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The effect of mechanical cues on the chondrogenic differentiation of bone marrow derived mesenchymal stem cells

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A thesis submitted to the University of Dublin in partial fulfilment of the requirements for the degree of

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Summary

Tissue engineering strategies utilising mesenchymal stem cells (MSCs) represent a promising treatment option for a range of injuries and diseases. If MSCs are to be implanted *in vivo*, understanding the intricacies of how their multipotent characteristics are controlled is vital. One regulator of MSC differentiation is biomechanical stimulation. A central hypothesis of this thesis is that mechanical stimulation can direct the chondrogenic differentiation and functional chondrogenesis of MSCs. Herein, the research examines the response of hydrogel encapsulated MSCs to dynamic compressive strain. The application of dynamic compression initiated at the onset of transforming growth factor-β3 (TGF-β3) induced differentiation was found to inhibit chondrogenesis of agarose encapsulated MSCs. However, allowing TGF-β3 mediated chondrogenic induction to occur for 21 days prior to the initiation of dynamic compression led to a more anabolic response to loading. This response was modulated by cell density, with dynamic compression leading to significant increases in cartilage specific matrix accumulation and construct mechanical properties at higher cell seeding densities.

The findings of these first studies provided motivation for the development of a tissue engineered construct with depth dependent zonal gradients analogous to articular cartilage. Through control of the oxygen tension and mechanical environment spatially within MSC seeded agarose constructs, a tissue with zonal gradients in biochemical composition mimicking aspects of normal articular cartilage was achieved. Finally, the effect of cell-matrix interactions and the external mechanical environment on MSC differentiation in response to TGF-β3 was assessed. Long term dynamic compression was found to regulate MSC differentiation fate in fibrin hydrogel; directing MSCs along a chondrogenic phenotype as opposed to the default myogenic route supported by the uncompressed fibrin clot.

This thesis demonstrates that the chondrogenic differentiation of MSCs can be mechano-regulated. Knowledge of the response of hydrogel encapsulated MSCs to dynamic compressive strain will improve our capacity to engineer more functional cartilage replacement tissues through the

development of optimum MSC conditioning regimes prior to implantation. Additionally, this work contributes to our general understanding of MSC mechanobiology which will ultimately impact upon numerous stem cell based therapies under development in the field of regenerative medicine.

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Nomenclature

α-SMA	α-smooth muscle actin
ab	Antibody
ACI	Autologous chondrocyte implantation
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
C	Right Cauchy-Green tensor
c	Oxygen concentration
$CaCl_2$	Calcium chloride
CFU-F	Colony forming unit-fibroblast
CO_2	Carbon dioxide
C_v	Viscous right Cauchy-Green tensor
D	Diffusion coefficient
DAB	Diaminobenzide
DAPI	4',6-diamidino-2-phenylinodole
DC	Dynamic compression
DDC	Delayed dynamic compression
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
D_{ag}	Diffusion coefficient for agarose
D_{H_2O}	Diffusion coefficient for water
ε	Strain
ECM	Extracellular matrix
EDTA	Ethylenediamine-tetraacetic acid
f	Force

F	Deformation gradient
FBS	Foetal bovine serum
FE	Finite element
F_e	Elastic portion of deformation gradient
F_v	Viscous part of deformation gradient
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FS	Free-swelling (unloaded)
g	Gravitational constant
GAG	Glycosaminoglycan
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
Hz	Hertz
H_2O	Water
H_2O_2	Hydrogen peroxide
Ig	Immunoglobulin G
IGF	Insulin growth factor
ITS	Insulin transferrin selenium
I_1	First principal invariant (trace)
I_3	Third principal invariant (determinant)
kPa	Kilo Pascal
K_m	Concentration at half the maximum consumption rate
µM	Micromolar
µL	Microlitre
M	Molar
MACI	Matrix-induced autologous chondrocyte implantation
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimetre

mM	Millimolar
MMP	Matrix metalloproteinase
MPa	Mega Pascal
MPC	Mesenchymal progenitor cell
mRNA	Messenger ribosomal nucleic acid
ms	Millisecond
MSC	Mesenchymal stem cell
MSC	Mesenchymal stromal cell
n	Number of observations
n	Cell density
N	Newton
Na	Sodium ion
ng	Nanogram
nM	Nanomolar
η_v	Viscosity function
O_2	Oxygen
OA	Osteoarthritis
PBS	Phosphate buffered saline
PCM	Pericellular matrix
PDMS	Polydimethylsiloxane
PEG	Poly(ethylene glycol)
PGA	Polyglycolic acid
PLGA	Poly(lactic-co-glycolic acid)
Q_m	Maximum consumption rate
RGD	Arginine-Glycine-Aspartic acid
RNA	Ribosomal nucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
s	Seconds
SD	Standard deviation

SEM	Standard error from the mean
SMC	Smooth muscle cell
sGAG	Sulphated glycosaminoglycan
t	Time
TGF-β	Transforming growth factor-beta
T_{ov}	Viscous overstress
w/w	Wet weight
ww	Wet weight
Ø	Diameter
∂	Partial derivative
ϕ_f	Fluid volume fraction
ψ_{eq}	Equilibrium free Helmholtz energy potential
2-D	2-dimensional
3-D	3-dimensional

Chapter 1 Introduction

1.1 General overview

Since the development of total joint replacements using inorganic materials in the 1950's, orthopaedic surgery has been able to provide improved quality of life for a great number of people, particularly the elderly and those suffering from end stage arthritis. However, many young and active patients still suffer from joint impairment and would benefit from a more lasting and effective articular therapy.

Articular cartilage is an avascular, neural deficient, highly organised load bearing tissue which provides a low friction, wear resistant bearing surface in diarthrodial joints. Damage to articular cartilage can occur through overloading of the joint (e.g. sporting injuries) or pathogenically through osteoarthritis; emerging in the form of focal lesions in addition to generalised structural and biological changes resulting in thinning of the tissue and loss of mechanical integrity. Once damage exceeds a certain threshold, cartilage is unable to repair itself. The most frequently affected sites are the hands, knees, hips and spine. The symptoms are often associated with significant functional impairment, in addition to signs and symptoms of inflammation, including pain, stiffness and loss of mobility (Felson 2006). Tissue engineering has been defined as "an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function" (Langer and Vacanti 1993), and offers the potential to generate cartilaginous constructs to repair such damaged tissue.

Articular cartilage defects may take the form of partial or full thickness lesions. Full thickness lesions may penetrate the subchondral bone beneath (Jackson and Simon 1999). Penetration of the subchondral bone allows mesenchymal stem cells (MSCs) resident in bone marrow to migrate into the cartilage defect (Shapiro et al. 1993) and as a result has formed the basis of a number of surgical procedures aimed at treating damaged articular cartilage such as microfracture. These

MSCs differentiate into cells which form a repair tissue. Clinical experience suggests that the repair after subchondral penetration can provide significant improvement of joint function and pain relief for many years (Blevins et al. 1998). However, the tissue formed is frequently of a fibrous nature, not the desired hyaline cartilage, and degrades with time (Jackson and Simon 1999; Redman et al. 2005). Various techniques and strategies have been employed to promote cartilage repair including:

- Arthroscopic repair procedures such as abrasion arthroplasty and micro-fracture, which stimulate bleeding from the bone marrow into the defect to form blood clots and subsequent MSC driven repair tissue
- Soft tissue grafts; periosteum or perichondrium tissue is transplanted into the defect
- Autogenic osteochondral graft transplantation
- Cell transplantation based repair; autologous chondrocyte implantation (ACI)
- Matrix-induced autologous chondrocyte implantation (MACI)

Success with the above techniques has been mixed with few instances of successful long term repair due to a range of complications including periosteal hypertrophy, delamination of the implant, arthrofibrosis, and implant failure (Marlovits et al. 2006). MACI holds more promise as chondrocytes are combined with resorbable biomaterials securing the cells within the defect and enhancing proliferation and differentiation (Toh et al. 2011). However, a major limiting factor in extending the use of therapies such as MACI is obtaining sufficient numbers of viable differentiated autologous chondrocytes, particularly in elderly and osteoarthritic patients. Current research is now exploring the potential use of mesenchymal stem cells (MSCs) for cartilage tissue engineering and regenerative therapies, in addition to the combination of such cells with three-dimensional scaffolds (Redman et al. 2005; Krampera et al. 2006; Shenaq et al. 2010).

1.2 MSC chondrogenic differentiation and mechanoregulation

MSCs are found in many adult skeletal tissues, most notably bone marrow, subcutaneous fat, joint fat pad, synovial membrane and the synovial fluid. Due to their distribution throughout the body,

MSCs may be isolated from tissue which is easily accessible for biopsy without causing the patient significant discomfort. MSCs have a good proliferative capacity in vitro and differentiation can be directed down various lineages, including the chondrogenic lineage (Caplan 1991; Pittenger et al. 1999; Tuan et al. 2003). Many growth factors have been implicated in the chondrogenic differentiation of MSCs in vitro, and include members of the transforming growth factor-β (TGF-β) superfamily (Johnstone et al. 1998; Indrawattana et al. 2004).

The same functional demands which put cartilage at risk of injurious damage and frequent degenerative changes must be considered in the design and appraisal of any therapeutic intervention. In the context of cell based tissue engineering therapies, mechanical factors are of particular importance. While chondrogenesis occurs in three-dimensional MSC systems, it has been shown that the matrix accumulation and the subsequent mechanical properties of MSC laden constructs are significantly lower than those of chondrocyte seeded controls (Mauck et al. 2006; Erickson et al. 2009). This suggests that further optimisation of the biochemical and biophysical environment is required if MSCs are to be used to engineer cartilaginous tissues with functional properties similar to those obtainable with healthy chondrocytes. Also, if MSCs are to be implanted into the joint as part of a clinical repair strategy, the biomechanical and biochemical influences of the joint environment on MSC differentiation and ECM synthesis must be determined.

1.3 Aims of this research

The overall objective of this work is to investigate the chondrogenic differentiation of mesenchymal stem cells (MSCs) in a 3-dimensional environment in response to mechanical stimuli. The main objectives are:

1. To investigate the hypothesis that dynamic compressive loading applied to MSC seeded agarose hydrogels in the presence of TGF-β3 will enhance chondrogenic differentiation and the consequent functional properties of the construct.

2. Having demonstrated that dynamic compression initiated at the onset of TGF-β3 induced differentiation can inhibit chondrogenesis of MSCs, it was hypothesised that the application of dynamic compression following cytokine induced differentiation will further enhance MSC chondrogenesis and functional maturation in agarose hydrogel culture.
3. Using the knowledge gained from objectives 1 and 2, the next objective was to engineer a cartilaginous construct with native-like zonal composition using MSCs by controlling both the oxygen tension and the mechanical environment through the depth of the developing tissue.
4. In addition to attempts to engineer a functional tissue using agarose encapsulated MSCs, the author will investigate the hypothesis that cell matrix interactions modulate MSC response to extrinsic mechanical signals; in this case dynamic compressive strain. As part of this study, the hypothesis that dynamic compression regulates the chondrogenic-myogenic fate decision in MSCs seeded within fibrin hydrogel will be investigated.

A number of studies are required to address these hypotheses. Hypothesis 1 will be tested through examination of the long-term effects of daily dynamic compressive strain on MSC seeded agarose chondrogenesis through measurement of cartilage specific extracellular matrix content and construct mechanical properties (Chapter 3). Objective 2 will investigate the effect of temporal application of dynamic compression and TGF-β3 stimulation (Chapter 4). Objective 3 will use the information obtained through completion of objectives 1 and 2. Oxygen tension and mechanical environment will be controlled through construct confinement and ensuing dynamic compression (Chapter 5). Objective 4 will investigate the effect of cellular attachment in a fibrin hydrogel on the MSC response to dynamic compression and altered TGF-β3 concentration. MSC differentiation along both the chondrogenic and myogenic pathways will be assessed in both agarose and fibrin hydrogels as TGF-β3 has been implicated in both differentiation pathways (Chapter 6).

In summary, this thesis aims to investigate the mechanoregulation of MSC chondrogenic differentiation and engineered tissue chondrogenesis. The first objective seeks to ascertain the response of agarose encapsulated MSCs to unconfined dynamic compressive strain. The second investigates whether the application of dynamic compression can have a pro-chondrogenic effect

with modification of its point of initiation. The knowledge gained from these two objectives will set the scene for the third objective which is to attempt to develop a MSC-agarose construct with depth dependent composition similar to native articular cartilage. The fourth and final objective investigates whether the inhibition of chondrogenesis with dynamic compression as observed in Chapter 3 and Chapter 4, is related to cell attachment and the nature of the MSC scaffold carrier.

Chapter 2 Literature Review

2.1 Articular cartilage

2.1.1 Composition and structure of articular cartilage

Chondrocytes; the sparsely distributed cells in articular cartilage, account for less than 10% of the tissue volume; a cell density of the order of five million per cm^3 (Stockwell 1967), compared to a few hundred million cells per cm^3 found in most tissues (Palsson 2004). The zonal arrangement of these cells is shown in Figure 2.1 below.

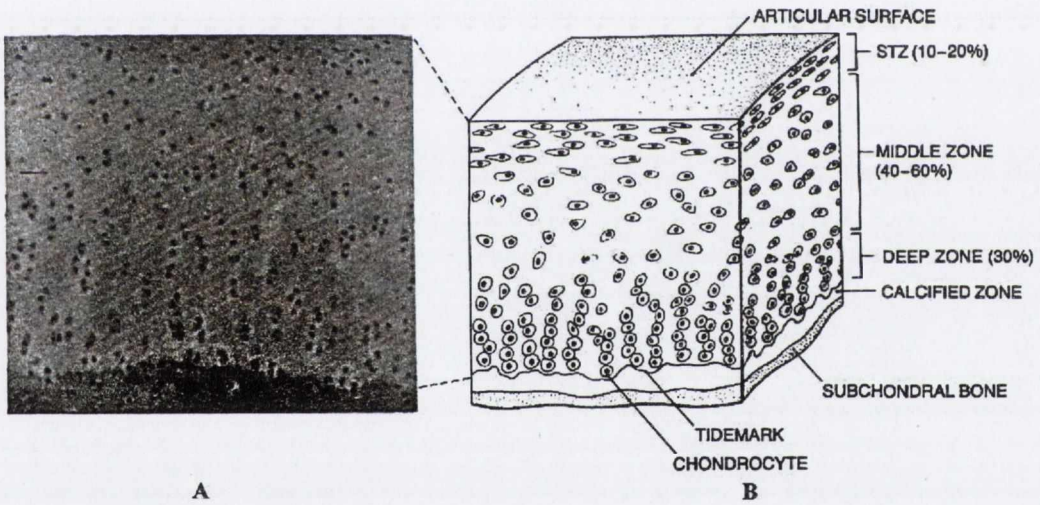

Figure 2.1: Pictograph (A) and schematic representation (B) of the chondrocyte arrangement throughout the depth of non-calcified articular cartilage. In the superficial tangential zone (STZ) chondrocytes are oblong with their long axes aligned parallel to the articular surface. In the middle zone, the chondrocytes are round and randomly distributed. Chondrocytes in the deep zone are arranged in columnar fashion orientated perpendicular to the tidemark, the demarcation between the calcified and non-calcified tissue (Mow et al. 1989).

Regardless of their sparse distribution, chondrocytes manufacture, secrete, and maintain the organic component of the extracellular matrix. The organic matrix is composed of a dense network of

collagen type II fibrils enmeshed in a concentrated solution of proteoglycans (PGs) (Eyre 1980; Muir 1983). The collagen content of cartilage tissue ranges from 10 to 30% by net weight and the PG content from 3 to 10% by net weight; the remaining 60 to 87% is water, inorganic salts, and small quantities of other matrix proteins, glycoproteins, and lipids (Mow and Ratcliffe 1997). Collagen fibrils and PGs are the structural components supporting the mechanical stresses resulting from applied loads (Broom and Silyn-Roberts 1990; Schmidt et al. 1990). It is these structural components, together with water which determine the biomechanical behaviour of this tissue.

Collagen is the most abundant protein in the body (Eyre 1980). The collagen in articular cartilage has a high level of structural organisation providing for a fibrous ultrastructure (Clarke 1971; Mow and Ratcliffe 1997). It is inhomogeneously distributed, giving the tissue a layered disposition. Numerous investigations using light, transmission electron, and scanning electron microscopy have identified three separate structural zones. Mow and Lai proposed a schematic arrangement for the collagen network shown in Figure 2.2 (1974). This inhomogeneity of fibre orientation is mirrored by zonal variations in the collagen content, which is greatest at the surface and remains relatively constant throughout the deeper zones (Lipshitz et al. 1975). This layering inhomogeneity serves an important biomechanical function, enhancing superficial interstitial fluid support and minimising solid matrix stress within the tissue (Setton et al. 1993; Krishnan et al. 2003).

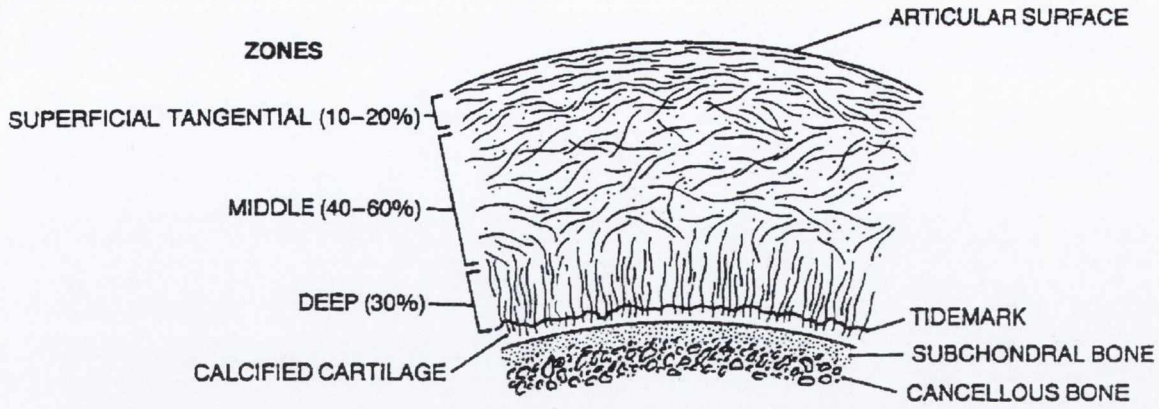

Figure 2.2: Schematic representation of the ultrastructural arrangement of the collagen network throughout the depth of articular cartilage. In the superficial tangential zone (STZ), collagen fibres are tightly woven into sheets arranged parallel to the articular surface. In the middle zone, randomly arrayed fibrils are less densely packed to accommodate the high density of proteoglycans and water. The collagen fibrils of the deep zone form larger, radially orientated fibre bundles which cross the tidemark, enter the calcified zone, and anchor the tissue to the underlying bone. Note the correspondence between this collagen fibre arrangement and that of the chondrocytes in Figure 2.1. (Mow and Lai 1974)

The most significant property collagen fibres exhibit is their tensile stiffness and strength (Figure 2.3). Although strong in tension, collagen fibres offer little resistance to compression due to their high slenderness ratio.

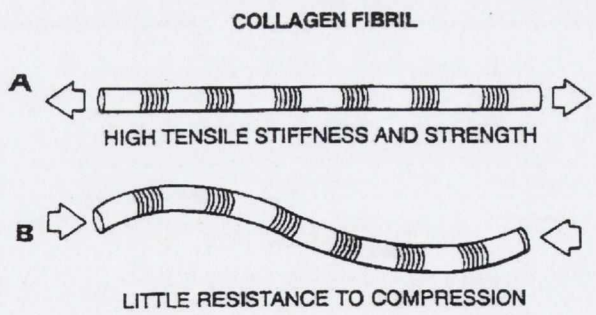

Figure 2.3: Illustration of a collagen fibril's mechanical properties. The fibril is stiff and strong in tension (A), but is weak and buckles easily in compression (B). (Mow et al. 1989)

Cartilage PGs are large protein-polysaccharide molecules consisting of a protein core to which one or more glycosaminoglycans (GAGs) are attached (Muir 1983). Aggrecan consists of an approximately 200 nm long protein core to which about 150 glycosaminoglycan (GAG) chains are covalently attached (Muir 1983). Keratan sulphate and chondroitin sulphate, the two sulphated glycosaminoglycans found in articular cartilage, are polymer chains of specific repeating disaccharide units. Aggrecan may be visualised as having a bottle-brush-like structural arrangement, with the GAGs attached to and radiating perpendicularly from the protein core (Figure 2.4A).

Most PG monomers in native cartilage associate with hyaluronate to form PG aggregates (macromolecules). These aggregates form when up to several hundred monomers non-covalently attach to a central hyaluronate core via their hyaluronic acid-binding region (HABR) (Figure 2.4B) (Muir 1983). The filamentous hyaluronic acid (HA) core molecule is a non-sulphated disaccharide chain. The attachment site between the HABR and the HA is stabilised by small glycoproteins; link proteins. It is widely accepted that PG aggregation promotes immobilisation of the PGs within the collagen meshwork, adding structural rigidity to the extracellular matrix (Hardingham and Muir 1974; Hascall 1977; Muir 1983).

