints were isolated overnight by pronase and collagenase digestion. Chondroprogenitor cells were isolated from the surface cell population with a 20 minute fibronectin adhesion assay[6,7] and cultured in monolayer for 7 days in 20% foetal calf serum (FCS), DMEM, HAMS F12 media and 50ug/ml ascorbate. Full depth chondrocytes were cultured likewise. 7-day-old bovine MCP articular cartilage explants were wounded with a defined-depth cutting tool to create 500um deep lesions. Chondroprogenitors and full depth chondrocytes were trypsinised from monolayer culture, resuspended in 10% FCS media and added to the wound in a 10ul droplet at 20,000 cells/ul. This was incubated at 37C overnight. The following day 2ml of 20% FCS media was added, submerging the explant. Media was replaced every 2 days and explants were cultured for 7, 14 and 28 days. Cryosections were cut at 12um intervals and stained with Safranin O.

RESULTS: Chondroprogenitors and full depth chondrocytes adhered to the articular cartilage lesion. Over a 28-day culture period the chondroprogenitor-enriched cell population was more successful at filling the 500µm deep wound. Both sets of cells secreted a Safranin O staining matrix, indicating glycosaminoglycan and proteoglycan content (figure 1). Media controls had a thin, acellular infilling at the base of the wound that did not stain with Safranin O until day 28 post-transplantation.

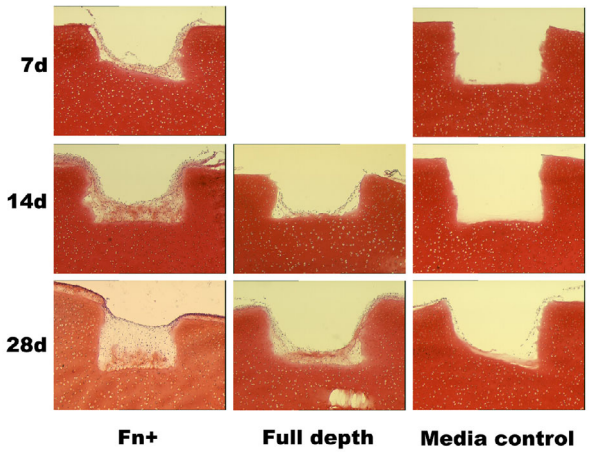

Fig. 1: Safranin O histology. Chondroprogenitors or chondrocytes have been transplanted to partial depth lesions in vitro, and cultured for 7, 14 and 28 days alongside media controls.

DISCUSSION & CONCLUSIONS: Chondrocytes readily adhere to an articular cartilage lesion. A transplanted chondroprogenitor enriched population performs better at filling a partial depth lesion in a short period of time than a typical population of chondrocytes taken from the full thickness of articular cartilage.

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ACKNOWLEDGEMENTS:

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Effects of mechanical strain on hyaluronan metabolism of cells cultured from the synovium of osteoarthritic knees.

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INTRODUCTION: Immobilization studies have shown that the removal of mechanical stimuli interferes with joint cavitation[2,3] suggesting that mechanically induced factors are likely to play a major regulatory role in joint morphogenesis. Hyaluronan (HA), a high molecular weight polysaccharide, is synthesized during joint cavitation[1.] Synthesis of HA persists in the adult joint helping to sustain friction free articulation of the joint. During osteoarthritis there is a loss of joint mobility accompanied by a decrease in HA synthesis[4]. This suggests that the role of mechanical stimuli in maintaining hyaluronan synthesis in the adult joint is likely to be essential.

We hypothesise that restoration of normal synovial HA concentrations is dependent upon appropriate mechanical cues and that OA synovial cells can respond to mechanical stimuli. Here we show that after a brief period of mechanical strain, cultured human OA synovial cells can increase and sustain media HA concentrations.

METHODS: Synovial cells from patients (both male and female, age range 57-77 years) undergoing total knee joint replacement surgery were isolated and grown to confluence.

Cells were trypsinised, counted and plated at a density of 15 x 10^3 cells per well of a 4-well strain plate. Confluent cells were serum deprived for 18 hours and fresh serum free media added 30 minutes prior to strain. Plates were strained at 4000, 6000 or 10000με at a frequency of 1 Hz for 10 minutes using a mechanical loading jig. All strains were carried out at 37°C. Control plates consisted of cells that did not undergo strain and cells that were subjected to media flow without strain. The media from each well was collected at 1 hour, 6 hours and 24 hours. The media HA levels were assayed using a plate based HA ELISA.