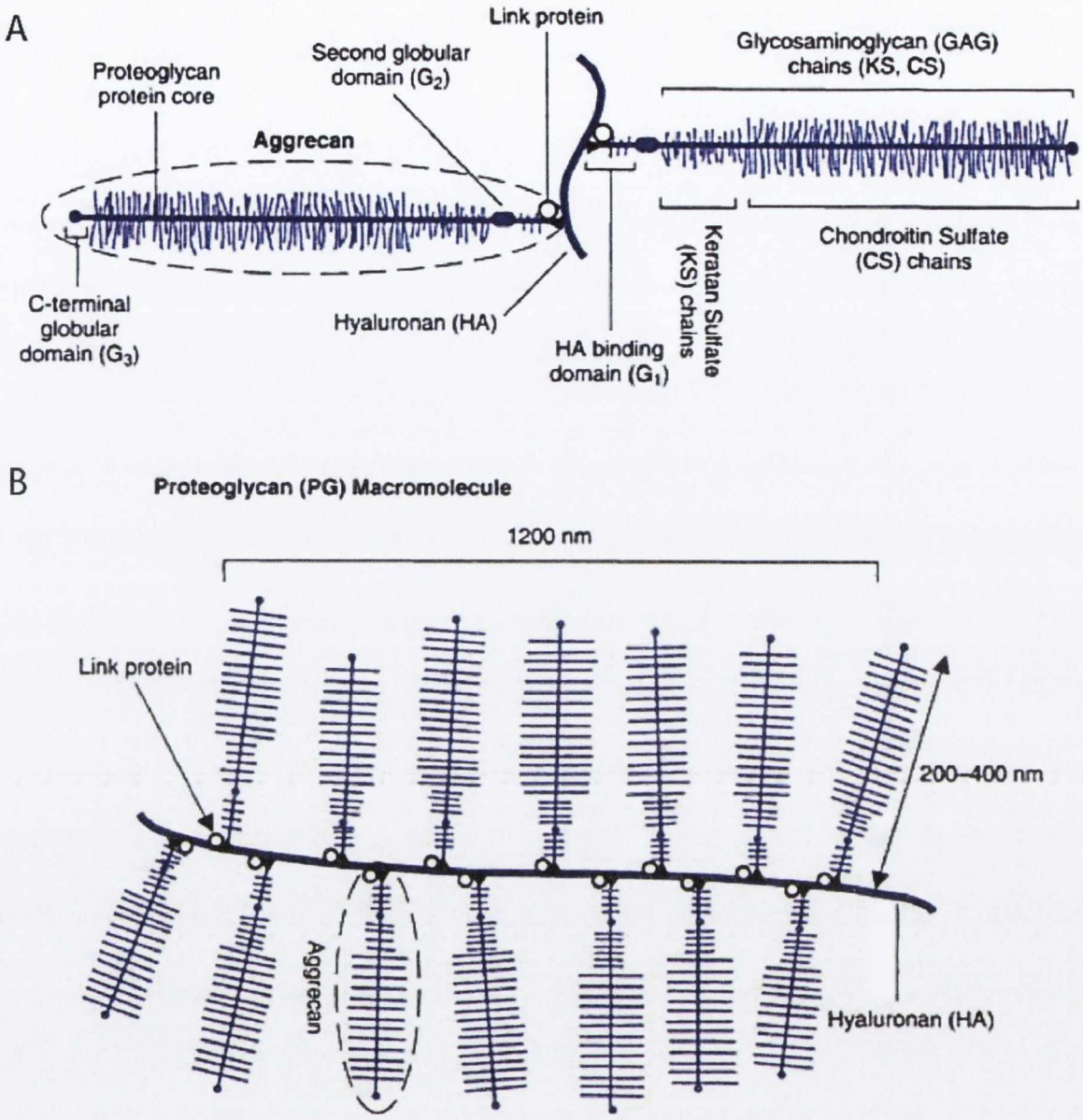

Figure 2.4: A: Schematic depiction of aggrecan, which is composed of keratin sulphate and chondroitin sulphate chains bound covalently to a protein core molecule. The proteoglycan protein has three globular regions as well as keratin sulphate rich and chondroitin sulphate rich regions. B: Schematic representation of a proteoglycan macromolecule. In the matrix, aggrecan non-covalently binds to HA to form a macromolecule with a molecular weight of approx. 200×10^6 Da. Link protein stabilises this interaction between the binding region of the monomer and the HA core molecule. (Mow and Hung 2001)

The most abundant component of articular cartilage is water. This is most concentrated near the articular surface (approximately 80%) and decreases in a nearly linear fashion with increasing

depth to a concentration of approximately 65% in the deep zone (Lipshitz et al. 1976). The fluid contains many mobile cations which influence the mechanical behaviour of the cartilage (Linn and Sokoloff 1965; Lai et al. 1991; Gu et al. 1998). This fluid component is essential to the health of this tissue, it being largely avascular, in that it facilitates the diffusion of gases, nutrients, and waste products between chondrocytes and the surrounding nutrient rich synovial fluid (Linn and Sokoloff 1965; Maroudas 1975; Albro et al. 2011). Only a small percentage of the fluid is intracellular, and about 30% is associated with the collagen fibrils (Torzilli et al. 1982). Therefore most of the water occupies the intermolecular space and is free to move when a load or pressure gradient is applied to the tissue. Theoretical and experimental studies have demonstrated that the interstitial fluid of cartilage pressurises considerably under loading, potentially supporting most of the applied load under various transient or steady state conditions (Ateshian 2009). About 70% of the water may be removed from the tissue under loading. This movement is crucial in controlling cartilage behaviour and joint lubrication (Hou et al. 1992; Hlavacek and Novak 1995; Ateshian 1997). It has been demonstrated that interstitial fluid load support plays a dominant role in regulating the frictional response of cartilage, with the understanding being that the friction coefficient remains low as long as the interstitial fluid load support is elevated (Ateshian 2009).

The chemical structure and interactions of the GAGs influence the properties and conformation of the PG aggregates. The closely spaced sulphate and carboxyl groups on the GAG disaccharide units dissociate in solution (Figure 2.5A), leaving fixed negative charges which create strong intramolecular and intermolecular charge repulsive forces. This charge repulsion extends and stiffens the macromolecules in the inter-fibrillar space formed by the collagen network. Mobile cations such as sodium and calcium are attracted to the anionic groups on the GAGs creating a substantial swelling pressure ranging from 0.05 MPa to 0.35 MPa (Maroudas 1976). The magnitude of this swelling pressure is related to the GAG density and is resisted and balanced by tension in the collagen network, confining PGs to only 20% of their free solution domain (Maroudas 1976). Consequently, the collagen network is subjected to a prestress, even in the absence of external loads (Setton et al. 1998). Additionally, PGs are inhomogeneously distributed

throughout the matrix depth, generally being most concentrated in the middle zone and least concentrated in the superficial and deep zones (Maroudas 1968; Lipshitz et al. 1976).

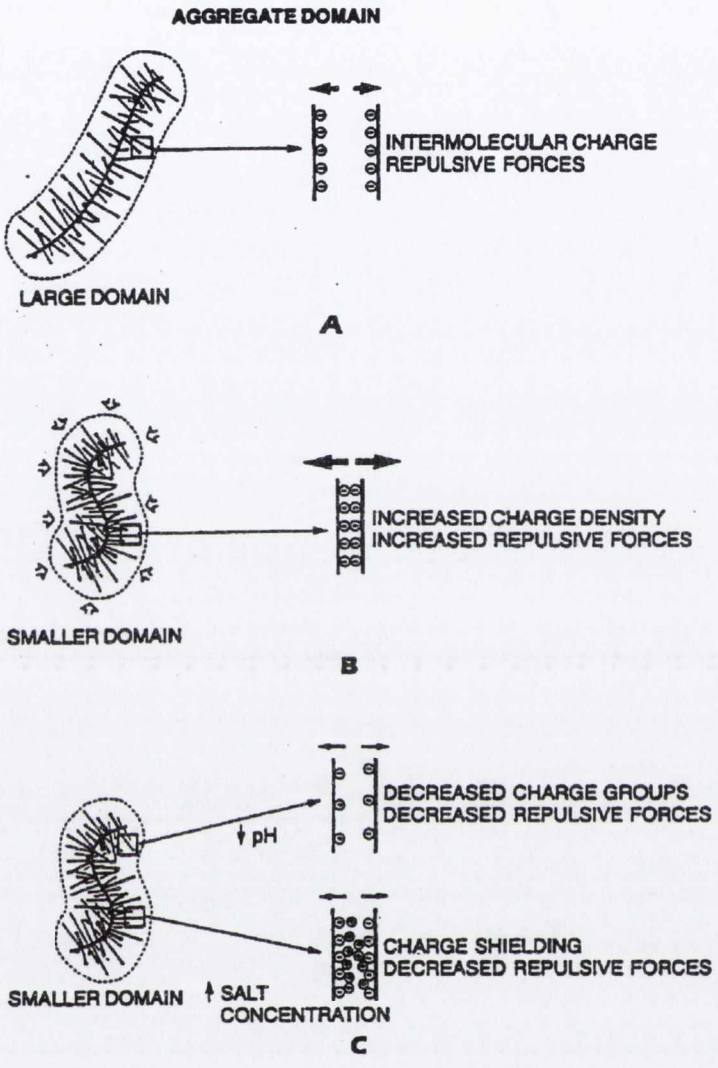

Figure 2.5: A: Schematic representation of a PG aggregate solution domain (left) and the repelling forces associated with the fixed negative charge groups on the GAGs of the monomer (right). These repulsive forces cause the aggregate to assume a stiffly extended conformation occupying a large solution domain. B: Applied compressive stress decreases the aggregate solution domain (left) which results in an increased charge density and thus the intermolecular repulsive forces (right). C: Lowering the solutions pH or increasing its ion concentration reduces the intermolecular charge repulsive forces (right), causing the PGs to assume a smaller aggregate domain (left). (Mow et al. 1989)

When a stress is applied to the cartilage surface, deformation occurs primarily due to a change in the PG molecular domain (Figure 2.5B). This external stress causes the internal matrix pressure to

exceed the swelling pressure, and liquid begins to exude from the tissue. As the fluid flows out, the PG concentration increases; this results in an increasing of the osmotic swelling pressure, charge-charge repulsive force, and compressive stress until equilibrium is achieved with the external stress. In addition, a change to the solution's pH or ion concentration will alter the PG intermolecular charge repulsive forces causing a change in the size of the aggregate domain (Figure 2.5C). In this way, the physiochemical properties of the PG trapped in the collagen network allow it to resist compression. This mechanism complements the role played by collagen which is strong in tension but weak in compression. We see that the ability of PGs to resist compression stems from two sources: the osmotic swelling pressure associated with the tightly packed fixed ionic groups on the GAGs and the bulk compressive stiffness of the PG aggregates tangled in the collagen network.

The structural macromolecules interact to form a porous, composite, fibre-reinforced matrix possessing the essential characteristics of a solid swollen with water and able to resist the stresses and strains of joint articulation (Mow et al. 1984). A schematic diagram depicting the structural arrangement within a small volume of articular cartilage is shown in Figure 2.6.

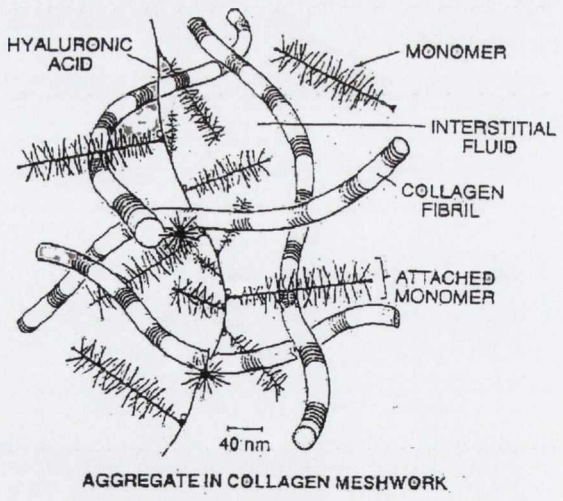

Figure 2.6: Schematic representation of the molecular organisation of cartilage. The structural components of cartilage, collagen and PGs, interact to form a porous composite fibre-reinforced organic solid matrix which is swollen with water. (Mow et al. 1989)

2.1.2 Biomechanical behaviour of articular cartilage

A viscoelastic material is one in which the mechanical response to a constant (time-independent) load or deformation varies (is time-dependent). Articular cartilage is such a material. The response of such a material can be modelled theoretically as a combination of the response of a viscous fluid and an elastic solid.

There are two fundamental responses of a viscoelastic material; creep and relaxation. Creep occurs in a viscoelastic material under the action of a constant load. Generally, the response of a viscoelastic solid is a rapid initial deformation followed by a slow (time-dependent), progressively increasing deformation known as creep, until equilibrium is reached. Stress relaxation occurs when such a material is subjected to a constant deformation. Typically, a viscoelastic solid responds with a high initial stress followed by a slow (time-dependent), progressively decreasing stress required to maintain the deformation.

It has been shown that the viscoelastic behaviour of articular cartilage is due primarily to the flow of interstitial fluid (Lai and Mow 1980; Mow et al. 1984), and in shear it is due primarily to the motion of long polymer chains such as collagen and PGs (Mow et al. 1984). In articular cartilage, the component of viscoelasticity due to interstitial fluid flow is known as biphasic viscoelastic behaviour (Mow et al. 1984), and the component due to macromolecular motion is known as flow-independent, or intrinsic, viscoelastic behaviour of the collagen-PG matrix.

Creep in articular cartilage is caused by exudation of the interstitial fluid. The biphasic creep response of articular cartilage in a one-dimensional confined compression test is shown in Figure 2.7(a). A constant compressive stress is applied to the tissue at a given time t_0 and the tissue is allowed to creep to its final equilibrium value. For articular cartilage, creep is caused by the exudation of the interstitial fluid. Creep ceases once the compressive stress within the solid matrix is sufficient to balance the applied stress alone. Since the rate of creep is governed primarily by the rate of fluid exudation, it can be used to determine the permeability coefficient of the tissue (Mow et al. 1984). At equilibrium, no fluid flow occurs, so the equilibrium deformation can be used to determine the intrinsic compressive modulus *Ha*, of the collagen-PG matrix. This has been found to

range from 0.1 to 2.0 MPa (Mow and Guo 2002). The biphasic stress relaxation response of articular cartilage is shown in Figure 2.7(b). A constant compression rate is applied to the tissue from t_0 to t_1 until a given deformation d_0, is reached. Beyond t_1 the deformation d_0 is maintained. During compression, the stress rises continuously until t_1, while during the stress relaxation phase, the stress continuously decays until equilibrium is reached. The stress rise in the compression phase is related to fluid exudation, while stress relaxation is related to fluid redistribution within the porous matrix. This stress relaxation ceases when the compressive stress within the solid matrix reaches the stress generated by the intrinsic compressive modulus of the solid matrix corresponding to d_0 (Mow et al. 1984).

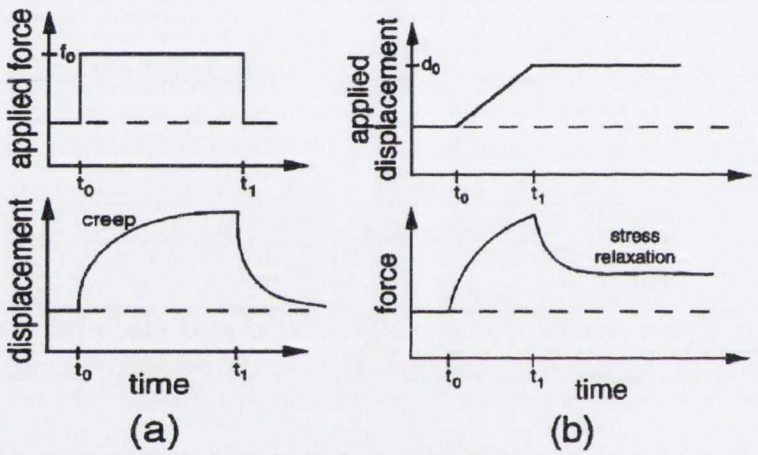

Figure 2.7: Schematics of load-deformation viscoelastic behaviours of articular cartilage. (a) In a creep test, a step force, f_0 applied onto a viscoelastic solid results in a transient increase of deformation or creep. Removal of f_0 at t_1 results in full recovery. (b) In a stress-relaxation test, a displacement is applied at a constant rate or ramped to t_0, until a desired compression is reached. This displacement results in a force rise followed by a period of stress relaxation for $t > t_1$, until an equilibrium force value is reached. (Mow and Guo 2002)

2.1.3 In vivo loading of articular cartilage

Past studies have reported on the magnitude of physiological loads within upper and lower extremity joints (Rydell 1965; Cooney and Chao 1977; Poppen and Walker 1978; Hodge et al. 1986). By and large, it has been found that the peak magnitudes of such loads in the lower extremities are a function of body weight, e.g. 2.5 to 4.9 × body weight in the hip during walking

(Rydell 1965; Armstrong et al. 1979; Brown and Shaw 1983; Hodge et al. 1986; Anderson et al. 2008) and 4 × body weight in the knee (Kurosawa et al. 1980). In the upper extremities peak magnitudes are comparable to body weight, e.g. 0.9 × body weight in the gleno-humeral joint during abduction (Poppen and Walker 1978). In general, the loading mechanism of diarthrodial joints is cyclical and/or intermittent (Dillman 1975). Joint loads produce contact stresses at the articular surfaces which have been comprehensively measured using various techniques (Fukubayashi and Kurosawa 1980; Ahmed 1983; Brown and Shaw 1983; Hodge et al. 1989; Manouel et al. 1992). Non-strenuous daily activities appear to produce mean contact stresses in the region of 2MPa, whilst strenuous activities can result in mean contact stresses in the region of 6MPa (Ahmed and Burke 1983; Ahmed et al. 1983; Brown and Shaw 1983; Brown and Shaw 1984; Huberti and Hayes 1984; Huberti and Hayes 1988). It has been estimated that the largest mean contact stress which could occur in non-traumatic conditions is of the order of 12.6MPa (Matthews et al. 1977), although in vivo measurements using instrumented femoral head prostheses have reported local contact stresses as high as 18MPa (Hodge et al. 1986; Hodge et al. 1989).

While comprehensive investigation of in vivo contact stresses has been performed, there is less known about in vivo cartilage deformation. Using various techniques such as radiographic analysis, ultrasound and magnetic resonance imaging, changes to cartilage thickness from 6-20% were observed for physiologic loading levels of 1 - 5 × body weight (Armstrong et al. 1979; Macirowski et al. 1994; Mukherjee and Wayne 1998; Eckstein et al. 2000). Theoretical contact analysis of biphasic cartilage layers under rolling or sliding motion have shown that in a congruent joint the cartilage layer thickness decreases by 6% under a contact load of 1 × body weight (Ateshian and Wang 1995). Hydrostatic fluid pressurisation occurs as a result of joint contact due to the high water content and low permeability of the tissue. With deformation, fluid is prevented from leaving the tissue and pressurises. This hydrostatic pressure has been shown both theoretically and experimentally to support over 90% of the applied stress even after many hundreds of seconds of stress application (Macirowski et al. 1994; Ateshian and Wang 1995; Soltz and Ateshian 1998; Soltz and Ateshian 2000). These findings are consistent with ex vivo measurements of the unconfined dynamic modulus of cartilage (Bian et al. 2008). The following example illustrates the

relevance of mechanical integrity for a tissue engineered construct. If a moderate contact stress of 3MPa was applied to a cartilage layer with dynamic modulus of 35kPa, it would result in a decrease in thickness of 8.6%. However, if a tissue engineered construct with a dynamic modulus less than 3MPa were implanted, the applied physiological compressive stress would simply crush the construct. This analysis provides a premise for the production of constructs with functional mechanical properties prior to implantation.

2.2 Cartilage tissue engineering

The attainment of certain design criteria is critical to the development of a successful repair strategy for both chondral and osteochondral defects. Firstly viable repair cells must be delivered within the defect site. Secondly, these cells must be regulated to form repair tissue with the desired site-specific characteristics of functional articular cartilage. A further goal is that the repair tissue integrates firmly with the surrounding tissue, especially the adjacent host cartilage. This has spurred the development of tissue engineering repair strategies which typically involve the following factors:

- Viable cells capable of producing the appropriate matrix molecules specific to the desired tissue
- A 3-D biomaterial which provides structural support and anchorage for cells, possibly representing their native extracellular matrix
- Biochemical (growth factor) and/or mechanical (deformation) stimulus to encourage cell differentiation and production of extracellular matrix

Chondrocytes have been the traditional cell source for cartilage tissue engineering approaches. However, chondrocytes are not abundant in the body, so taking a cartilage biopsy creates further lesions in the joint surface. When culture expanded in 2-D they de-differentiate toward a fibrous phenotype producing collagen type I as opposed to collagen type II (Benya and Shaffer 1982). Therefore this cell type requires a 3-D environment to maintain their phenotype and function. An

age related loss in chondrocyte yield, proliferation and chondrogenic capacity has been observed in culture-expanded chondrocytes (Barbero et al. 2004) while chondrocytes isolated from osteoarthritic patients exhibit reduced collagen synthesis (Tallheden et al. 2005). This may partially be explained by changes in the responsiveness of aged or OA chondrocytes to cytokines and growth factors (Martin et al. 1997; Fan et al. 2004; Fan et al. 2005). In addition, osteoarthritic chondrocytes are likely to have significant DNA damage among other cellular degenerative alterations (Aigner et al. 2007).

2.2.1 Biomaterials

Tissue engineering strategies use biomaterials both to study cell metabolism under various culture conditions, and to create a functional tissue with a view toward implantation. As chondrocytes require a 3-D morphology to maintain their phenotype, the biomaterials used should try to replicate this. Ideally, a scaffold should be three-dimensional, biodegradable, non-toxic, non-immunogenic and easily manufactured (Cancedda et al. 2003). Scaffolds must have both high porosity and permeability to permit the transport of nutrients and wastes to cells located at the scaffold core. Scaffolds must be able to withstand biomechanical loading, provide a mechanism by which the cells and any neo-matrix are kept within the scaffold, be it through cell adhesion as seen in fibrous scaffolds or through cell encapsulation as in gels, and may represent native extra-cellular matrix (ECM) (LeBaron and Athanasiou 2000).

2.2.2 Agarose hydrogel

Hydrogels are a class of scaffold that are commonly used in cartilage tissue engineering and include alginate, agarose, poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), pluronics, chitosan, collagen and fibrin as examples (Lum and Elisseeff 2003; Elisseeff et al. 2005). A significant benefit of hydrogels is their potential use as an in situ forming scaffold for cartilage defect repair. In addition, their swelling nature means they provide an aqueous environment comparable to soft tissue for encapsulated cells and their high water content facilitates the exchange of nutrients and gases (Lum and Elisseeff 2003).

Agarose, a linear polysaccharide extracted from marine red algae, is a thermosetting hydrogel that undergoes gelation in response to a reduction in temperature. Chondrocytes cultured in agarose will maintain their phenotype and synthesise near normal levels of collagen II and proteoglycan (Benya and Shaffer 1982; Aydelotte and Kuettner 1988; Aulthouse et al. 1989). Over time, chondrocyte seeded agarose hydrogels have been shown to produce a functional ECM in free swelling culture (Buschmann et al. 1992). For these and other reasons, numerous investigators have utilised agarose hydrogels for cartilage tissue engineering applications, specifically to assess the elaboration of both peri-cellular matrix (PCM) (Quinn et al. 2002) and extra-cellular matrix (Miyata et al. 2004; Miyata et al. 2005; Mouw et al. 2005), the chondrogenic differentiation of MSCs (Awad et al. 2004; Mauck et al. 2006), and the interaction of biochemical stimulants (Coleman et al. 2007; Ng et al. 2007). Furthermore, agarose has also been used in animal models of cartilage defect repair (Rahfoth et al. 1998; Weisser et al. 2001).

Agarose is commonly used to provide a 3-D environment to investigate chondrocyte mechano-transduction pathways in vitro. Typically such studies involve encapsulating cells within agarose and subjecting the cell-seeded hydrogel to defined levels of mechanical deformation or loading and observing the resulting changes in cellular structure and organisation (Freeman et al. 1994; Knight et al. 1998; Lee et al. 2000; Sawae et al. 2004). The mechanobiology of chondrocytes and chondrocyte progenitor cells is also commonly investigated using the agarose model. The application of appropriate levels of dynamic compressive loading (Buschmann et al. 1995; Lee and Bader 1997; Lee et al. 1998; Lee et al. 2000; Mauck et al. 2000; Chowdhury et al. 2001; Mauck et al. 2002; Mauck et al. 2003b; Mauck et al. 2003c; Kelly et al. 2004; Ng et al. 2006) or hydrostatic pressure (Toyoda et al. 2002; Toyoda et al. 2003; Elder et al. 2006) to chondrocytes cultured in agarose hydrogels has also been shown to enhance their biosynthetic activity. Furthermore the role of mechanical loading in regulating chondrogenesis of mesenchymal stem or progenitor cells is commonly investigated using the agarose hydrogel culture system (Walker et al. 2000; Huang et al. 2004a; Huang et al. 2004b; Finger et al. 2007; Mauck et al. 2007). In a number of these studies, computational techniques such as the finite element method are used to determine spatial variations in the local mechanical environment within the agarose hydrogel due to the applied levels of

loading (Freeman et al. 1994; Hunter and Levenston 2002; Mauck et al. 2003b; Huang et al. 2004a; Lima et al. 2004; Ng et al. 2006).

2.2.3 Fibrin hydrogel

Fibrinogen, and its polymerised form fibrin, are natural components of blood and represent the major extracellular component of this fluid. As a polymer unit, fibrinogen exists physiologically in a liquid state within the blood stream, with activation of polymerisation via thrombin only in instances of vascular lesioning or other pathological situations. It then forms a three-dimensional solid matrix (fibrin) to prevent blood loss from the intra-vascular space (Brandstedt et al. 1980; van Hinsbergh et al. 2001). Fibrin is pro-inflammatory, inducing its own degradation and substitution by cellular components of the extracellular matrix. Its physiological degradation products are non-toxic. Due to its ready availability from both autologous and allogeneic sources, fibrin glues have long been used as surgical sealants and more recently fibrin has been employed as a carrier for cells and growth factors (Hunziker 2002). The ability of fibrin gels to support survival, proliferation, and/or differentiation of MSCs has been demonstrated (Catelas et al. 2006; Ho et al. 2006).

Fibrin glues usually consist of two constituents, highly concentrated fibrinogen and thrombin (Janmey et al. 2009). The fibrinogen buffer solution typically includes factor XIII, anti-fibrinolytic agent (aprotinin or tranexamic acid), fibronectin, and traces of other plasma proteins such as plasminogen. Thrombin buffer solution contains calcium and sodium chlorides. High fibrinogen concentrations have been shown to reduce MSC proliferation (Ho et al. 2006). Other components such as calcium chloride, thrombin concentration, pH, and sodium chloride are known to affect gel properties such as fibre thickness and porosity which modulate gel stiffness and long-term stability (Eyrich et al. 2007; Potier et al. 2010b; Davis et al. 2011).

Fibrin has been shown to maintain the chondrogenic phenotype leading to functional chondrogenesis in long term culture when seeded with chondrocytes (Eyrich et al. 2007). Chondrogenic differentiation of MSCs is also supported in fibrin hydrogel (Li et al. 2009; Pelaez et al. 2009), but it similarly facilitates osteogenic (Catelas et al. 2006; Weinand et al. 2006) and myogenic (Nieponice et al. 2007; O'Cearbhaill et al. 2010) differentiation.

2.2.4 Chondrocyte mechanobiology

An important requirement for healthy cartilage homeostasis is mechanical loading. Spatially variable mechanical stimuli are generated within articular cartilage during normal physiological loading. Shear stress and high fluid flow is most dominant at the surface whereas high hydrostatic pressure and low fluid flow occurs close to the tidemark in the deep zone. These stimuli are perceived and transduced by chondrocytes and regulate cartilage maintenance and development.

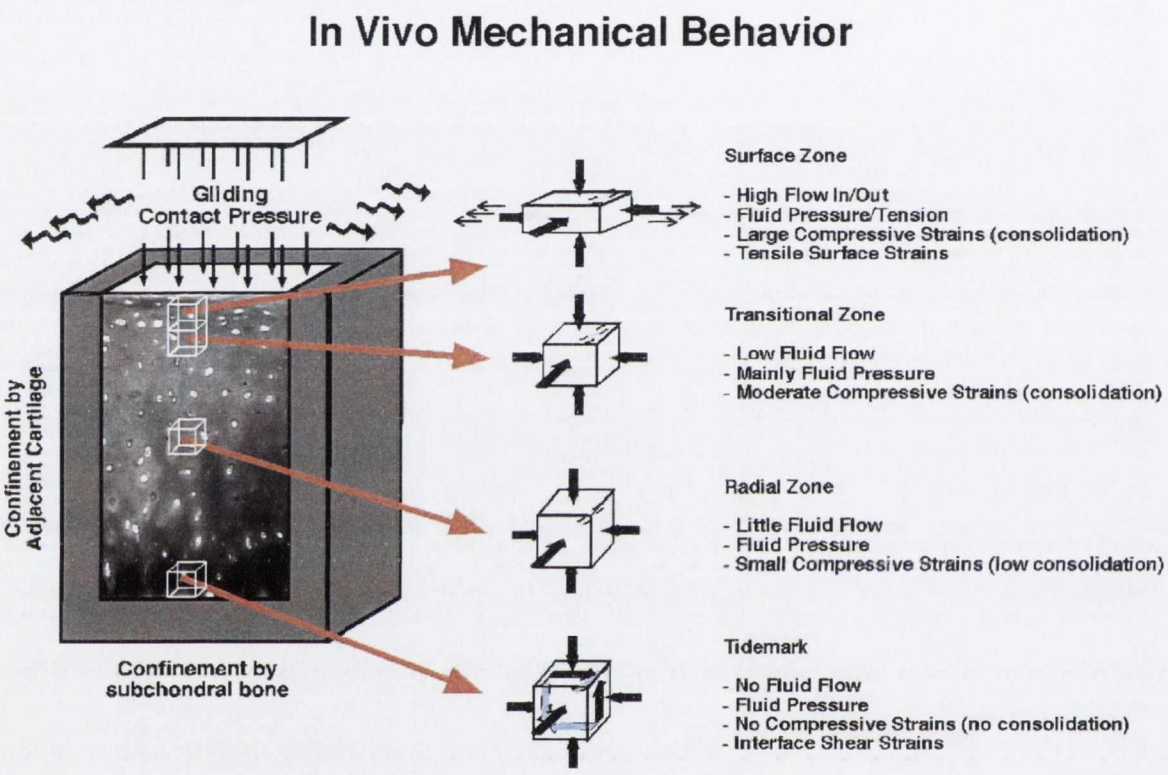

Figure 2.8: Schematic representation of the *in vivo* mechanical environment of articular cartilage under intermittent joint loading and motion (Wong and Carter 2003).

The biosynthetic activity of chondrocytes during *in vitro* culture has been shown to be dependent on both the biochemical and biophysical stimuli the cells experience. The application of appropriate levels of dynamic compressive loading (Buschmann et al. 1995; Lee and Bader 1997; Lee et al. 1998; Lee et al. 2000; Mauck et al. 2000; Chowdhury et al. 2001; Mauck et al. 2002; Mauck et al. 2003b; Kelly et al. 2004; Ng et al. 2006) or hydrostatic pressure (Toyoda et al. 2002; Toyoda et al. 2003) to chondrocytes encapsulated in agarose hydrogels has been shown to enhance cartilage specific matrix production.

Furthermore, it has been shown that temporal application of TGF-β3 for up to 14 days prior to the initiation of dynamic compression culminates in the achievement of glycosaminoglycan (GAG) content and equilibrium moduli surpassing that of native cartilage (Demarteau et al. 2003; Lima et al. 2007). This allows for the cartilage cells to develop a certain amount of peri-cellular and extra-cellular matrix which may result in a more anabolic response to compression.

Much work has focused on the optimisation of dynamic compression protocols for chondrocyte based cartilage tissue engineering. Variables including loading duration, loading frequency, applied dynamic strain and static strain offset are all seen to have an effect on the biosynthetic activity of chondrocytes; be they in their native extracellular environment (cartilage explant) or seeded in a scaffold. Table 2.1 outlines key studies investigating the effects of static and dynamic compression on chondrocyte and cartilage explant cultures. Although a positive effect was observed in response to many of the various dynamic compression protocols employed in Table 2.1, in terms of tissue chondrogenesis, only the protocol employed by Lima *et al.* (2007) where 10% dynamic strain was applied at 1 Hz for 3 hours/day, 5 days/week initiated after 2 weeks unloaded culture in the presence of transforming growth factor-3 (TGF-β3) resulted in the attainment of tissue engineered constructs with sulphated glycosaminoglycan levels and mechanical properties comparable to native articular cartilage.

Table 2.1: Studies investigating compressive mechanical stimulation of chondrocyte seeded constructs or cartilage explants

Mechanical Stimulus	*Loading*	*Matrix*	*Reference*
Static compression	Graded levels up to 50% strain for 12 hours	Cartilage explant	Kim *et al.* (1994)
	Graded levels up to 50% strain for 16 hours	Agarose	Buschmann *et al.* (1995)
	Graded levels up to 24 MPa for 2-24 hours	Cartilage explant	Torzilli *et al.* (2011)
	50% strain for 24 hours	PGA	Davisson *et al.* (2002)
	0%, 25% and 50% strain for 24 hours	Collagen type I gel	Hunter *et al.* (2002)
Dynamic compression	0.63%-10.4% strain at 0.001-0.1 Hz for 23 hours	Cartilage explant	Kim *et al.* (1994)
	2% strain superimposed on a ~25% strain static offset at 0.001, 0.01, 0.1 and 1 Hz for 10 hours	Agarose	Buschmann *et al.* (1995)
	0.5-24 MPa at 1 Hz for 2-24 hours	Cartilage explant	Torzilli *et al.* (2011)
	15% strain at 0.3, 1 and 3 Hz for 48 hours	Agarose	Lee and Bader (1997)
	10% strain at 1 Hz for 3 hours/day, 5 days/week for 4 weeks	Agarose	Mauck *et al.* (2000)
	15% strain at 0.3, 1 and 3 Hz for 48 hours	Agarose	Lee *et al.* (2000)
	2-3% strain at 0.1 Hz for up to 48 hours	Cartilage explant	Bonassar *et al.* (2001)
	5% strain superimposed on a 10% or 50% static offset at 0.001 or 0.1 Hz for 24 hours	PGA	Davisson *et al.* (2002)

	50% static strain for 24 hours followed by 25%±4% strain at 1 Hz for 24 hours	Collagen type I gel	Hunter *et al.* (2002)
	10% strain at 1 Hz for 3 hrs/day, 5 days/week for 4 weeks	Agarose	Mauck *et al.* (2002)
	5% strain superimposed on a 5% static offset at 0.1 Hz for 2 hours/day for 3 days following 2, 7 or 14 days unloaded culture	PGET/PBT	Démarteau *et al.* (2003)
	3% strain superimposed on a 10% static offset at 0.1 Hz for 24 hours following 2, 7, 14 or 30 days of unloaded culture	Collagen type II scaffold	Lee *et al.* (2003)
	10% strain at 1 Hz for 3 hrs/day, 5 days/week for 56 days	Agarose	Mauck *et al.* (2003c)
	10% strain tapering to 2% stain by day 42 at 1 Hz for 3 hours/day, 5 days/week to day 56 following 2 weeks of unloaded culture	Agarose	Lima *et al.* (2007)

2.3 Mesenchymal stem cells

Stem cells are an undifferentiated cell type capable of undergoing self-renewal and multi-lineage differentiation. Stem cells originate from embryonic and postnatal tissues. Embryonic stem cells can be derived from the blastocyst and are described as pluripotent since they are capable of developing into lineages of all three embryonic germ layers; endoderm (liver, pancreas, thymus, thyroid and lung), mesoderm (bone, cartilage, muscle, heart and kidneys) or ectoderm (skin, brain, eyes and neural tissue) (Duplomb et al. 2007). However, much controversy surrounds the use of human embryonic stem cells. They carry many social and ethical issues, and legal constraints prevent the use of these cells in numerous countries at present. An alternative source of stem cell is of postnatal origin, and of particular relevance to orthopaedics and other disciplines are the mesenchymal progenitor cells (MSCs). These are pluripotent cells present in many adult mesenchymal tissues such as bone marrow, adipose tissue, muscle and synovial membrane. Alexander Freidenstein (1976) discovered the non-haematopoietic MSCs of the bone marrow, and developed the plastic adherence technique for the isolation of such precursor cells to mechanocytes, enabling them to be separated from the non-adherent haematopoietic cells present in bone marrow. These cells exhibit a homogeneous population of spread, fibroblast morphologies which formed colonies during 2-D monolayer expansion. This gave rise to the original term of "colony forming unit-fibroblast" (CFU-F) or "marrow stromal fibroblasts" (Minguell et al. 2001). However, there are still various different terms by which researchers refer to this population of cells including 'mesenchymal stem cells' (MSCs), 'marrow stromal cells' (MSCs) and 'mesenchymal progenitor cells' (MPCs) (Minguell et al. 2001). Debate still remains over whether these cells truly are stem cells and hence what they should be called (Bianco et al. 2008; Caplan 2008; Caplan 2009). Regardless of the term used to describe this cell group, directed differentiation of MSCs has been demonstrated down the adipogenic (fat), osteogenic (bone) and chondrogenic (cartilage) lineages (Caplan 2005). Ultimately the function of MSCs in the body is to direct and control the re-modelling, repair and rejuvenation of various tissues (Caplan and Bruder 2001).

2.3.1 Characteristics of MSCs

Bone marrow derived MSCs have good proliferative potential and have the ability to differentiate into a range of tissues. It has been stated that only 0.001-0.01% of cells resident in adult bone marrow comprise MSCs (Pittenger et al. 1999). Identification of a range of surface markers allows for the characterisation of these cells. Various techniques are available which utilise these surface markers to isolate and sort the cells including flow cytometry performed on a fluorescent activated cell sorter (FACS) and magnetic bead isolation. Many researchers however continue to use the plastic adherence technique to isolate MSCs from the other cell types present in bone marrow.

2.3.2 Chondrogenic differentiation of MSCs

The growth-factor induced differentiation of bone marrow MSCs from many different species has been well documented. The multi-lineage potential of MSCs makes them an attractive cell source for engineering tissues such as cartilage and bone (Figure 2.9). The ability of human MSCs to differentiate along the osteogenic, chondrogenic and adipogenic lineages was first shown by Pittenger et al (1999).

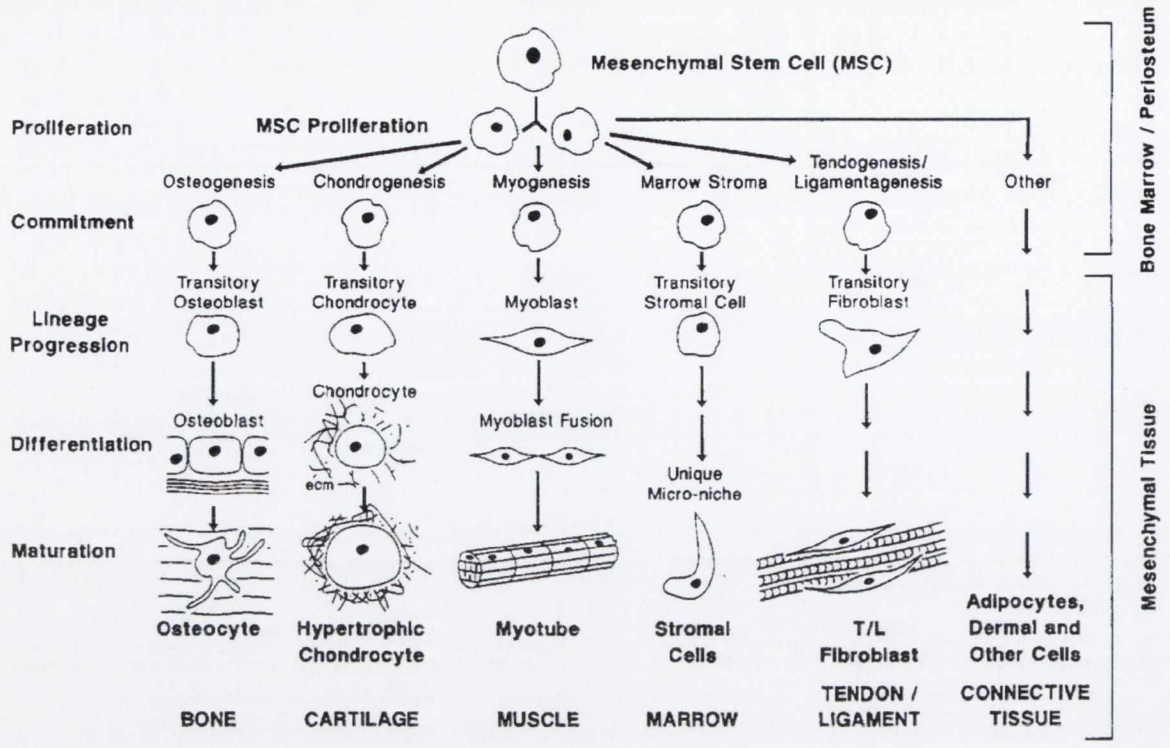

Figure 2.9: Mesenchymal stem cells (MSCs) have the capacity to differentiate into bone, cartilage, muscle, marrow stroma, tendon/ligament, fat, and other connective tissues when signalled with the appropriate stimuli (Caplan 2005).

The differentiation of MSCs along the chondrogenic lineage has been shown in vitro using a number of growth factors in various 3-D environments primarily pellet culture, and in various scaffold types including hydrogels (Johnstone et al. 1998; Aung et al. 2002; Awad et al. 2004; Bosnakovski et al. 2004; Bosnakovski et al. 2006; Mouw et al. 2007; Erickson et al. 2009). Chondrogenic differentiation can be directed by different growth factors or cytokines. These include the bone morphogenic proteins (BMPs) (Majumdar et al. 2001; Schmitt et al. 2003; Zeiter et al. 2009), members of the transforming growth factor-β (TGF-β) superfamily (Iwasaki et al. 1993; Johnstone et al. 1998; Mackay et al. 1998; Yoo et al. 1998; Nishimura et al. 1999; Zuk et al. 2001; Huang et al. 2004b; Palmer et al. 2005), fibroblast growth factors (FGFs), insulin like growth factor (IGF-1), Indian hedgehog and others (de Crombrugghe et al. 2000).

A timescale for the synthesis of a cartilage matrix as a result of TGF-β induced chondrogenesis in pellet culture is shown in Figure 2.10. The synthesis of matrix components is divided into three

stages, 0-6 days, 6-8 days and 8-21 days, and is described in terms of gene expression, protein synthesis, glycosaminoglycan (GAG) synthesis and GAG accumulation (Barry et al. 2001).

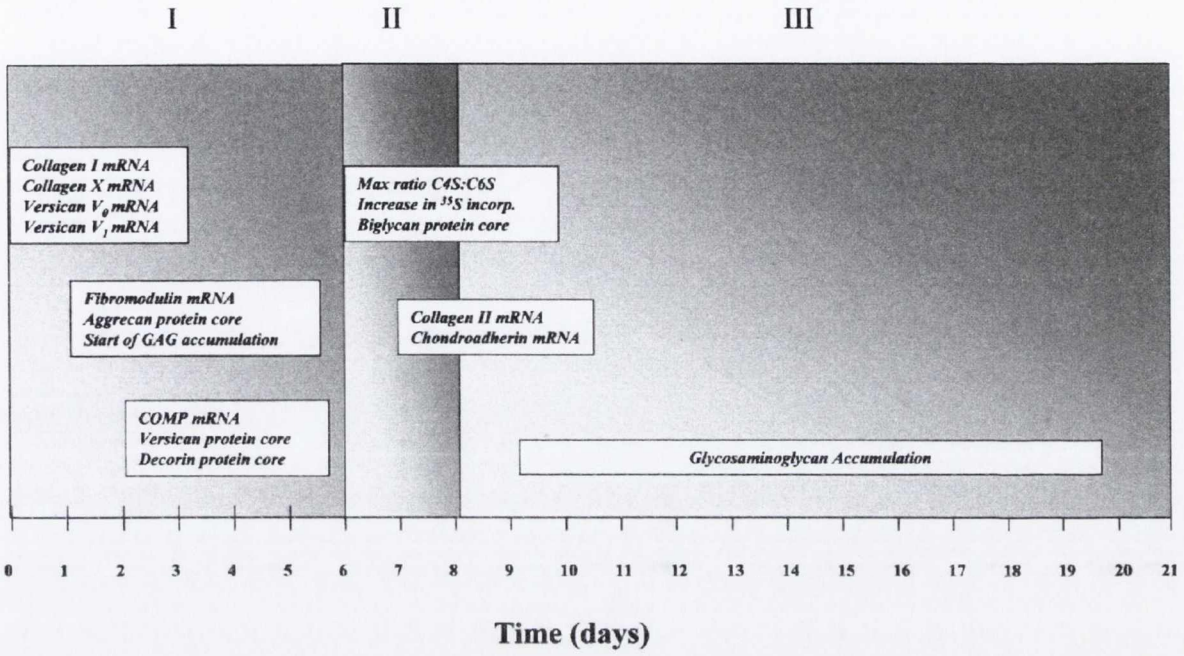

Figure 2.10: Defined events in chondrogenic differentiation of mesenchymal stem cells determined by the sequential expression of matrix components during pellet culture. The process is divided into three stages: Stage 1 (0-6 days) shows the expression of GAG, mRNA expression and protein synthesis; Stage 2 (6-8 days) shows collagen type II gene expression and increases in ^{35}S sulphate incorporation; Stage 3 (8-21 days) shows accumulation of glycosaminoglycan leading to formation of mature chondrocytes (Barry et al. 2001).