RESULTS: Firstly, the HA media levels from several cell lines from static controls could be seen to increase with time. Comparisons of static control cells and flow control cells showed no significant differences in their media hyaluronan concentrations.

Of the three strains tested the lowest strain of 0.4% gave the greatest significant increase in HA synthesis over static control cells, at all time points (Fig.1.). The highest strain (1%) produced a significant decrease in hyaluronan media levels at 24 hours. All data were analysed using a one-way ANOVA followed by Tukey post hoc test.

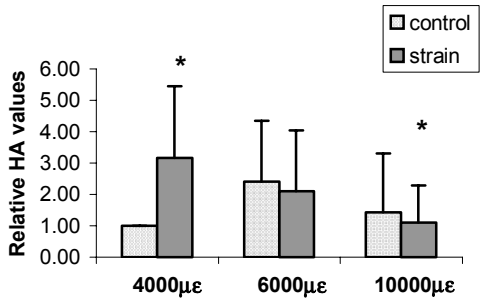

*Fig. 1: Relative media hyaluronan levels 24 hours post strain. *p<0.05*

DISCUSSION & CONCLUSIONS: These results demonstrate that although the synovial cells have been cultured from the synovium of osteoarthritic patients that show reduced levels of hyaluronan, they are able to alter their hyaluronan synthesis when subjected to mechanical strain. The upregulation of HA levels was seen predominately in the cells subjected to the lowest strain while a decrease in HA levels was seen at a higher strain. This result suggests that the loss of mobility in osteoarthritic joints could be a contributory factor in the decrease of hyaluronan. The determination of an appropriate strain and frequency required to restore hyaluronan levels to those of the normal synovial joint will be important in generating exercise based therapies for osteoarthritis.

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Induction of multicellular aggregate formation *via* periodate oxidation of cell surface sialic acids.

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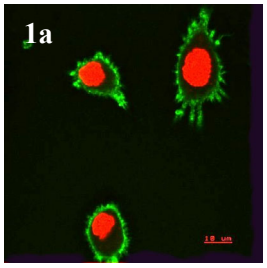
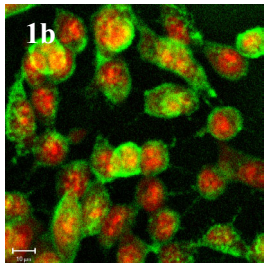
INTRODUCTION: One of the primary aims of tissue engineering is to generate functional, three-dimensional tissue. To achieve this, intimate cell-cell and cell-matrix interactions must occur, which are frequently controlled by cell surface molecules. The non-natural aggregation of cells *via* surface engineering presents a facilitatory tool to control/complement naturally occurring interactions. We describe the remodeling of living myoblast surfaces by mild oxidation of terminal sialic acids with sodium periodate. The resulting non-natural aldehyde groups can subsequently be used as "handles" to attach suitably functionalized molecules as desired. This cell surface engineering technique was optimized, and then extended to induce the rapid formation of multicellular aggregates. Additionally, we have achieved similar results using HepG2 hepatocytes highlighting the versatility of this technique.

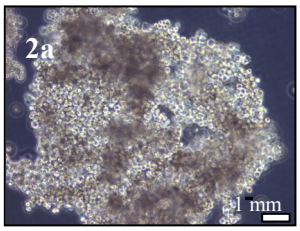
METHODS: Oxidation of myoblast surfaces was performed using a low (1 mM) concentration of sodium periodate. This process for aldehyde generation could be achieved rapidly in times as short as 1 minute when performed at room temperature. The mild oxidation was followed by biotinylation of the modified sialic acid residues using biotin hydrazide. The extent of biotinylation was then determined by the immobilization of FITC-Avidin on cell surface biotin molecules as visualized using confocal microscopy (figure 1) and measured by flow cytometry. The resultant fluorescence shift by flow cytometry was concentration dependent with an EC_{50} of 377 µM. Furthermore, this treatment had no effect on myoblast viability, and a small, but significant, effect on myoblast cell number as measured over a period of 72 hours. This method of cell surface modification was then used to form multicellular aggregates. Myoblasts were treated with sodium periodate and subsequently biotinylated in suspension to ensure the entire cell surface was modified. Addition of avidin to the suspension caused cross-linking of cells, resulting in multicellular aggregates on the millimetre scale (figure 2). All procedures were later performed using HepG2 cells in place of myoblasts.