2.3.3 MSC mechanobiology

There is much evidence that chondrocytes when subjected to physiological levels of dynamic compressive loading show enhanced cartilage-specific macromolecule biosynthesis (Palmoski and Brandt 1984; Sah et al. 1989; Kim et al. 1994; Buschmann et al. 1995; Grodzinsky et al. 2000; Mauck et al. 2000; Davisson et al. 2002; Mauck et al. 2002). Six months after MSCs were transplanted into full-thickness cartilage defects in rabbit knee joints, it was shown that different local mechanical environments resulted in considerable differences in mechanical properties of the repair tissue on the anterior and posterior aspects of the defect (Wakitani et al. 1994). This would suggest that the differentiation pathway of MSCs is at least partially regulated by the local

mechanical environment. As a result, MSCs are being cultured in bioreactors designed to mimic certain aspects of in vivo joint loading, most commonly dynamic compression or hydrostatic pressure (Knothe Tate et al. 2008).

Protocols employed for the dynamic compression of MSC seeded scaffolds and hydrogels have been based on those used in chondrocyte studies. Table 2.2 outlines key studies investigating the effects of dynamic compression on MSC chondrogenic differentiation and the subsequent chondrogenesis of MSC seeded constructs.

It has been demonstrated that dynamic compressive loading in the absence of TGF-β family members can increase chondrogenic gene expression (Huang et al. 2004a; Huang et al. 2005; Campbell et al. 2006; Park et al. 2006; Mauck et al. 2007; Terraciano et al. 2007) and stimulate the accretion of cartilage-like ECM components relative to unloaded controls (Park et al. 2006; Mauck et al. 2007; Terraciano et al. 2007; Kisiday et al. 2009). Increased sox9 and aggrecan gene expression has been seen after only 1 hour dynamic compressive loading in PEG hydrogels (Terraciano et al. 2007). However, the combined effects of dynamic compression and chondrogenic growth factor supplementation (TGF-β) on chondrogenesis of MSCs are generally more complex. Increases in both chondrogenic gene expression and ECM secretion have been demonstrated with dynamic compression in the presence of TGF-β (Angele et al. 2004; Huang et al. 2004a; Terraciano et al. 2007). Other studies, however, indicate the contrary, with the combination of dynamic compression and TGF-β resulting in a down-regulation of chondrogenic gene-expression (Campbell et al. 2006) and inferior ECM accumulation (Kisiday et al. 2009) when compared to unloaded controls.

Table 2.2: Studies investigating compressive mechanical stimulation on MSC seeded constructs

Loading	*Growth factors*	*Matrix*	*Reference*
10% strain at 1 Hz for 4 hours/day for 3, 7 or 14 days with 5 days subsequent culture in unloaded conditions	Serum free ±TGF-β1	Agarose	Huang *et al.* (2004a)
7994 Pa peak stress (~40% strain) at 0.33 Hz for 4 hours/day for 7 days with subsequent analysis at day 1, 7, 14 or 21.	Serum free +TGF-β1	Hyaluronan-gelatin composite	Angele *et al.* (2004)
15% strain at 1 Hz for 1, 2 or 4 hours/day for 1 or 2 days	Serum free	Agarose	Huang *et al.* (2005)
Unknown strain at 1 Hz for 10 min twice daily for 7 days	Serum free	Alginate	Park *et al.* (2006)
15% strain at 1 Hz in cycles of 1.5 hours followed by 4.5 hours unloaded culture (6 hours/day) for 4 or 8 days.	Serum free ±TGF-β3	Alginate	Campbell *et al.* (2006)
10% strain at 0.33, 1 or 3 Hz for 1 hour with subsequent analysis at 1 or 3 days; 10% strain at 1 Hz for 1 or 3 hours with subsequent analysis at 1 or 3 days; 1 Hz for 3 hours/day for 5 days with subsequent analysis at day 0, 3, 7, 14 and 28 (-TGF-β3)	Serum free ±TGF-β3	Agarose	Mauck *et al.* (2007)
10% strain at 1Hz for 1, 2, 2.5 or 4 hours/day for 1, 2 or 3 weeks	Serum free ±TGF-β1	PEGDA	Terraciano *et al.* (2007)
10±3% strain at 1 Hz for 3 hours initiated after 8 or 16 days unloaded culture	Serum free ±TGF-β1	Agarose	Mouw *et al.* (2007)
7.5±2.5% strain at 0.3 Hz in daily cycles of 45 min. load followed by 45 min unloaded	Serum free ±TGF-β1	Agarose	Kisiday *et al.* (2009)

culture (12 hours/day) for 15 days, or in cycles of 45 min. load followed by 5 hours 15 min. unloaded culture on alternate days for 21 days			
5%, 10% or 20% strain superimposed on a 10% static offset at 0.1 or 1 Hz for 1 hour/day for 7 days initiated after 7 days unloaded culture	Serum free +TGF-β1	Fibrin-polyurethane	Li *et al.* (2010b)
10% strain superimposed on a 10% static offset at 1 Hz for 1 hour/day for 7 days initiated after 7 days unloaded culture	Serum free ±TGF-β1	Fibrin-polyurethane	Li *et al.* (2010a)
10% strain at 1 Hz for 4 hours/day for 3 weeks with and without 3 weeks pre-culture in unloaded conditions; 10% strain at 1 Hz for 1 or 4 hours for 3 weeks initiated after 3 weeks unloaded culture; 10% strain at 0.01 or 1 Hz for 4 hours for 3 weeks initiated after 3 weeks unloaded culture	Serum free ±TGF-β3	Agarose	Huang *et al.* (2010a)

Campbell *et al.* (2006) found a down regulation in Sox-9 expression compared to day 0 for all treatment conditions (Figure 2.11). However there was a tendency for a higher expression with the application of dynamic compression in the absence of TGF-β3. The opposite was seen in the presence of TGF-β3 with the unloaded samples showing higher expression. Similar trends were seen for aggrecan expression (Figure 2.11).

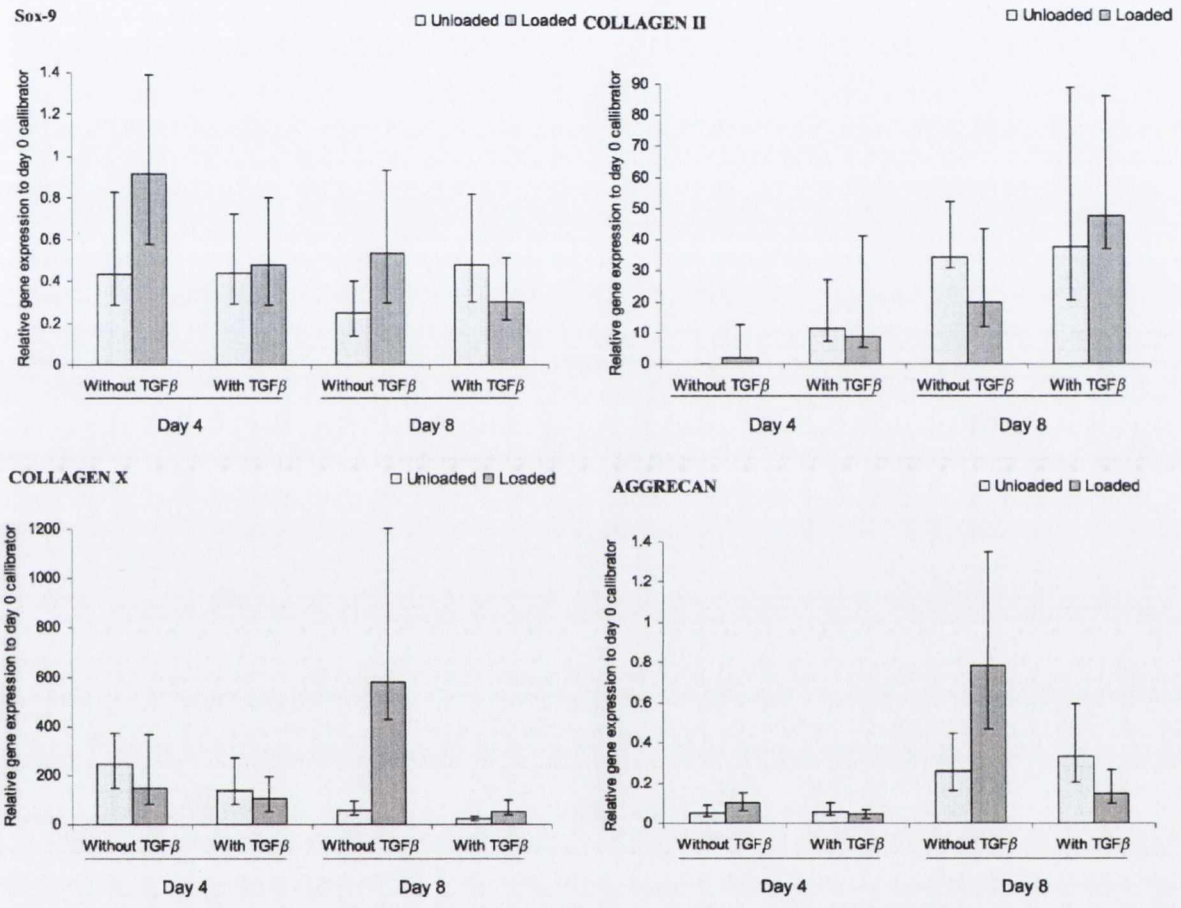

Figure 2.11: The gene expression of Sox-9, collagen type II, collagen type X and aggrecan (normalised to individual sample GAPDH values and relative to the day 0 control) by MSCs seeded in alginate constructs subjected to 15% intermittent dynamic compression at 1Hz for 4 and 8 days in medium supplemented with 0 or 10 ng/mL TGF-β3. Each value represents the median ± inter-quartile range. Adapted from Campbell *et al.* (2006).

Kisiday *et al.* (2009) found that in the presence of TGF-β1, biosynthesis in loaded MSC agarose constructs was less than in unloaded controls. Extracellular matrix synthesis and toluidine blue staining were compared to unloaded constructs in the presence of TGF-β1 for MSCs from three donor horses after 15 days of culture (Figure 2.12). ^{3}H-proline and ^{35}S-sulphate incorporation in

dynamic compression cultures were 74% and 55% of unloaded cultures respectively (Figure 2.12A). Similarly, GAG accumulation in dynamic compression cultures was 64% of free-swelling samples. Toluidine blue staining in dynamically compressed constructs was homogeneously distributed as a function of depth, with less intensity than that exhibited by free-swelling constructs (Figure 2.12B).

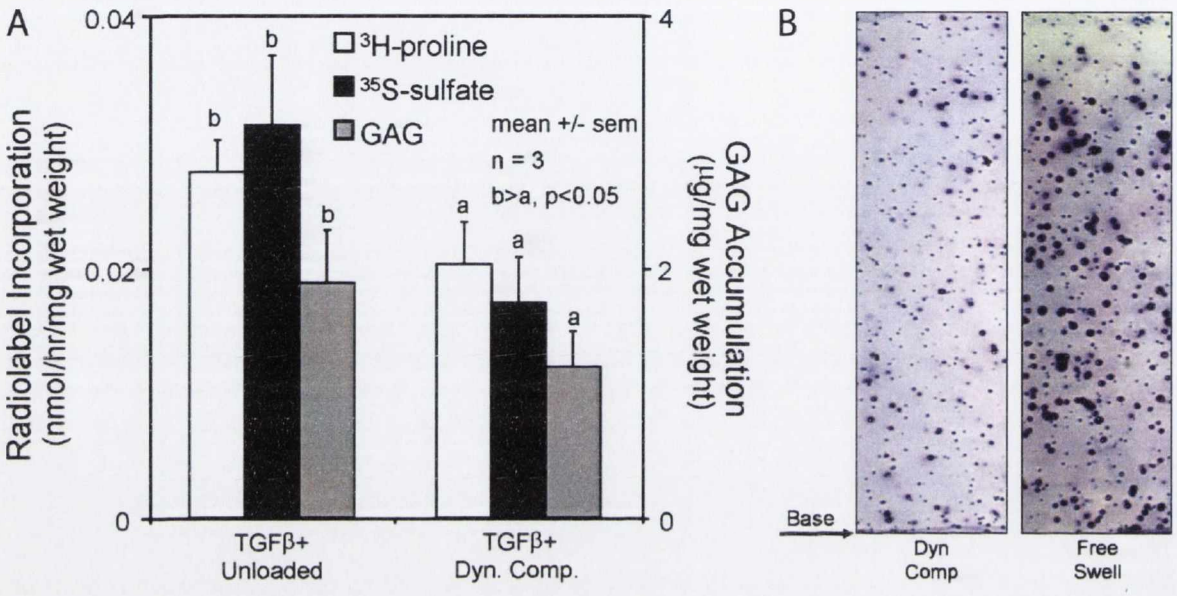

Figure 2.12: A: Extracellular matrix synthesis in response to dynamic compression in the presence of TGF-β1 after 15 days of culture. For each assay, significant differences are denoted by the labels 'a' and 'b'. B: Toluidine blue staining for proteoglycan deposition over full thickness (3mm) sections of samples maintained in medium containing TGF-β1 (Day 15). Dynamic compression samples were loaded for 12 hours per day in cycles of 45 mins loading followed by 45 mins free-swelling culture. Adapted from Kisiday *et al.* (2009).

Mouw *et al.* (2007) have shown that dynamic compression in the presence of TGF-β1 applied at day 8 resulted in a decrease in aggrecan gene expression. However when loading was applied at day 16, an increase in chondrogenic gene expression was observed. This suggests that the mechano-sensitivity of MSCs changes depending on the stage of chondrogenesis.

While many protocols for the application of dynamic compression have been implemented (Table 2.2) with varying success in terms of eliciting pro-chondrogenic gene expression, it is only in recent times that dynamic compression conditions conductive to matrix accumulation have been

investigated (Huang et al. 2010a; Li et al. 2010b). As these studies appeared within the period covered by this thesis, the consequences of these findings were implemented in the later studies presented.

At the outset of this thesis, the effect of mechanical stimulus on the long term chondrogenesis of MSC seeded constructs had not been previously investigated. Previous studies on the effect of dynamic compression had either taken place in the absence of chondrogenic cytokines such as TGF-β, or were typically no longer than 14-21 days and focused on gene expression, not measures of tissue elaboration and function. Therefore, the first objective of this thesis as outlined in Section 1.3 was to ascertain the response of agarose encapsulated MSCs to unconfined dynamic compressive strain. The results of this study led to the formation of the hypothesis tested in the second objective which was partially based on chondrocyte studies where the delayed application of dynamic compression led to the enhancement of chondrogenesis for chondrocyte seeded constructs (Demarteau et al. 2003; Lima et al. 2007). As previous attempts to engineer a cartilaginous tissue with depth dependent properties were largely unsuccessful, a new approach to this problem was explored in the third objective of this thesis (Chapter 5). This approach attempts to recapitulated aspects of the microenvironment which may be responsible for the creation of a zonal cartilage tissue during development. While many studies have examined the effect of varying biomaterial on MSC differentiation, this has not been examined in combination with the application of dynamic compression. Therefore in Chapter 6, the effect of MSC scaffold carrier was examined in the context of the cellular response to dynamic compression.

Chapter 3 Dynamic compression can inhibit chondrogenesis of mesenchymal stem cells

For the purposes of this thesis, the study presented is adapted from the original article published in Biochemical and Biophysical Research Communications, 2008, Vol. 377, No 2 pp. 458-462.

3.1 Abstract

The objective of this study was to investigate the influence of dynamic compressive loading on mesenchymal stem cell (MSC) chondrogenic differentiation in the presence of TGF-β3. Isolated porcine MSCs were suspended in 2% agarose and subjected to intermittent dynamic compression (10% strain) for a period of 42 days in a dynamic compression bioreactor. After 42 days in culture, the free-swelling specimens exhibited more intense alcian blue staining for proteoglycans, while immunohistochemical analysis revealed increased collagen type II immunoreactivity. Glycosaminoglycan (GAG) content increased with time for both free-swelling and dynamically compressed constructs. By day 42 GAG was significantly higher in both the core (2.5±0.21 %w/w vs. 0.94±0.03 %w/w) and annulus (1.09±0.09 %w/w vs. 0.59±0.08 %w/w) of free-swelling constructs compared to dynamically compressed constructs. This result suggests that further optimisation is required in controlling the biomechanical and/or the biochemical environment if such stimuli are to have beneficial effects in generating functional cartilaginous tissue.

3.2 Introduction

Articular cartilage has a limited capacity for repair. This has motivated the development of cell based therapies such as autologous chondrocyte implantation for the repair of cartilage defects (Brittberg et al. 1994; Peterson et al. 2000). A major limiting factor in extending the use of such

therapies is obtaining sufficient numbers of differentiated autologous chondrocytes, particularly in elderly and more osteoarthritic (OA) patients. An age-related loss in chondrogenic capacity has been observed in culture-expanded chondrocytes (Barbero et al. 2004), while collagen synthesis is lower in chondrocytes obtained from OA patients (Tallheden et al. 2005). This may be partially explained by changes in the responsiveness of aged or OA chondrocytes to cytokines and growth factors (Martin et al. 1997; Fan et al. 2004; Fan et al. 2005). In addition, OA chondrocytes are likely to have significant DNA damage among other cellular degenerative alterations (Aigner et al. 2007).

Mesenchymal stem cells (MSCs) are a promising alternative cell source for cartilage repair due to both their ease of isolation and expansion, and their chondrogenic differentiation potential (Caplan 1991). Chondrogenic differentiation of MSCs from different tissue sources has been demonstrated in the presence of growth factors from the transforming growth factor-β (TGF- β) superfamily (Iwasaki et al. 1993; Johnstone et al. 1998; Mackay et al. 1998; Yoo et al. 1998; Nishimura et al. 1999; Zuk et al. 2001). A tissue engineering approach to repair damaged cartilage tissues would involve seeding MSCs into a scaffold and either implanting following minimal in vitro pre-culture, or to culture these constructs for longer periods of time to engineer a more functional cartilaginous tissue. While chondrogenesis does occur in these different systems, it has been demonstrated that the amount of cartilage matrix production and the subsequent mechanical properties of the tissue is lower with MSCs compared to chondrocytes (Mauck et al. 2006), leading to the suggestion that further optimization may be required if MSCs are to be used to engineer cartilaginous tissues with similar functional properties to that obtainable with healthy chondrocytes.

It is well established that the application of appropriate levels of dynamic compressive loading can enhance the biosynthetic activity of chondrocytes (Palmoski and Brandt 1984; Sah et al. 1989; Kim et al. 1994; Buschmann et al. 1995; Mauck et al. 2000; Davisson et al. 2002; Mauck et al. 2002). It is also believed that the differentiation pathway of MSCs is at least partially regulated by the local mechanical environment. In the absence of chondrogenic growth factors, it has been demonstrated that dynamic compressive loading can enhance chondrogenesis of bone marrow derived MSCs (Huang et al. 2004a; Campbell et al. 2006; Mauck et al. 2007). For example, increased aggrecan

gene expression has been observed with as few as three 4 hour loading cycles (Huang et al. 2004a), however other studies have observed little dynamic compression induced stimulation of gene expression or matrix synthesis in the absence of chondrogenic growth factors (Mouw et al. 2007). The combined effects of dynamic compression and chondrogenic growth factors (TGF-β) on chondrogenesis of MSCs are generally more complex (Campbell et al. 2006; Mouw et al. 2007). Dynamic compression has been shown to up-regulate aggrecan gene expression in the absence of TGF-β, but to down-regulate it in the presence of TGF-β (Campbell et al. 2006). In both the presence and absence of chondrogenic growth factors, it has been demonstrated that short-term exposure (≤ 1 week) to intermittent dynamic compression leads to enhanced GAG synthesis in the proceeding weeks of culture compared to free swelling controls (Angele et al. 2004; Mauck et al. 2007). What remains unclear is what influence the long-term application of dynamic compression has on the differentiation of MSCs in a chondrogenic environment. This is an important question not only for engineering functional cartilaginous tissues from MSCs, but also to understand how MSCs will respond once implanted into load bearing defects. The goal of this study was to determine how the daily application of dynamic compressive loading influences cartilage specific matrix production by MSCs undergoing chondrogenic differentiation in long-term agarose culture. Our initial hypothesis was that dynamic compression would enhance matrix synthesis, leading to increases in the mechanical properties of MSC seeded agarose hydrogels.

3.3 Methods

The commercial sources of reagents are listed in Appendix B.

3.3.1 Cell isolation, expansion and agarose hydrogel encapsulation

Femora from a 4 month old porcine donor (~50 kg) were sawn and the gelatinous bone marrow removed under sterile conditions. Porcine MSCs were isolated and expanded according to a modified method developed for human MSCs (Lennon and Caplan 2006). Cultures were expanded in high-glucose Dulbecco's Modified Eagle Medium (DMEM GlutaMAX™) supplemented with 10% foetal bovine serum (FBS), and penicillin (100 U/mL)-streptomycin (100 μg/mL). Culture-

expanded MSCs (3rd passage) were encapsulated in agarose (Type VII) at ~40°C, to yield a final gel concentration of 2% and a cell density of 15×10^6 cells/mL. Agarose was used as the cell carrier due to the success with this material in cartilage tissue engineering studies utilising chondrocytes as outlined in Section 2.2.2. The agarose-cell suspension was cast in a stainless steel mould to produce cylindrical discs (Ø6 mm × 4 mm thickness). Constructs were maintained in a chemically defined chondrogenic medium (CM) consisting of DMEM GlutaMAX™ supplemented with penicillin (100 U/mL)-streptomycin (100 µg/mL), 100 µg/ml sodium pyruvate, 40 µg/ml L-proline, 50 µg/ml L-ascorbic acid-2-phosphate, 1 mg/ml BSA, 1× insulin–transferrin–selenium, 100 nM dexamethasone and 10 ng/ml recombinant human transforming growth factor-β3 (TGF-β3; R&D Systems, UK). Constructs were allowed to equilibrate for 4 days before the addition of dexamethasone and TGF-β3, and the initiation of mechanical compressive loading.

3.3.2 Application of dynamic compression

Intermittent unconfined dynamic compression (DC) was carried out in a custom pneumatic based compressive loading bioreactor with constructs immersed in CM. The dynamic compression protocol consisted of ~10% strain amplitude superimposed on a 0.01 N preload at a frequency of 0.5 Hz. This loading regime was employed for a period of 1 hour, 5 days/week and is consistent with protocols used to elicit pro-chondrogenic gene expression from MSCs in other studies outlined in Section 2.3.3. Free swelling (FS) controls were maintained in the same amount of medium adjacent to the loading device during loading periods. A 50% medium exchange was performed every 2-3 days.

3.3.3 Mechanical and biochemical analysis

Constructs were mechanically tested in unconfined compression between impermeable platens using a standard materials testing machine with a 5N load cell (Zwick Z005, Roell, Germany). Stress relaxation tests were performed, consisting of a ramp and hold cycle with a ramp displacement of 1 µm/s until 10% strain was obtained and maintained until equilibrium was reached (~30 minutes). Dynamic tests were performed immediately after the stress relaxation cycle.

Cyclic strain amplitude of 1% superimposed upon the 10% strain was applied for 10 cycles at 1Hz. The compressive equilibrium modulus and dynamic modulus were determined from these tests.

The biochemical content of constructs was assessed at each time point (0, 14 and 42 days); constructs were cored using a 3mm biopsy punch, the wet mass of both annulus and core was recorded and then frozen for subsequent analyses. Annuli and core samples were digested with papain (125μg/ml) in 0.1 M sodium acetate, 5 mM L-cysteine HCl, 0.05 M EDTA, pH 6.0 (all from Sigma–Aldrich, Ireland) at 60°C under constant rotation for 18hours. DNA content was quantified using the Hoechst Bisbenzimide 33258 dye assay as described previously (Kim et al. 1988), with a calf thymus DNA standard. Proteoglycan content was estimated by quantifying the amount of sulphated glycosaminoglycan (GAG) in constructs using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Northern Ireland), with a chondroitin sulphate standard. Total collagen content was determined by measuring the hydroxyproline content (Kafienah and Sims 2004), using a hydroxyproline-to-collagen ratio of 1:7.69 (Ignat'eva et al. 2007).

3.3.4 Histology and immunohistochemistry

At each time point, at least one sample per group was fixed in 4% paraformaldehyde overnight, rinsed in PBS, and embedded in paraffin. The constructs were embedded such that sectioning at 8 μm produced a cross section perpendicular to the disc face. Sections were stained with 1% alcian blue 8GX (Sigma–Aldrich, Ireland) in 0.1M HCl which stains sulphated mucins, and picro-sirius red to stain collagen. The deposition of collagen type II was identified by immunohistochemical analysis. A rabbit anti-human polyclonal antibody (3 mg/mL) that binds to collagen type II was used. The presence of the collagen type II antibody was subsequently detected by the secondary antibody, biotin-labelled goat anti-rabbit polyclonal (1 mg/mL). Positive and negative controls of cartilage and trachea were included.

3.3.5 Statistical Analysis

Statistical analyses were performed using GraphPad Prism (Version 4.03) software with 3-4 samples analysed for each experimental group. Two-way ANOVA was used for analysis of

variance with Bonferroni post-tests to compare between groups. Numerical and graphical results are displayed as mean ± standard deviation. Significance was accepted at a level of $p < 0.05$.

3.4 Results

There were no statistical differences in the DNA content between the FS and DC groups at any time point; or between core and annular regions within any group of constructs (Figure 3.1A).

The GAG content was significantly higher after both 14 and 42 days in culture compared to the corresponding samples on day 0 (Figure 3.1B). No difference was found between the FS and DC groups for annulus and core respectively at day 14. However, at day 42 both annulus (1.09±0.09 %w/w vs. 0.59±0.08 %w/w) and core (2.5±0.21 %w/w vs. 0.94±0.03 %w/w) regions of FS constructs had significantly greater GAG ($p<0.001$) than DC constructs. GAG content in the core region of all construct groups was significantly greater than that in the annulus (Figure 3.1B). Collagen content was also higher in FS constructs when compared to DC constructs at day 42 (Figure 3.1C).

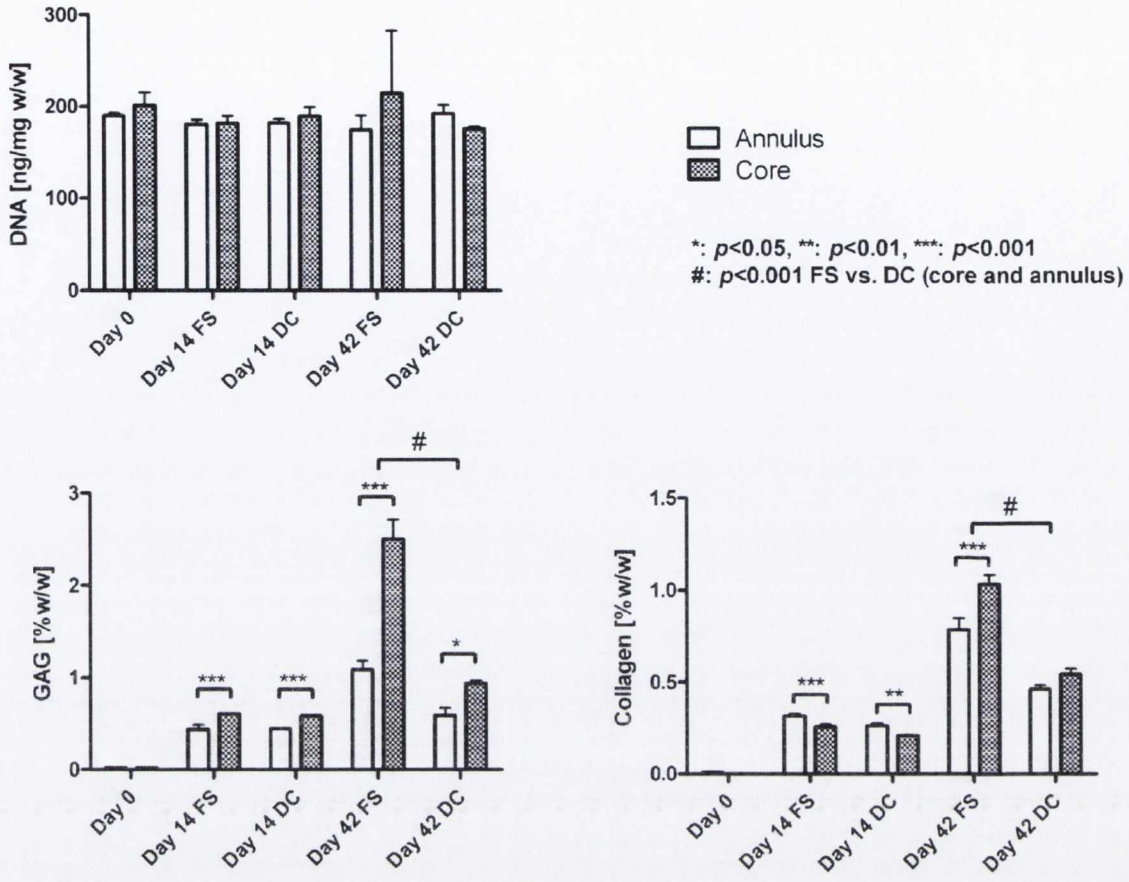

Figure 3.1: Biochemical composition of both core and annulus regions for FS and DC groups; (A): DNA content (ng DNA/mg construct w/w), (B): GAG content (% construct w/w), (C): Collagen content (% construct w/w).

Staining of construct sections with alcian blue for sulphated proteoglycan revealed a steady accretion of positive staining for both FS and DC conditions (Figure 3.2). At days 14 and 42 the FS constructs exhibited more intense positive staining for GAG than the DC constructs. Similar trends were seen in picro sirius staining for collagen (data not shown). This staining corresponded to biochemical results, with less intense staining around the periphery of the construct. Collagen type II immunohistochemistry (Figure 3.2) provides evidence of chondrogenic differentiation, again showing stronger staining away from the construct edge. The DC constructs exhibit a more homogeneous spatial staining in each case than the FS constructs.

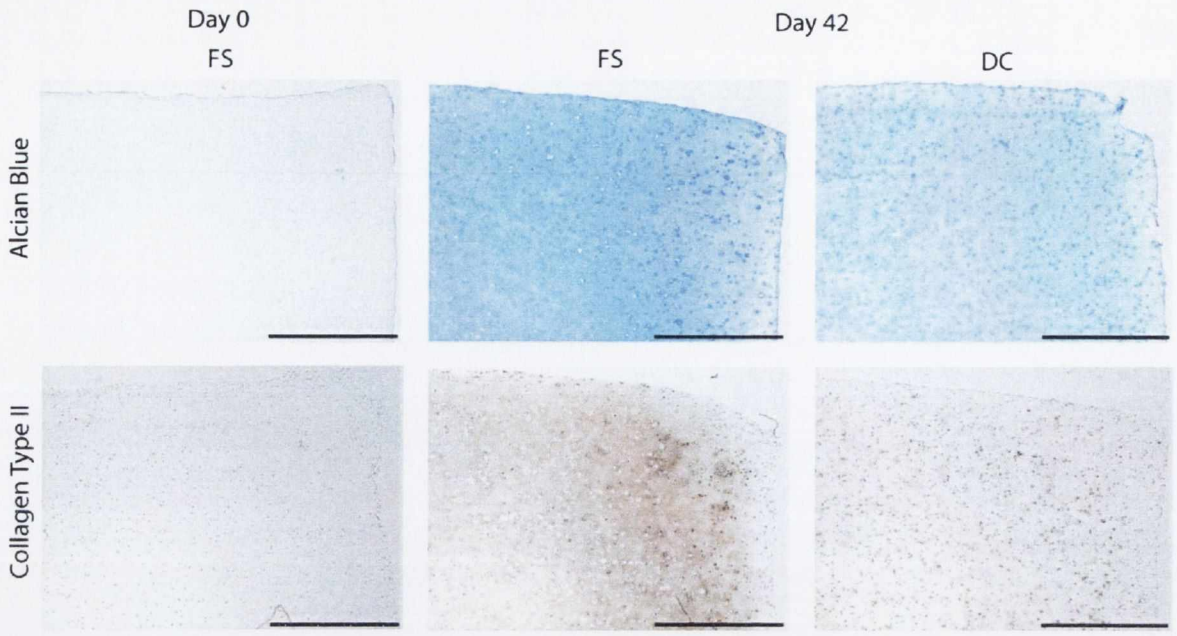

Figure 3.2: Representative alcian blue staining (top) and collagen type II immunohistochemistry (bottom) at day 0 and day 42 for both FS and DC construct groups. Scale bar: 1 mm.

The equilibrium and dynamic mechanical properties were dependent on time in culture ($p<0.001$) and mechanical stimulation ($p<0.001$) (Fig.3). However, significant differences between FS and DC were only seen at day 42 ($p<0.001$); the equilibrium modulus of day 42 FS constructs reached 49.9±0.6 kPa compared to 24.4±1.5 kPa for the DC constructs. The 1 Hz dynamic modulus followed a similar trend with day 42 FS constructs reaching 230.7±3.4 kPa compared to 111.4±4.1 kPa for the DC constructs. Notably, the mechanical properties of DC constructs did not increase between day 14 and 42.

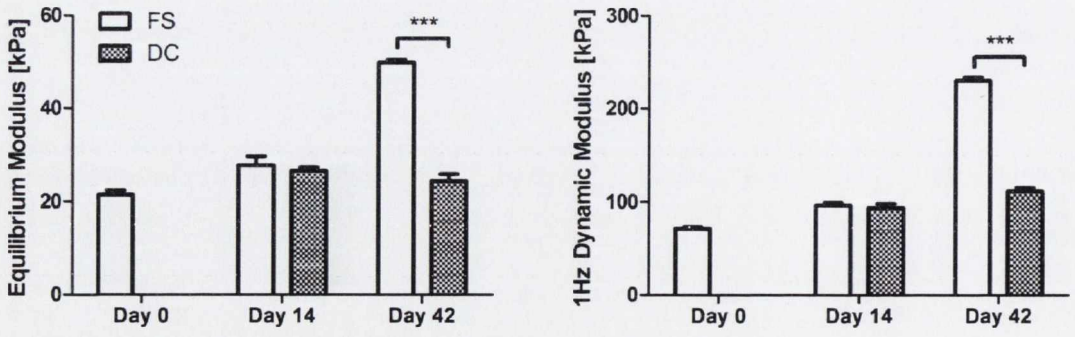

Figure 3.3: Equilibrium compressive modulus (left) and 1 Hz dynamic modulus (right) of FS and DC constructs at each time point. ***: $p<0.001$.

3.5 Discussion

Whether injected directly in-vivo, or cultured ex-vivo on scaffolds prior to implantation, understanding the response of MSCs to physical forces is of critical importance when developing cell based therapies for cartilage repair. Our hypothesis was that intermittent dynamic compression applied daily to MSCs in agarose culture in a chemically defined medium supplemented with TGF-β3 would enhance chondrogenesis, resulting in the formation of a more functional cartilaginous tissue. No evidence was found to support this hypothesis. Rather dynamic compressive loading was observed to inhibit chondrogenesis of the engineered tissue in the presence of TGF-β3.

There are a number of factors that could explain the observed results. Our initial hypothesis was based on the observation that dynamic compressive loading of chondrocytes in agarose culture stimulates cartilage specific gene expression and protein synthesis (Buschmann et al. 1995; Mauck et al. 2000). In this study, dynamic compressive loading was applied shortly after the MSCs had been exposed to chondrogenic growth factors. At this early stage in the differentiation process, it is perhaps not surprising that MSCs do not respond to biophysical stimulation in a similar manner to fully differentiated articular chondrocytes. Mouw *et al.* (2007) observed that the response of bone marrow derived MSCs in agarose culture to dynamic compression varied during the process of chondrogenesis, with dynamic compression only enhancing chondrogenesis following 16 days of pre-culture in free swelling conditions in the presence of TGF-β1. It was suggested that the developing pericellular and extracellular matrix may be regulating mechano-stimulation of MSCs undergoing chondrogenesis. Cartilage matrix synthesis by articular chondrocytes in dynamically compressed agarose gels is greater at later time points than earlier time points (Buschmann et al. 1995), implying that a well-developed inter-territorial matrix may be required for the transduction of mechanical compression to a cellular biosynthetic response.

Another possible explanation is that the type, magnitude, frequency or duration of the applied mechanical stimulus in this study (unconfined compression at 10% strain at 0.5 Hz for 1 hour/day) inhibits the chondrogenic differentiation of MSCs. The application of this loading protocol produces a complex mechanical environment within the agarose hydrogel, with magnitudes of

deformation, hydrostatic pressure and fluid velocities that vary spatially and temporally. Theoretical models have suggested that the mechanical environment can regulate the differentiation pathway of MSCs (Prendergast et al. 1997; Kelly and Prendergast 2005), and it is possible that excessive levels of deformation or fluid flow in the agarose hydrogel, particularly in the absence of a fully developed pericellular matrix to regulate mechano-transduction, could be promoting the formation of a more fibroblastic phenotype. Immunohistochemical analysis also revealed the presence of type I collagen in both FS and DC constructs (data not shown). Comparisons of different bioreactor systems for cartilage tissue engineering suggest that fluid flow plays a role in regulating chondrogenesis (Vunjak-Novakovic et al. 1999). Chondrocyte differentiation has also been shown to be modulated by the frequency and duration of cyclic compressive loading (Elder et al. 2001). Altering parameters of the dynamic compression loading protocol such as the strain magnitude and frequency would allow this hypothesis to be investigated further.

These factors do not explain the results of similar studies where mechanical loading in the absence of TGF-β enhances chondrogenesis (Huang et al. 2004a; Campbell et al. 2006; Mauck et al. 2007), even when applied shortly after agarose encapsulation. This would suggest that a more complex interplay exists between the biophysical and biochemical environment in regulating chondrogenesis of MSCs, leading to the inhibition of cartilage-specific matrix production observed in this study. There are many potential interactions that need to be considered. In free swelling cultures, it was observed that total GAG synthesis was greater in the core compared to the annulus of agarose hydrogels (Figure 3.1). This may be partially explained by diffusion of GAG from the annulus of the construct into the surrounding media. Another explanation may be diffusion limitations or cell consumption at the periphery of the hydrogel producing a radial gradient in the molecules responsible for regulating chondrogenesis. For example, lower concentrations of TGF-β3 or oxygen towards the core may be promoting chondrogenesis. If this is the case, the enhanced transport and concentration of these molecules due to dynamic compression (Mauck et al. 2003a) may explain the inhibition of chondrogenesis in this study. For example, hypoxia has been shown to promote chondrogenesis of MSCs in the presence of chondrogenic growth factors (Kanichai et al. 2008), however it has also been shown that diffusional limitations in oxygen supply can result in

more anaerobic conditions resulting in markedly reduced rates in the growth of chondrocyte seeded polymer scaffolds (Obradovic et al. 1999). The biosynthetic response of cartilage explants to TGF-β treatment has been shown to be dose dependant, reaching saturation at 10ng/ml (Morales and Roberts 1988). Chondrogenic differentiation of MSCs would also appear to depend on the dose of TGF-β, with 5ng/ml of TGF-β1 reported as optimal in monolayer culture (Worster et al. 2001). In pellet culture it has been reported that at a concentration of 1ng/ml of TGF-β1, chondrogenesis is limited to the outer third of the cell aggregate, with more homogenous chondrogenesis at 10ng/ml (Johnstone et al. 1998). In addition, stimulation of the TGF-β signalling pathway could modulate mechano-transduction, or alternatively, mechanical stimulation may modulate TGF-β signalling (Mouw et al. 2007). Interestingly, dynamic compressive loading of chondrocyte seeded agarose hydrogels applied concurrently with exposure to 10 ng/ml of TGF-β3 has also been shown to inhibit GAG synthesis (Lima et al. 2007). In the same study (Lima et al. 2007), deformational loading initiated after culturing for two weeks in the presence of TGF-β3 yielded significantly stiffer constructs than free–swelling controls. Adopting a similar loading protocol with MSCs undergoing chondrogenesis may produce a more anabolic response to dynamic deformational loading. Allowing agarose encapsulated MSCs to undergo TGF-β3 mediated differentiation in unloaded conditions may result in enhanced chondrogenesis on the delayed initiation of dynamic compression. This will be the focus of Chapter 4.

Chapter 4 The response of bone marrow derived mesenchymal stem cells to dynamic compression following TGF-β3 induced chondrogenic differentiation

For the purposes of this thesis, the study presented is adapted from the original article published in Annals of Biomedical Engineering, September 2010, Vol. 38, No. 9 pp. 2896-2909.

4.1 Abstract

The objective of this study was to investigate the hypothesis that the application of dynamic compression following transforming growth factor-β3 (TGF-β3) induced differentiation will further enhance chondrogenesis of mesenchymal stem cells (MSCs). Porcine MSCs were encapsulated in agarose hydrogels and cultured in a chemically defined medium with TGF-β3 (10 ng/ml). Dynamic compression (1 Hz, 10% strain, 1 hour/day) was initiated at either day 0 or day 21 and continued until day 42 of culture; with TGF-β3 withdrawn from some groups at day 21. Biochemical and mechanical properties of the MSC-seeded constructs were evaluated up to day 42. The application of dynamic compression from day 0 inhibited chondrogenesis of MSCs. This inhibition of chondrogenesis in response to dynamic compression was not observed if MSC-seeded constructs first underwent 21 days of chondrogenic differentiation in the presence of TGF-β3. Spatial differences in sGAG accumulation in response to both TGF-β3 stimulation and dynamic compression were observed within the constructs. sGAG release from the engineered construct into the surrounding culture media was also dependent on TGF-β3 stimulation, but was not affected by dynamic compression. Continued supplementation with TGF-β3 appeared to be a more potent chondrogenic stimulus than the application of 1 hour of daily dynamic compression following cytokine initiated differentiation. In the context of cartilage tissue engineering, the results of this

study suggest that MSC seeded constructs should be first allowed to undergo chondrogenesis *in vitro* prior to implantation in a load bearing environment.

4.2 Introduction

Once damaged articular cartilage has a limited reparative capacity and thus lesions often progress to arthritis (Buckwalter and Mankin 1997). This has motivated the development of cell based therapies for the repair of cartilage defects such as autologous chondrocyte implantation (Brittberg et al. 1994; Peterson et al. 2000). A major limiting factor in extending the use of such therapies is obtaining sufficient numbers of viable chondrocytes, particularly in elderly and more osteoarthritic patients. An age related loss in chondrocyte yield, proliferation and functional capacity has been observed in culture-expanded chondrocytes (Barbero et al. 2004), while chondrocytes isolated from osteoarthritic patients exhibit reduced collagen synthesis (Tallheden et al. 2005). Mesenchymal stem cells (MSCs) are a promising alternative cell source for cartilage repair due to both their ease of isolation and expansion, and their chondrogenic differentiation capacity (Caplan 1991; Pittenger et al. 1999; Tuan et al. 2003). Chondrogenic differentiation of MSCs from different tissue sources has been shown in the presence of members of the transforming growth factor-β (TGF-β) superfamily (Iwasaki et al. 1993; Johnstone et al. 1998; Mackay et al. 1998; Yoo et al. 1998; Nishimura et al. 1999; Zuk et al. 2001; Huang et al. 2004b; Palmer et al. 2005).