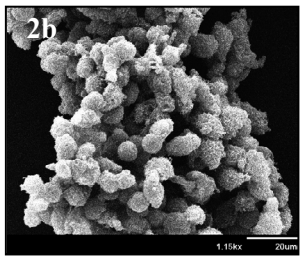
RESULTS:





Figures 1a and 1b show confocal microscopy visualisation of immobilization of FITC-Avidin (green) on the cell surface (a = L6 myoblasts, b = HepG2 hepatocytes).





Figures 2a and 2bshowlight microscopy and SEM images of myoblast aggregates respectively.

DISCUSSION & CONCLUSIONS: These data show that myoblast surfaces can be modified *via* sodium periodate treatment providing "handles" for the attachment of a desired ligand. Our studies with HepG2 hepatocytes demonstrate that this technique can be applied to other cell types. This cell surface can be employed to create cell-cell interactions resulting in the formation of millimeter sized multicellular aggregates. Furthermore, the aggregates of HepG2 cells were typically larger than their myoblast counterparts. The reasons for this are not clear but may be due to either increased levels of natural cell-cell interactions, or higher surface densities of sialic acids on HepG2 cells. This technique is noncytotoxic and has considerable potential for tissue engineering, cell biology and drug delivery applications.

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Seeding Efficiency and Distribution of Primary Osteoblasts in 3D Porous Poly(L-lactide) Scaffolds

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INTRODUCTION: Three-dimensional porous polymer scaffolds can be fabricated to convey spatial information to seeded cells. As the initial step in tissue engineering utilising 3D constructs, cell seeding efficiency and distribution is of paramount importance. Resultant construct cellularity, matrix composition and mechanical integrity are greatly influenced by cell distribution[1]. In this study, the effects of initial seeding conditions and culture techniques were investigated with respect to porous poly(L-lactide) scaffolds.

METHODS: The cylinder shaped (ϕ 9 x 4 mm) porous scaffolds were made from medical grade poly(L-lactide) (PLLA) using a salt-leaching technique, resulting in 250-350 μm pore size and 90% porosity[2]. To encourage cell attachment, the 3D scaffolds were coated by collagen type I (Sigma). Cell seeding efficiency in the scaffolds was investigated for both static and dynamic seeding techniques by counting the number of cells remaining in the culture media in which constructs were suspended. Primary bone cells were labelled with PKH26 red fluorescent linker (Sigma) prior to seeding, allowing cell distribution within the construct to be observed using confocal microscopy.In long-term experiments, constructs were cultured statically for 4 weeks or perfused for the last week of the 4-week culture period, then embedded in Technovit 7100 resin. Cross-sections were taken from the cylindrical constructs and cell distribution calculated.

RESULTS: Dynamic seeding, where 1 million primary osteoblasts suspended in 50μl of media were seeded directly onto the scaffold surface, placed in a conical tube and rotated at approximately 60rpm on a cortical shaker for 3 hours, resulted in 91-92% cell seeding efficiency.

Static seeding techniques, where the same amount of cell suspension was applied in the same manner, but constructs were not agitated in media, resulted in lower seeding efficiency. Cell distribution throughout the scaffold was more uniform when using the dynamic seeding technique, where cells penetrated throughout the construct. Static seeding resulted in cell distribution being limited to the upper portion and middle of the scaffold, with few osteoblasts observed at the lowermost face of the construct.

Long-term experiments, where osteoblasts were initially applied to the scaffold by static seeding, indicated cells penetrated through scaffolds, however the distribution was asymmetrical, with the majority of cells contained within the upper portion of the construct, supporting the cell seeding observations at early times under static conditions. Furthermore, constructs subjected to perfusion had improved cell distribution when compared to static control.

DISCUSSION & CONCLUSIONS: A 91-92% seeding efficiency for primary osteoblasts in porous PLLA 3D scaffolds was recorded for dynamic seeding techniques after 3 hours. Static seeding techniques resulted in lower efficiency and less well distributed cells throughout the construct at the same time period. Long-term experiments, where primary osteoblasts were seeded using the static technique, and cultured for 4 weeks demonstrated that the cells migrate into the centre of the scaffolds, but do not penetrate the lower portion of the scaffold, resulting in asymmetrical distribution. With 1 week of perfusion culture following the 3-week static culture, the asymmetrical distribution was improved, suggesting that in perfusion culture, where nutrient and gas diffusion is enhanced, osteoblasts can migrate and proliferate more readily than cells in static culture conditions.

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