The same functional demands which put cartilage at risk of injurious damage and frequent degenerative changes must be considered in the design and appraisal of any therapeutic intervention. In the context of cell based tissue engineering therapies, mechanical factors are of particular importance. While chondrogenesis of MSCs has been demonstrated in different three-dimensional scaffolds and hydrogels (Williams et al. 2003; Mauck et al. 2006; Coleman et al. 2007; Kisiday et al. 2008; Erickson et al. 2009; Li et al. 2009), it has been shown that matrix accumulation and the subsequent mechanical properties of MSC laden constructs are lower than those of chondrocyte seeded controls (Mauck et al. 2006; Erickson et al. 2009). This suggests that further optimisation of the cellular environment is required if MSCs are to be used to engineer

cartilaginous tissues with functional properties similar to those obtainable with chondrocytes. Furthermore, if MSCs are to be implanted into the joint as part of a clinical repair strategy, the biomechanical and biochemical influences of the joint environment on MSC differentiation and extracellular matrix (ECM) synthesis must be determined.

Chondrocytes generally respond to physiological levels of dynamic compressive loading through enhanced cartilage-specific macromolecule biosynthesis (Palmoski and Brandt 1984; Sah et al. 1989; Buschmann et al. 1992; Kim et al. 1994; Grodzinsky et al. 2000; Mauck et al. 2000; Davisson et al. 2002). The differentiation pathway and biosynthetic activity of MSCs is also at least partially regulated by the biophysical environment (Knothe Tate et al. 2008; Kelly and Jacobs 2010). For example, the mechanical properties of repair tissue generated by MSCs transplanted into full-thickness cartilage defects have been shown to depend on the location of the injury (Wakitani et al. 1994), suggesting that the local mechanical environment is regulating cellular activity. MSCs have also been cultured in vitro in bioreactors designed to mimic certain aspects of in vivo joint loading, most commonly dynamic compression or hydrostatic pressure (Angele et al. 2003; Huang et al. 2004a; Mauck et al. 2007). It has been demonstrated that dynamic compressive loading in the absence of TGF-β family members can increase chondrogenic gene expression (Huang et al. 2004a; Huang et al. 2005; Campbell et al. 2006; Park et al. 2006; Mauck et al. 2007; Terraciano et al. 2007) and stimulate the accretion of cartilage-like extra-cellular matrix (ECM) components (Park et al. 2006; Mauck et al. 2007; Kisiday et al. 2009) relative to unloaded controls. However, the combined effects of dynamic compression and chondrogenic growth factor supplementation on chondrogenesis of MSCs are generally more complex (Campbell et al. 2006). Dynamic compression in the presence of TGF-β has resulted in increases in both chondrogenic gene expression (Angele et al. 2004; Huang et al. 2004a; Terraciano et al. 2007) and ECM secretion (Angele et al. 2004; Terraciano et al. 2007). Other studies however, indicate the contrary, with the combination of dynamic compression and TGF-β resulting in a down-regulation of chondrogenic gene-expression (Campbell et al. 2006) and inferior ECM accumulation (Thorpe et al. 2008; Kisiday et al. 2009) when compared to unloaded controls. Previous work in our laboratory investigating the effects of long term application of dynamic compression on MSC differentiation

in the presence of TGF-β3 showed that chondrogenesis was significantly inhibited as evidenced by lower ECM deposition and resulting mechanical properties when compared to unloaded controls (Thorpe et al. 2008).

It has been shown that the application of dynamic compression to MSC seeded constructs in the presence of TGF-β1 applied at early time-points (day 8) results in decreased aggrecan gene expression; while loading at later time-points (day 16) leads to increases in chondrogenic gene expression (Mouw et al. 2007). This suggests that the mechano-sensitivity of MSCs changes depending on the stage of chondrogenesis. In similar bioreactor studies, it has been shown that delaying the application of dynamic compression to chondrocyte seeded constructs allowing ECM accumulation can enhance subsequent sulphated glycosaminoglycan (sGAG) synthesis (Demarteau et al. 2003) or the mechanical properties of the engineered tissue (Lima et al. 2007). In this study MSCs were allowed to undergo differentiation in the presence of TGF-β3 for 21 days prior to initiation of delayed dynamic compression. The objective of this study was to test the hypothesis that the application of dynamic compression following TGF-β3 induced differentiation would further enhance chondrogenesis of MSCs in agarose hydrogel culture. To comprehensively address this hypothesis, the study (i) investigated the spatial accumulation of ECM within agarose hydrogels; (ii) compared continued TGF-β3 supplementation and dynamic compression as potential regulators of post-cytokine initiated MSC chondrogenic differentiation; and (iii) assessed the effect of compression and TGF-β3 supplementation on sGAG retention within, and release from, MSC seeded agarose hydrogels.

4.3 Materials and Methods

The commercial sources of reagents are listed in Appendix B.

4.3.1 Experimental design

This study comprised of two parts. The first, termed "transient TGF-β3" received TGF-β3 supplementation for the first 21 days of culture, after which it was removed. The second termed

"continuous TGF-β3" received continuous TGF-β3 supplementation for the 42 day culture period. Both parts involved dynamic compression applied after a 21 day period of unloaded free-swelling (FS) culture in the presence of TGF-β3. Within each part, half the samples taken through 21 days of FS culture were subjected to dynamic compressive culture for a further 21 days; termed delayed dynamic compression (DDC). The remainder were kept in FS conditions as a control. An additional group also received dynamic compression culture from day 0 through to day 42, termed continuous dynamic compression (CDC). (Details of loading magnitude and duration are provided below. The term 'continuous' refers to samples loaded from day 0). The timeline and conditions are illustrated in Figure 4.1. Samples were assessed at day 0, day 21 and day 42.

4.3.2 Cell isolation and expansion

MSCs were isolated from the femora of three porcine donors (4 month old; ~50 kg) within 3 hours of sacrifice. Porcine MSCs were isolated and expanded according to a modified method developed for human MSCs (Lennon and Caplan 2006). MSCs were plated at a seeding density of 5×10^3 cells/cm^2 in high-glucose Dulbecco's modified eagles medium (4.5 mg/mL D-Glucose, 200mM L-Glutamine; hgDMEM) supplemented with 10% foetal bovine serum (FBS) and penicillin (100 U/mL)-streptomycin (100 µg/mL) and expanded to passage three in a humidified atmosphere at 37°C and 5% CO_2.

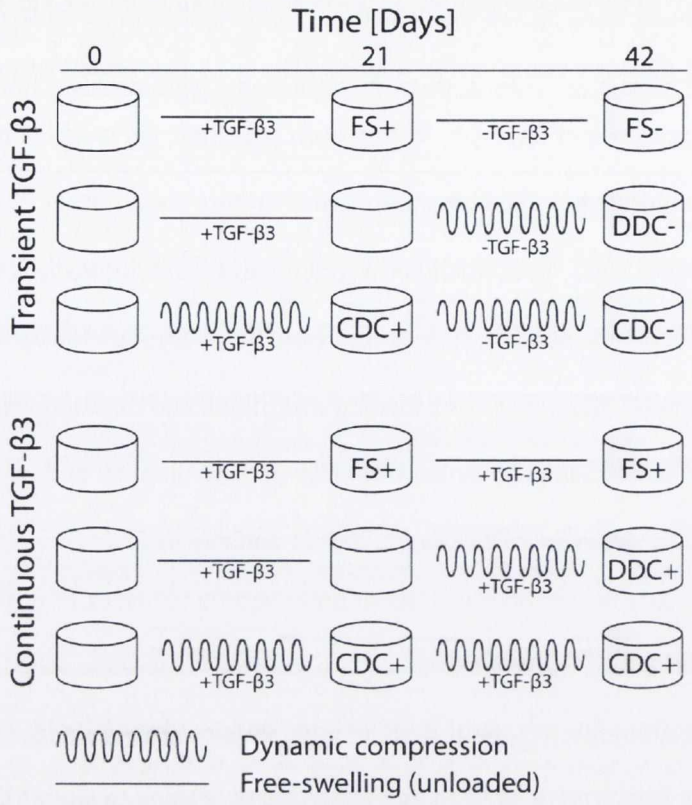

Figure 4.1: Schematic of experimental design. -TGF-β3: without TGF-β3 supplementation. +TGF-β3: with TGF-β3 supplementation. FS: free-swelling unloaded samples. DDC: delayed dynamic compression (maintained in free-swelling conditions for 21 days prior to dynamic compressive loading). CDC: continuous dynamic compression. -: transient TGF-β3 supplementation. +: continuous TGF-β3 supplementation. *n* = 5 or 6 constructs per group per time point.

4.3.3 Agarose hydrogel encapsulation

MSCs from 3 donors were pooled, suspended in hgDMEM and mixed with 4% agarose (Type VII) in phosphate buffered saline (PBS) at a ratio of 1:1 at ~40°C, to yield a final gel concentration of 2% and a cell density of 15×10^6 cells/mL. The agarose-cell suspension was cast in a stainless steel mould to produce cylindrical constructs (Ø5 mm × 3 mm thickness). Constructs were maintained separately in 12-well plates with 2.5 mL chondrogenic medium (CM) which consisted of hgDMEM supplemented with penicillin (100 U/mL)-streptomycin (100 µg/mL), 100 µg/ml sodium pyruvate, 40 µg/mL L-proline, 1.5 mg/mL bovine serum albumin, 4.7 µg/mL linoleic acid, 1× insulin–transferrin–selenium, 50 µg/mL L-ascorbic acid-2-phosphate. 100 nM dexamethasone and 10 ng/mL TGF-β3 were added 72 hours after construct fabrication; the day 0 time point. Medium was exchanged every 3 or 4 days with 500 µL samples taken from three wells for biochemical analysis.

4.3.4 Dynamic compression application

Dynamic compressive loading was applied to constructs as outlined in Figure 4.1. Intermittent dynamic compression (DC) was carried out in an incubator-housed, custom-built compressive loading bioreactor, as shown in Figure 4.2. Medium used for loading was the same as that in which the constructs were maintained (enabling media sampling for sGAG release as described below). Axial compression was applied via impermeable platens using an electric linear actuator with 0.05 μm resolution (Zaber Technologies Inc., Vancouver, Canada). A 1000 g load cell (RDP Electronics Ltd, Wolverhampton, UK) positioned beneath the constructs sensed the load applied. The system was controlled and data logged using LabVIEW 7 control and data acquisition software (National Instruments Corp., Newbury, UK). The dynamic compression protocol consisted of 10% strain amplitude superimposed on a 0.01 N/construct preload at a frequency of 1 Hz employed for a period of 1 hour/day, 5 days/week.

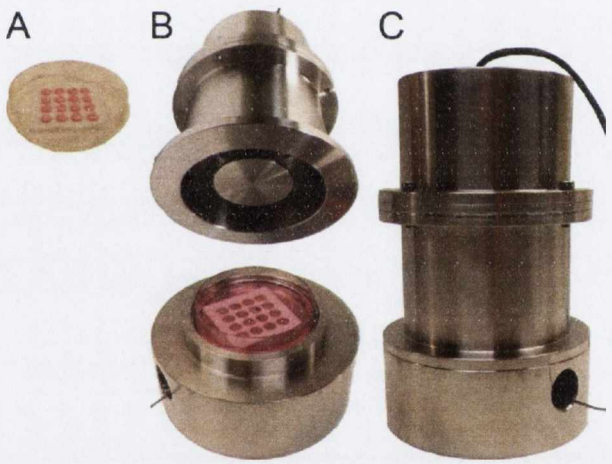

Figure 4.2: Bioreactor setup; A: Petri dish with 1.5mm thick PTFE spacer holding up to 16 constructs in position for loading. B: Constructs surrounded by medium in bioreactor for loading. C: Bioreactor during dynamic compression.

4.3.5 Mechanical testing and analysis of physical parameters

Constructs were mechanically tested (n = 4 or 5) in unconfined compression between impermeable platens using a standard materials testing machine with a 5N load cell (Zwick Roell Z005, Herefordshire, UK) as previously described (Buckley et al. 2009b). Briefly, constructs were kept hydrated through immersion in a PBS bath maintained at room temperature. A preload of 0.01N

was applied to ensure that the construct surface was in direct contact with the impermeable loading platens. Stress relaxation tests were performed consisting of a ramp displacement of 1 μm/s up to 10% strain, which was maintained until equilibrium was reached (~30 min.). This was followed by a dynamic test where cyclic strain amplitude of 1% (10%-11% total strain) was applied for 10 cycles at 1 Hz.

4.3.6 Biochemical content

The biochemical content of constructs (n = 3 or 4) was assessed at each time point. Constructs were cored using a 3 mm biopsy punch; the wet mass of each component recorded and the construct frozen at -85 °C for later analyses. Samples were digested with papain (125 μg/ml) in 0.1 M sodium acetate, 5 mM L-cysteine HCl, 0.05 M EDTA, pH 6.0 (all Sigma–Aldrich) at 60°C under constant rotation for 18 hours. DNA content was quantified using the Hoechst Bisbenzimide 33258 dye assay as previously described (Kim et al. 1988). The proteoglycan content was estimated by quantifying the amount of sulphated glycosaminoglycan (sGAG) in constructs using the dimethylmethylene blue dye-binding assay, with a shark chondroitin sulphate standard. Total collagen content was determined through measurement of the hydroxyproline content (Kafienah and Sims 2004). A hydroxyproline-to-collagen ratio of 1:7.69 was used (Ignat'eva et al. 2007). Samples of cell culture medium taken for analysis at each media exchange (n = 3) were analysed for sGAG secreted to the media. Total media volume was accounted for and the data is presented as the average sGAG released into the media per construct.

4.3.7 Histology and immunohistochemistry

Constructs (n = 2) were fixed in 4% paraformaldehyde, wax embedded and sectioned at 5 μm to produce a cross section perpendicular to the disc face. Sections were stained for sGAG with 1% alcian blue 8GX in 0.1M HCl, and for collagen with picro-sirius red. The deposition of collagen type I and type II was identified through immunohistochemistry. Briefly, sections were quenched of peroxidase activity, rinsed with PBS before treatment with chondroitinase ABC in a humidified environment at 37°C. Slides were rinsed with PBS and non-specific sites were blocked with goat serum. Sections were then incubated overnight at 4°C with the primary antibody; mouse

monoclonal collagen type I antibody (1:400; 1.4 mg/mL) or mouse monoclonal anti-collagen type II (1:100; 1 mg/mL). After washing in PBS, sections were incubated for 1 hour in the secondary antibody; anti-mouse IgG biotin antibody produced in goat (1:400; 1 mg/mL). Colour was developed using the Vectastain ABC reagent followed by exposure to peroxidase DAB substrate kit. Negative and positive controls of porcine ligament and cartilage were included for each batch.

4.3.8 Statistical analysis

The study was repeated twice. Unless otherwise stated, results presented are from a single replicate which is representative of both in terms of the main study findings. Statistics were performed using MINITAB 15.1 software package (Minitab Ltd., Coventry, UK). Groups were analysed for significant differences using a general linear model for analysis of variance with factors of group, construct region, growth factor supplementation, dynamic compression condition and interactions between these factors examined. Tukey's test for multiple comparisons was used to compare conditions. Significance was accepted at a level of $p \leq 0.05$. Numerical and graphical results are presented as mean ± standard deviation.

4.4 Results

Differences in construct physical parameters were observed across groups (Table 4.1). Removal of growth factor at day 21 had no effect on construct mass, while loading did have a significant effect with FS and DDC in both studies having greater wet weights than constructs receiving dynamic compression from day 0 (CDC) at days 21 ($p<0.05$) and 42 ($p<0.0001$). Loading condition had an effect on thickness with continuous dynamic compression (CDC) resulting in inhibition of axial swelling ($p<0.0001$).

Table 4.1: Construct physical parameters of wet weight (mg), diameter (mm), thickness (mm), volume (μL), and density (kg/m^3).

	Day 0	*Day 21*		*Day 42*					
				Transient TGF-β3			*Continuous TGF-β3*		
		FS+	*CDC+*	*FS-*	*DDC-*	*CDC-*	*FS+*	*DDC+*	*CDC+*
Wet weight [mg]	42.8±4.9	60.3±1.5[a,c]	51.4±2.9[a]	60.2±2.0[a,c]	59.9±1.8[a,c]	52.5±2.0[a]	62.0±1.4[a,c]	60.9±1.4[a,c]	52.4±4.2[a]
Diameter [mm]	5.024±0.043	5.046±0.038	5.001±0.030	5.029±0.048	5.118±0.070	5.063±0.052	5.195±0.093[a,c]	5.103±0.063	5.030±0.049
Thickness [mm]	2.395±0.124	3.063±0.073[a,c]	2.792±0.028[c]	2.979±0.132[a,c]	2.990±0.055[a,c]	2.664±0.090[a]	3.112±0.043[a,c]	3.027±0.097[a,c]	2.746±0.045[a]
Volume [μL]	47.29±1.91	61.32±2.05[a,c]	54.87±1.20[a]	59.14±3.22[a,c]	61.05±2.43[a,c]	53.73±2.89[a]	65.44±2.23[a,c]	62.69±2.63[a,c]	55.16±1.03[a]
Density [kg/m^3]	867.8±55.2	983.1±22.1[a]	936.3±53.0	1023.8±21.9[a]	996.5±38.2[a]	980.7±29.3[a]	956.6±47.5	975.4±24.4[a]	986.1±36.8[a]

a: $p<0.05$ vs. day 0; b: $p<0.05$ vs. day 21; c: $p<0.05$ vs. corresponding CDC group.

Staining of construct sections for sGAG and collagen revealed a steady accretion of positive staining for all conditions at each time point beyond day 0. At day 21, stronger straining for proteoglycan, collagen and specifically collagen type II was observed in the free swelling constructs (FS+) when compared to the continuous dynamic compression constructs (CDC+) (Figure 4.3). This staining was especially prevalent in the pericellular region. At day 42, both FS constructs and constructs subjected to delayed dynamic compression (DDC) exhibited more intense staining for proteoglycan than continuously loaded (CDC) constructs (Figure 4.4). Pericellular staining for collagen was evident in all regimes. A significant amount of extra-cellular matrix (ECM) staining was evident in FS and DDC constructs which was not present in the CDC constructs (Figure 4.4).

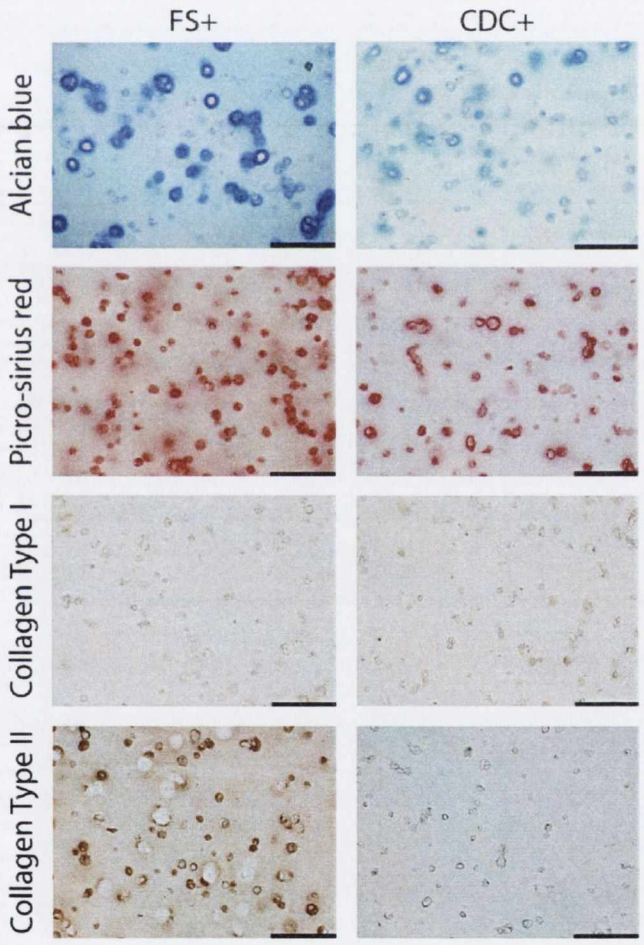

Figure 4.3: Histological and immunohistochemical analysis for FS+ and CDC+ at day 21 with alcian blue staining for sulphated proteoglycan (top), picro-sirius red for collagen (second from top), collagen type I immunochemistry (third from top) and collagen type II immunohistochemistry (bottom). Representative images taken at the centre of each construct section. Scale bar: 100 μm.

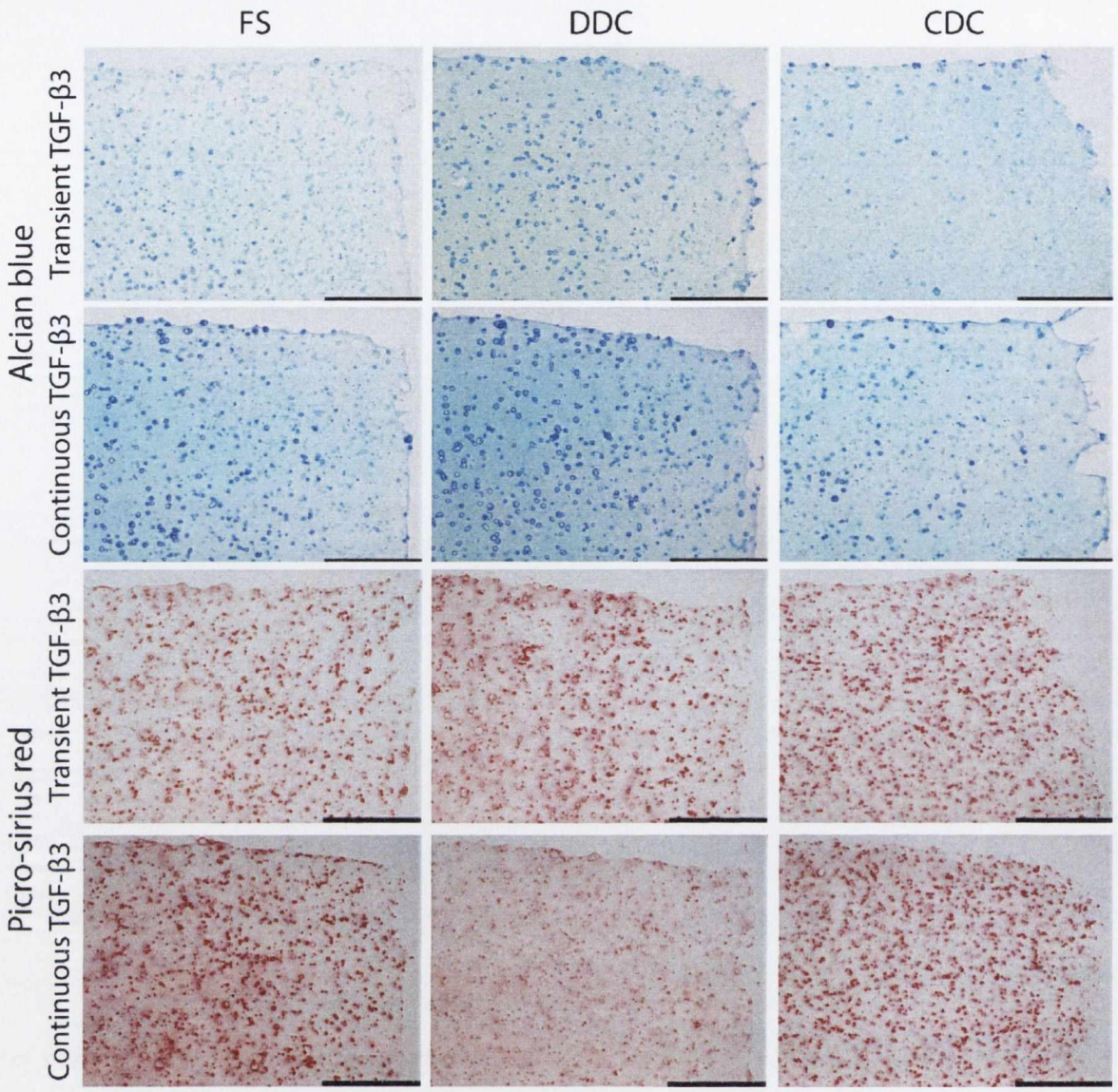

Figure 4.4: Histological analysis of constructs at day 42 with alcian blue staining for sulphated proteoglycan (top) and picro-sirius red for collagen (bottom). FS: free-swelling. DDC: delayed dynamic compression, initiated on day 21. CDC: continuous dynamic compression, initiated on day 0. Representative images taken of approx. $^1/_4$ of each construct section. Scale bar: 500 μm.

Immunohistochemistry indicated a strong presence of collagen type II in both the pericellular matrix (PCM) and the ECM of both FS and DDC groups for either TGF-β3 supplementation condition. Weaker collagen type II staining was evident in the CDC group with transient TGF-β3. For FS groups, the core region exhibited more intense staining than the annular region (Figure 4.5). A more intense collagen type II staining was evident in constructs receiving continued TGF-β3 supplementation when compared to transient supplementation. These same constructs stained weakly for collagen type I, while FS and DDC constructs receiving transient TGF-β3

supplementation exhibited stronger staining for collagen type I, especially in the annular region (Figure 4.5).

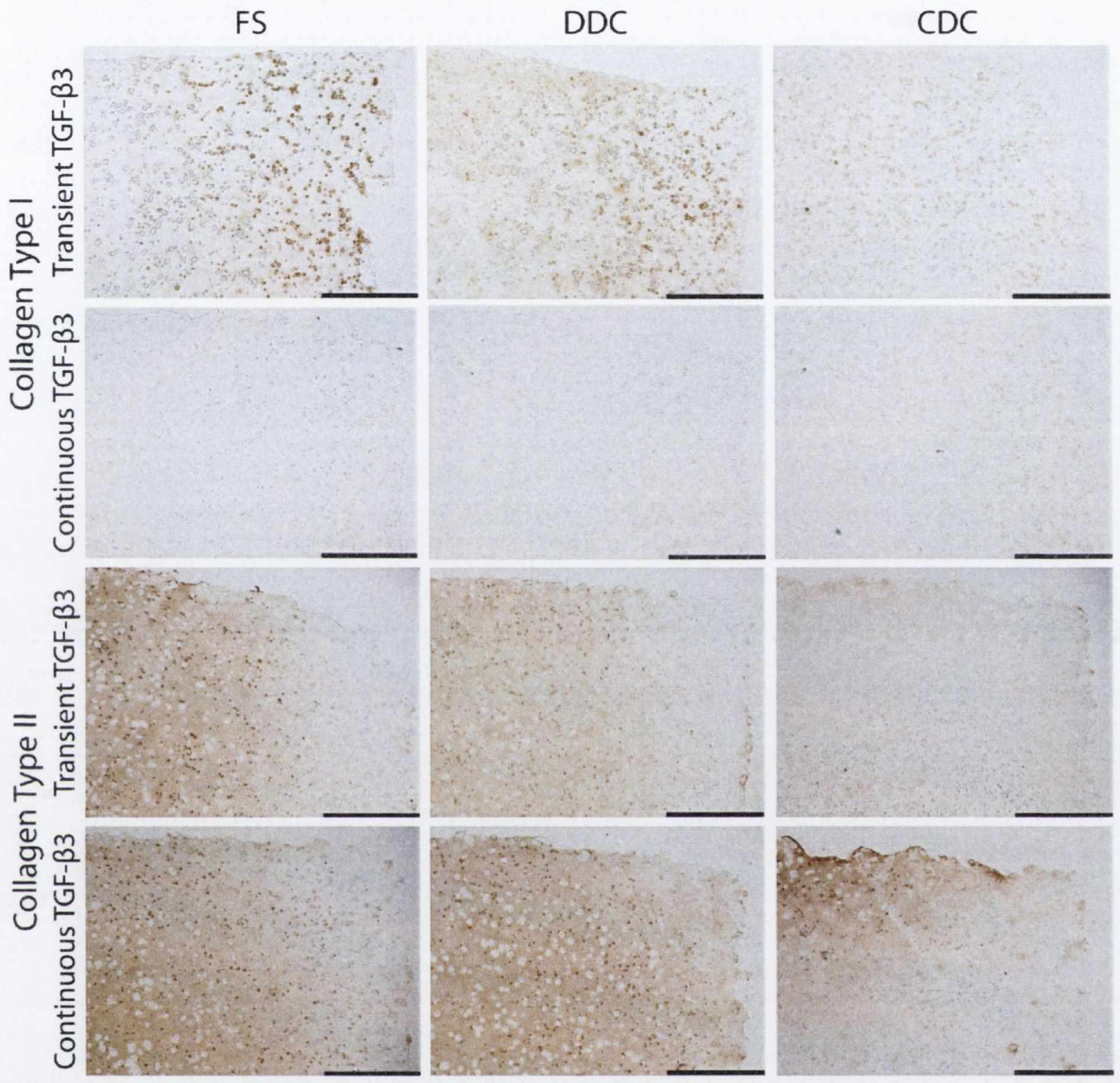

Figure 4.5: Immunohistochemical analysis of constructs at day 42 with collagen type I immunohistochemistry (top) and collagen type II immunohistochemistry (bottom). FS: free-swelling. DDC: delayed dynamic compression, initiated on day 21. CDC: continuous dynamic compression, initiated on day 0. Representative images taken of approx. $^1/_4$ of each construct section. Scale bar: 500 µm.

There was a net increase in DNA content indicating cell proliferation at day 42 in groups receiving continued TGF-β3 supplementation (p=0.0059); resulting in significantly higher DNA content than groups where TGF-β3 was removed (transient TGF-β3; p<0.0001; Figure 4.6A). sGAG content

increased over day 0 values for all experimental conditions ($p<0.05$; Figure 4.6B & C, and Figure 4.8B). Removal of TGF-β3, loading condition and construct region all had a significant effect on sGAG accumulation ($p<0.001$). Regardless of TGF-β3 supplementation condition, both FS and DDC constructs had significantly greater sGAG content than CDC constructs ($p<0.002$; Figure 4.6B & C). At day 42, sGAG accumulation (% wet weight) was significantly greater in constructs continuously supplemented with TGF-β3 (Figure 4.6B). Following the removal of TGF-β3 (transient TGF-β3), delayed dynamic compression (DDC-) resulted in a non-significant trend towards higher sGAG/DNA in comparison to free-swelling conditions (FS-; $p=0.067$; Figure 4.6C). This difference was significant in the construct cores ($p<0.05$; Figure 4.7B & C). There was no difference between FS+ and DDC+ groups at day 42.

Collagen content increased with time for all constructs ($p<0.0001$; Figure 4.6D& E). As with sGAG, loading condition and construct region were seen to have a significant effect on collagen content; however TGF-β3 supplementation had no effect on collagen accumulation (% wet weight). Collagen accumulation was no different in FS or DDC constructs; however both FS- and DDC- constructs contained higher collagen/DNA than the corresponding CDC- constructs ($p<0.05$; Figure 4.6E, transient TGF-β3). Increases in collagen accumulation were greatest in the construct core ($p<0.0002$; Figure 4.7D & E).

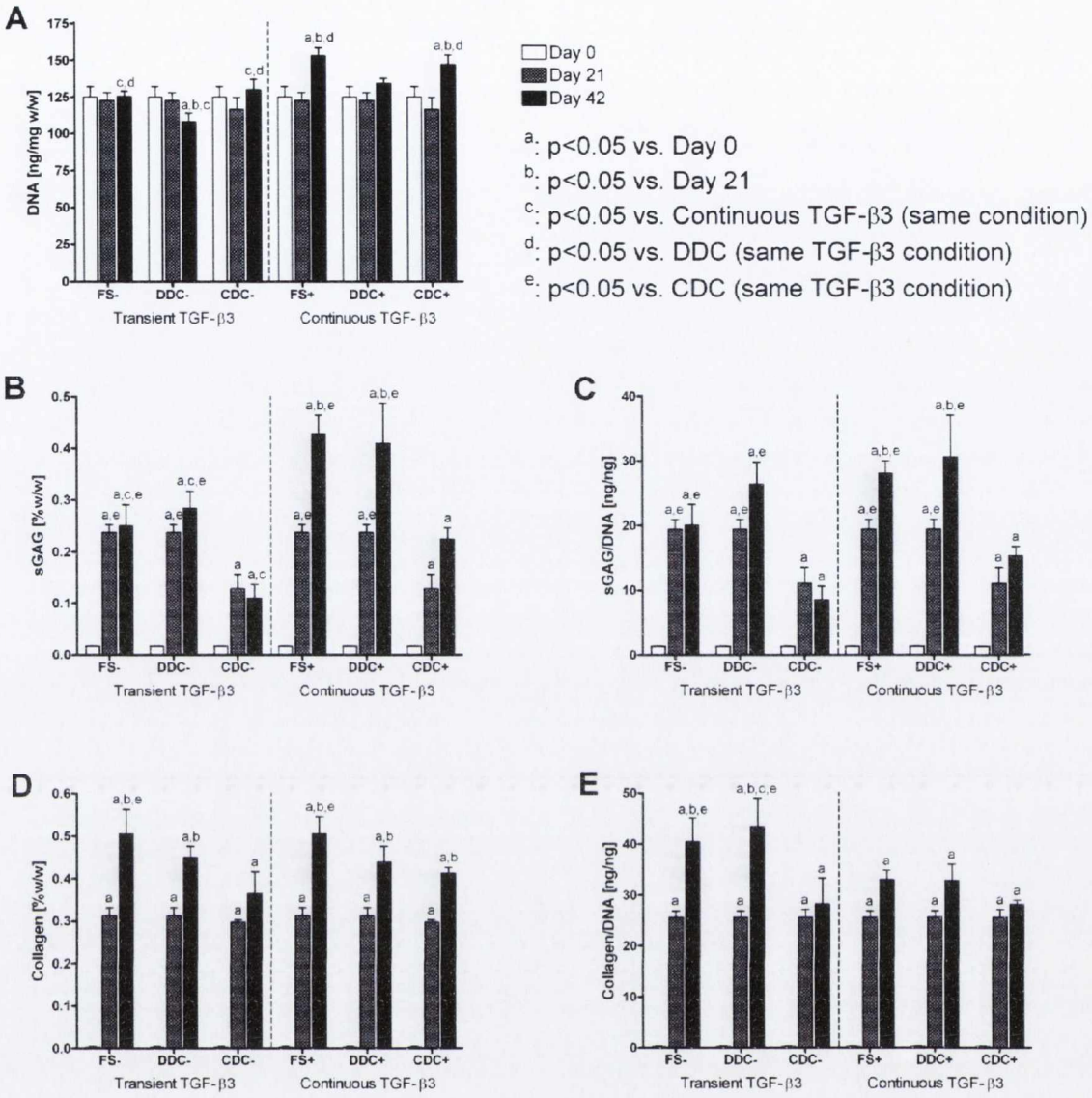

Figure 4.6: Total biochemical composition of constructs for transient TGF-β3 (left) and continuous TGF-β3 (right). A: DNA content (ng/mg w/w); B: sGAG content (%w/w); C: sGAG/DNA (ng/ng); D: Collagen content (%w/w); and E: Collagen/DNA (ng/ng).

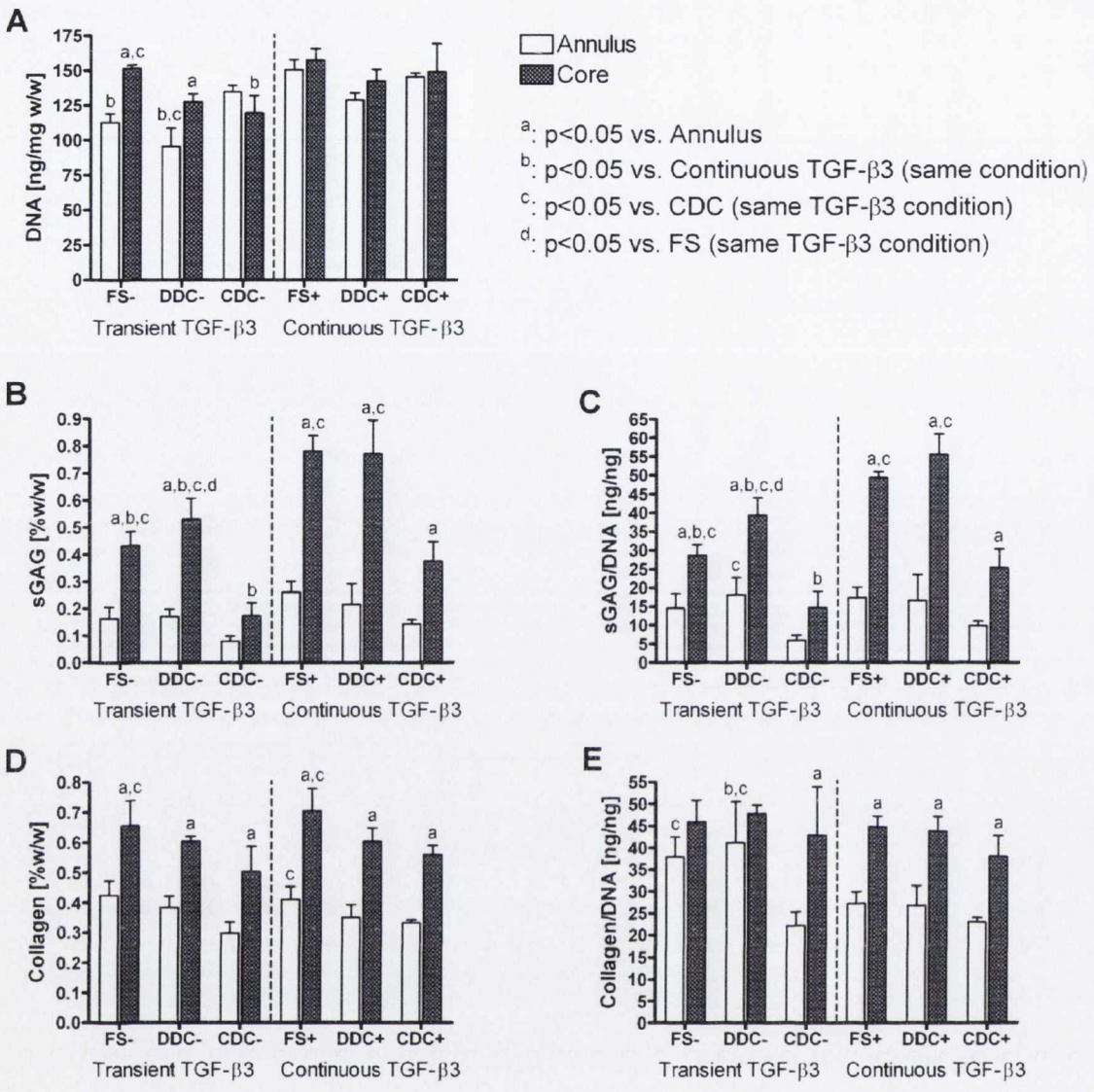

Figure 4.7: Biochemical composition of annulus and core at day 42 for constructs with transient TGF-β3 (left) and continuous TGF-β3 (right). A: DNA content (ng/mg w/w); B: sGAG content (%w/w); C: sGAG/DNA (ng/ng); D: Collagen content (%w/w); and E: Collagen/DNA (ng/ng).

sGAG secreted to the media increased with time in culture with significantly more sGAG secreted for all experimental conditions from day 21 to day 42 than day 0 to day 21 ($p<0.001$; Figure 4.8A). Significantly more sGAG was secreted to the media from samples where TGF-β3 supplementation ceased at day 21 (transient TGF-β3), than constructs receiving continuous supplementation; $p<0.0001$ (Figure 4.8A). Both FS and DDC constructs under both TGF-β3 conditions secreted significantly more sGAG to the medium than the corresponding constructs compressed from day 0 (CDC; $p<0.05$; Figure 4.8).

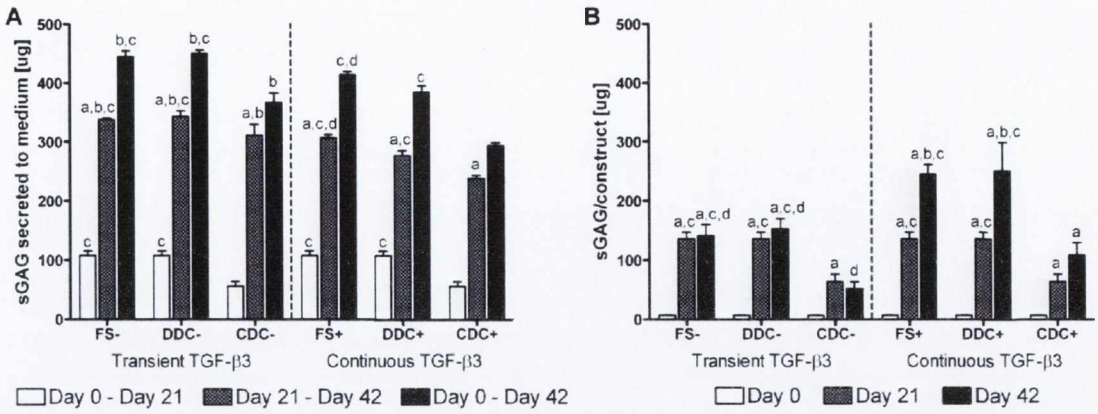

Figure 4.8: A: sGAG secreted per construct to medium over the experimental time period (μg). a: $p<0.05$ vs. Day 0-Day 21; b: $p<0.05$ vs. continuous TGF-β3; c: $p<0.05$ vs. CDC (same TGF-β3 condition); d: $p<0.05$ vs. DDC (same TGF-β3 condition). B: sGAG retained per construct (μg). a: $p<0.05$ vs. day 0; b: $p<0.05$ vs. day 21; c: $p<0.05$ vs. CDC; d: $p<0.05$ vs. continuous TGF-β3. Medium data cannot be matched to individual construct data as media is pooled during bioreactor culture. Data presented in this figure is pooled from multiple experimental runs.

The compressive equilibrium modulus increased with time for all conditions relative to day 0 ($p<0.05$; Figure 4.9A). On day 42, the compressive equilibrium modulus of FS- constructs reached a value of 16.17±1.21 kPa; significantly greater than either continuous dynamic compression (CDC) group. There were no differences between FS and DDC constructs. Increases in the 1Hz dynamic modulus with time were only seen at day 42 for FS and DDC constructs ($p<0.05$; Figure 4.9B). The 1Hz dynamic modulus was not different for both FS and DDC constructs, however both were significantly greater than CDC constructs ($p<0.0001$). The 1Hz dynamic modulus was effected by TGF-β3 removal, with continued TGF-β3 supplementation resulting in greater dynamic moduli than transient TGF-β3 supplementation ($p=0.031$).

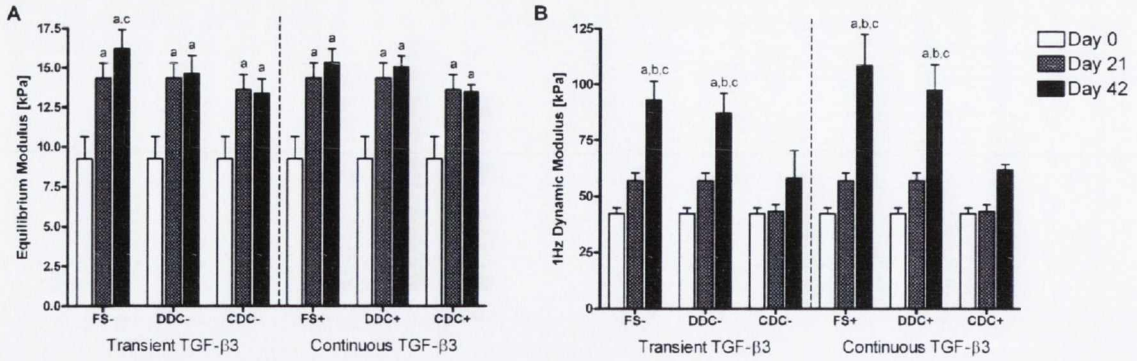

Figure 4.9: Construct mechanical properties. A: Compressive equilibrium modulus and B: 1Hz dynamic modulus for transient TGF-β3 (left) and continuous TGF-β3 (right). a: $p<0.05$ vs. Day 0; b: $p<0.05$ vs. Day 21; c: $p<0.05$ vs. CDC (same TGF-β3 condition).

4.5 Discussion

The chondrogenic potential of MSCs appears to be regulated by their mechanical environment, but this response depends on the stage of chondrogenesis. In the presence of TGF-β3, continuous dynamic compression was observed to inhibit chondrogenesis, corroborating our previous finding (Thorpe et al. 2008). By delaying the application of dynamic compression for 3 weeks until chondrogenic differentiation had been initiated (as evidenced by histological and immunohistochemical staining for proteoglycan and type II collagen), this inhibition of chondrogenesis in response to dynamic compression was not observed. There are a number of possible explanations for this temporal response to dynamic compression. The phenotype of the MSCs on initiation of dynamic compression at day 0 (CDC) and day 21 (DDC) may be one such explanation; as a greater level of chondrogenic differentiation may result in a more anabolic response to loading similar to that of chondrocytes (Palmoski and Brandt 1984; Sah et al. 1989; Kim et al. 1994; Buschmann et al. 1995; Grodzinsky et al. 2000; Mauck et al. 2000; Davisson et al. 2002; Mauck et al. 2002). Mouw *et al.* (2007) demonstrated that dynamic compression applied at day 8 resulted in a decrease in aggrecan gene expression in MSCs; however when loading was applied at day 16, an increase in chondrogenic gene expression was observed. Related to this, the different levels of matrix accumulation at day 0 and day 21 could also be responsible for the

differential response to dynamic compression in CDC and DDC constructs. The level of PCM and ECM accumulation would affect both the local mechanical stimuli developed during loading (Guilak and Mow 2000), and biochemical signalling from the ECM. For example, in a study of dynamically compressed single chondrocytes and chondrons (chondrocytes with attached PCM), the presence of a PCM regulated the response to mechanical compression (Wang et al. 2009b). It has also been demonstrated that the stiffness of isolated chondrons seeded into agarose is higher than that of the surrounding extracellular agarose environment, leading to stress shielding of the chondrocytes during loading (Knight et al. 1998).

Akin to our previous studies, sGAG and collagen accumulation in MSC laden constructs was greatest in the construct core (Thorpe et al. 2008; Buckley et al. 2010). This may be due to a lower oxygen tension in the construct core, as low oxygen tension has been shown to promote chondrogenesis in chondrocyte seeded constructs (Clark et al. 1991; Obradovic et al. 1999; Grimshaw and Mason 2000; Obradovic et al. 2000). Another explanation for this spatial difference in matrix deposition is that a large percentage of the sGAG produced in the annulus is simply secreted into the culture medium. Indeed, significant levels of sGAG were measured in the culture media. Similar levels of sGAG release to the media from MSC seeded constructs have been observed in other studies (Babalola and Bonassar 2010; Li et al. 2010a). Interestingly, while sGAG accumulation at day 42 was greater in constructs continuously maintained in TGF-β3; sGAG secreted to the culture medium was greatest in constructs transiently supplemented with TGF-β3. This suggests that continued supplementation of TGF-β3 encourages retention of sGAG within the construct. This may be due to an increase in type I collagen production following removal of TGF-β3, and possibly the formation of a collagen network less capable of retaining sGAG within the construct. Intense collagen type II staining was seen throughout the centre of both FS+ and DDC+ constructs continuously supplemented with TGF-β3. However when TGF-β3 supplementation ceased at day 21, a slight reduction in type II staining was evident by day 42; with significant type I staining observed, particularly in the annular region. Future studies will investigate whether this in indicative of a failure to achieve a stable chondrogenic phenotype following 21 days of culture in the presence of TGF-β3.

sGAG release into the media was lower in the continuous dynamic compression (CDC) groups compared to FS controls. This demonstrates that the inhibition of chondrogenesis observed due to CDC from day 0 is not simply due to greater sGAG release into the media due to loading. Similarly the application of delayed dynamic compression did not lead to increases in sGAG release to the media, indicating that mechanical loading does not negatively influence sGAG retention in these immature chondrogenically primed constructs.

The application of 1 hour of daily dynamic compression to MSC-laden constructs following three weeks of TGF-β3 induced differentiation did not enhance overall levels of cartilage matrix accumulation within the constructs. While a trend towards higher levels of sGAG/DNA was observed in constructs subjected to delayed dynamic compression, these differences were not significant. We were therefore unable to corroborate the initial hypothesis of this study. A more complex picture emerges from the spatial analysis of sGAG accumulation within constructs subjected to delayed dynamic compression. In the construct core, dynamic compression was seen to have a small positive effect on sGAG accumulation following the withdrawal of TGF-β3. No statistically significant differences were observed between construct annuli. These differences may be due in part to spatial variations in the mechanical signals developed in cell seeded agarose hydrogels in response to dynamic unconfined compression (Huang et al. 2004a; Mauck et al. 2007). Even in a homogenous tissue or hydrogel, MSCs in the annulus of the construct will experience higher levels of fluid flow in response to dynamic unconfined compression (Mauck et al. 2007). Furthermore, as greater tissue maturation is observed in the construct core compared to the annulus, this could potentially alter cell-level deformation and lead to increased fluid pressurisation within the construct core due to dynamic compression. Such hydrostatic fluid pressure is known to promote chondrogenesis of MSCs (Angele et al. 2003; Miyanishi et al. 2006a; Miyanishi et al. 2006b; Wagner et al. 2008). However, uncoupling the exact role of mechanical signals in regulating MSC chondrogenesis in this model system is further complicated by spatial gradients in oxygen, nutrients and growth factors that will develop within the engineered construct.

When TGF-β3 was continuously maintained in the culture media, no significant differences in spatial sGAG accumulation were observed in DDC constructs compared to FS controls. The

finding that MSC response to loading depends on TGF-β3 supplementation agrees with the observation of Li *et al.* (2010a), who demonstrated greater differences between loaded and FS constructs at 0 or 1 ng/mL TGF-β1 concentrations. They suggest that surface motion superimposed on 10% dynamic compression promotes chondrogenesis of MSCs seeded onto fibrin-polyurethane scaffolds through the TGF-β pathway by up-regulating TGF-β gene expression and protein synthesis. This may explain why delayed dynamic compression with the continued supplementation of TGF-β3 did not enhance sGAG accumulation in construct cores in the present study; as there may have been an overabundance of the growth factor above what was required to stimulate maximal chondrogenesis, rendering any TGF-β production stimulated through dynamic compression ineffective.

Large increases in the equilibrium modulus of any of the experimental groups were not observed despite increases in total sGAG and collagen within the constructs. This may be due to the inhomogeneous accumulation of matrix; with the majority of sGAG and collagen accumulation in the construct core. Inhomogeneous tissue development has been shown to influence the apparent mechanical properties of engineered cartilaginous tissue (Kelly and Prendergast 2004). In addition, an important finding of this study is that the application of delayed dynamic compression did not lead to increases in the mechanical properties of the engineered tissue in either the continuous or transient TGF-β supplementation groups. This is in contrast to the results of studies subjecting chondrocytes seeded in agarose to delayed dynamic compression following pre-culture in a chemically defined medium supplemented with TGF-β (Lima et al. 2007). There are a number of possible explanations for this finding. If cell mediated reorganisation of the engineered tissue in response to dynamic compression is responsible for the superior mechanical properties in chondrocyte seeded hydrogels, it may be that MSCs are unable to actively remodel their ECM in a similar manner in response to only 1 hour of daily dynamic compression. Perhaps longer durations of loading are necessary for improvements in the mechanical properties of cartilaginous constructs engineered using MSCs. A recent study using bovine MSCs in a similar agarose model system demonstrated that 4 hours of delayed dynamic compression is necessary for improvements in tissue mechanical properties to be observed (Huang et al. 2010a). Another possible explanation is that

other matrix molecules not investigated here such as cartilage oligomeric matrix protein (COMP), which may play a key role in determining the functional properties of engineered cartilage tissues (Ng et al. 2009b), may be differentially expressed by MSCs and chondrocytes in response to loading. Alternatively, it may simply be that the beneficial effects of dynamic compression on the mechanical properties of tissue engineered cartilage will only become apparent once a certain minimum threshold of ECM is accumulated. Given that the total biochemical content of the cartilaginous tissues generated in this study are significantly lower than that observed with primary chondrocytes cultured under similar conditions (Lima et al. 2007; Byers et al. 2008), future studies will investigate if altered culture conditions and MSC sources will lead to greater ECM accumulation prior to the application of dynamic compression.

To conclude, in the presence of TGF-β3, the application of 10% dynamic compression for 1 hour per day from day 0 to day 42 inhibited chondrogenesis. In contrast, the application of 1 hour of daily dynamic compression from day 21 to day 42 following TGF-β3 induced differentiation did not inhibit chondrogenesis of MSCs; however neither did it lead to any increases in the functional properties of the tissue. In the context of cartilage tissue engineering, this study also demonstrated superior chondrogenesis in MSC-laden constructs maintained in TGF-β3 for the duration of the 42 day culture period; suggesting that continued supplementation with TGF-β3 at supra-physiological levels is a more potent chondrogenic stimulus than the dynamic compression regime utilised in this study. It has yet to be established if this is true for other types of mechanical loading regimes. Finally, this study could also be viewed as an *in vitro* model of how MSC-laden constructs might respond to a load bearing environment. In this case, allowing for a period of TGF-β induced differentiation produced a tissue that responded more positively to a load bearing environment.

Chapter 5 Engineering zonal cartilage using mesenchymal stem cells by controlling the local environment through the depth of the developing tissue

For the purposes of this thesis, the study presented is adapted from the original article which is currently under review.

The computational models presented in this chapter were contributed as a personal communication from Mr Thomas Nagel.

5.1 Abstract

The objective of this study was to engineer a cartilaginous construct with a native-like zonal composition by controlling both the oxygen tension and mechanical environment thorough the depth of agarose hydrogels seeded with mesenchymal stem cells (MSCs). The bottom halves of these constructs were radially confined and subjected to dynamic compression. Computational models indicated that confinement reduced oxygen levels in the bottom of the construct, and when combined with dynamic compression increased both the pore pressure in the bottom and tensile strain across the top of the engineered construct. At lower densities (20×10^6 cells/mL), confinement increased sulphated glycosaminoglycan (sGAG) accumulation in the bottom of constructs, leading to depth-dependant changes in compressive equilibrium properties mimicking native cartilage. Furthermore, confinement coupled with dynamic compression increased collagen accumulation in the top of the construct such that a depth-dependent gradient in sGAG and collagen content similar to normal articular cartilage was achieved. At higher cell seeding densities (50×10^6 cells/mL), zonal gradients mimicking articular cartilage were not achieved, although dynamic compression did enhance both sGAG accumulation and mechanical function. In conclusion, by controlling the

environment through the depth of developing constructs, tissues with zonal gradients analogous to normal articular cartilage can be engineered using MSCs.

5.2 Introduction

As demonstrated in Chapter 3, the application of dynamic compression inhibits chondrogenesis of MSC seeded agarose constructs. However, with the initiation of dynamic compression post TGF-β3 mediated differentiation in unloaded conditions, a more positive response to dynamic compression is observed (Chapter 4). This dynamic compression regime will now be used to engineer an MSC seeded agarose construct with depth dependent composition somewhat similar to native articular cartilage.

Adult articular cartilage consists of three separate structural zones; the superficial tangential, middle and deep zones. This depth dependent composition and organisation is fundamental to the normal physiological function of articular cartilage as outlined in Section 2.1.1 (Setton et al. 1993; Krishnan et al. 2003). Not only does cell morphology and arrangement change with depth, but each zone has distinct extra-cellular matrix (ECM) composition, architecture and mechanical properties. The dominant load carrying structural components of the ECM are collagen (~75% tissue by dry weight) and proteoglycan (20% – 30% tissue by dry weight), the concentrations of which vary with depth from the articular surface (Brocklehurst et al. 1984; Mow and Hung 2001; Mow and Guo 2002). Collagen content is highest in the superficial zone, and decreases by ~20% in the middle and deep zones (Mow and Hung 2001; Mow and Guo 2002). Proteoglycan content is inversely related to the collagen content, being lowest at the surface and increasing by as much as 50% into the middle and deep zones (Brocklehurst et al. 1984; Mow and Guo 2002). The zonal composition and structural organisation of the ECM result in biomechanical properties which vary through the tissue depth; such that the compressive modulus increases from the superficial zone to the deep zone (Schinagl et al. 1997; Laasanen et al. 2003), while the tensile modulus decreases from the superficial surface to the deep zone (Akizuki et al. 1986).

An on-going challenge in the field of cartilage tissue engineering is the attainment of this stratified zonal structure. Classical tissue engineering approaches focus primarily on forming homogeneous tissues by embedding chondrocytes or stem cells in various scaffolds, and do not mimic the organised zonal architecture of articular cartilage. One approach toward this aim is to utilise different cell types, such as zonal chondrocytes in specific regions of the construct (Kim et al. 2003; Klein et al. 2003; Sharma et al. 2007). It has been shown that chondrocytes from different zones demonstrate different biosynthetic activities (Klein et al. 2003; Sharma et al. 2007). Layering such zonal chondrocytes in a photo-polymerising hydrogel has been shown to result in increased sGAG accumulation in the bottom of the construct when compared to the top (Sharma et al. 2007). However collagen content was also significantly higher in the bottom (Sharma et al. 2007), resulting in a gradient unlike that found in articular cartilage. An alternative approach to engineering zonal cartilage is to vary biomaterial properties such as pore size (Woodfield et al. 2005), stiffness (Ng et al. 2005; Ng et al. 2006) or composition (Hwang et al. 2007; Nguyen et al. 2011a; Nguyen et al. 2011b) through the depth of the scaffold or hydrogel. For example, combining layers of 2% and 3% agarose leads to zonal differences in the initial mechanical properties of the construct, however chondrocyte matrix elaboration in such bi-layered constructs was inferior to that in uniform 2% agarose (Ng et al. 2005). Dynamic compressive strain applied to the same constructs did increase overall matrix elaboration and compressive stiffness; however this occurred predominantly in the 2% top layer, such that it became the stiffest region in compression (Ng et al. 2006). A combined approach, varying both the cell source and mechanical properties has also been explored resulting in gradients in sGAG and compressive modulus which were qualitatively similar to those in articular cartilage, although collagen accumulation remained lower in the superficial region (Ng et al. 2009a). While promising, there are potential limitations associated with such an approach including the development of a distinct boundary between gel layers which could delaminate as a result of shear stress (Lee et al. 2007). Additionally, isolation of chondrocytes from separate zones of articular cartilage can be difficult, particularly in damaged and diseased human tissue where the zonal differences are less distinct and potential biopsies are limited in size.

An alternative strategy to engineering zonal cartilage is to attempt to recapitulate aspects of the micro-environment which may be responsible for the creation of depth-dependent properties in articular cartilage during development and maturation. A prerequisite for such an approach is the identification of a cell source phenotypically similar to that present during articular cartilage development. Bone marrow derived mesenchymal stem cells (MSCs) may represent such a cell type (Hunziker et al. 2007; Kelly and Jacobs 2010), and are not restricted by ethical concerns associated with embryonic stem cells. MSCs have been shown to respond to environmental factors known to regulate the chondrocyte phenotype. For example, low oxygen tension, characteristic of avascular articular cartilage, has been shown to enhance chondrogenesis of MSCs (Kanichai et al. 2008; Krinner et al. 2009). Culture of MSCs seeded into agarose constructs in a 5% oxygen environment has been shown to result in increased sGAG and collagen accumulation compared to normoxic conditions (Meyer et al. 2010). The distribution of ECM was found to be inhomogeneously accumulated throughout such constructs (Meyer et al. 2010), suggesting that gradients in oxygen concentration could modulate ECM synthesis locally in the construct. In addition to oxygen, it has long been proposed that mechanical signals guide the differentiation of mesenchymal stem cells (Pauwels 1960; Carter et al. 1988; Prendergast et al. 1997). Hydrostatic pressure has been shown to promote a chondrogenic phenotype (Angele et al. 2003; Miyanishi et al. 2006b) and enhance collagen and sGAG accumulation for agarose encapsulated MSCs (Meyer et al. 2011). Dynamic compressive strain has been shown to promote MSC chondrogenesis (Angele et al. 2004; Huang et al. 2004a; Campbell et al. 2006; Mauck et al. 2007; Kisiday et al. 2009) and positively modulate the functional development of cartilaginous constructs engineered using MSCs encapsulated in agarose hydrogels (Huang et al. 2010a). It has also been demonstrated that intermittent cyclic tensile strain applied to MSC seeded constructs increases collagen accumulation (Connelly et al. 2010; Baker et al. 2011).

The objective of this study was to engineer a cartilaginous construct with native-like zonal composition using MSCs by controlling both the oxygen tension and mechanical environment thorough the depth of the developing tissue. In an attempt to create a gradient in oxygen tension through the depth of the construct mimicking that in normal articular cartilage (Zhou et al. 2004),

the bottom half of MSC seeded agarose constructs was radially confined to limit oxygen transport into this region of the construct. Furthermore, by subjecting these radially confined constructs to dynamic compression it is possible to modulate the mechanical environment throughout the depth of the tissue, with higher levels of fluid pressure in the bottom of the construct and greater strains across the top of the construct. It is hypothesized that such a depth dependent microenvironment will lead to the development of zonal cartilage tissues with a composition mimicking that of normal articular cartilage.

5.3 Methods

The commercial sources of reagents are listed in Appendix B.

5.3.1 Cell isolation and expansion

Porcine MSCs were isolated and maintained as previously described (Thorpe et al. 2010). Briefly, mononuclear cells were isolated from the femora of 4 month old porcine donors (~50 kg) within 2 hours of sacrifice and plated at 10×10^6 cells per 75cm^2 flask allowing colony formation. MSCs were maintained in high-glucose Dulbecco's modified eagles medium (4.5 mg/mL D-Glucose, 200 mM L-Glutamine; hgDMEM) supplemented with 10% foetal bovine serum (FBS), penicillin (100 U/mL)-streptomycin (100 µg/mL) and amphotericin B (0.25 µg/mL). Cultures were washed in Dulbecco's phosphate buffered saline (PBS) after 72 hrs. When passaged, MSCs were plated at 5×10^3 cells/cm^2 and expanded to passage two in a humidified atmosphere at 37 °C and 5% CO_2. Separate donors were used for each study.

5.3.2 Agarose hydrogel encapsulation and construct confinement

MSCs were suspended in defined chondrogenic medium (CM) consisting of hgDMEM supplemented with penicillin (100 U/mL)-streptomycin (100 µg/mL), 0.25 µg/mL amphotericin B, 100 µg/ml sodium pyruvate, 40 µg/mL L-proline, 1.5 mg/mL bovine serum albumin, 4.7 µg/mL linoleic acid, 1× insulin–transferrin–selenium, 50 µg/mL L-ascorbic acid-2-phosphate, 100 nM dexamethasone and 10 ng/mL TGF-β3. This cell suspension was mixed with agarose (Type VII) in

PBS at a ratio of 1:1 at approx. 40 °C, to yield a final agarose concentration of 2% and a cell density of either 20×10^6 cells/mL or 50×10^6 cells/mL. The agarose-cell suspension was cast between stainless steel plates, one of which was overlaid with a patterned PDMS layer, allowed cool to 21 °C for 30 min., and cored to produce cylindrical constructs (Ø6 mm × 4 mm thickness) which were patterned on one surface. Constructs remained patterned side up throughout culture and were maintained in approx. 1mL CM per 1×10^6 cells/day with medium exchanged every 3 or 4 days. Media was sampled at each exchange for biochemical analysis. Either at fabrication or at day 21 of culture, constructs were press-fitted into custom made PTFE confinement chambers (Fig 1) where they remained for the outstanding culture duration.

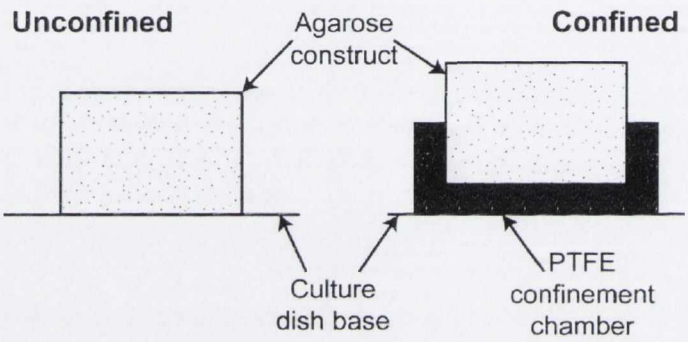

Figure 5.1: Constructs were press-fitted into custom made PTFE wells such that the bottom 2 mm of the construct thickness was confined.

5.3.3 Dynamic compression application

Dynamic compressive loading was applied as described previously (Thorpe et al. 2010) to constructs from days 21 to 42 of culture. Briefly, intermittent dynamic compression (DC) was carried out in an incubator-housed, dynamic compression bioreactor under strain control. The dynamic compression protocol consisted of 10% strain amplitude superimposed on a 1% pre-strain, with a 0.01 N per construct preload at a frequency of 1 Hz for 4 hours/day, 5 days/week.

5.3.4 Mechanical testing and analysis of physical parameters

On removal from culture, construct diameter and wet weight (ww) were measured. Constructs were mechanically tested (n = 3 or 4) in unconfined compression between impermeable platens using a standard materials testing machine (Bose Electroforce 3100; Bose Corporation, Gillingham, UK) as

previously described (Buckley et al. 2009b). A preload of 0.01N was applied to ensure that the construct surface was in direct contact with the impermeable loading platens. Stress relaxation tests were performed consisting of a ramp displacement of 0.025% /s up to 10% strain, which was maintained until equilibrium was reached (~30 min.). This was followed by a dynamic test where cyclic strain amplitude of 1% (10% – 11% total strain) was applied for 10 cycles at 1Hz. Samples were subsequently sliced into top and bottom regions using a custom built rig and each region was tested simultaneously on separate, randomly assigned material testing machines (Zwick Roell Z005; Zwick Testing Machines Ltd., Herefordshire, UK) as above.

5.3.5 Biochemical constituents

The biochemical content of constructs (n = 3 or 4) was assessed at each time point as previously described (Thorpe et al. 2010). After mechanical testing of top and bottom construct regions, the wet weight (ww) of each was recorded and the construct frozen at -85 °C for later analyses. Samples were digested with papain (125 μg/mL) in 0.1 M sodium acetate, 5 mM L-cysteine HCl, 0.05 M EDTA, pH 6.0 at 60 °C under constant rotation for 18 hours. DNA content was quantified using the Hoechst Bisbenzimide 33258 dye assay as previously described (Kim et al. 1988). The sulphated glycosaminoglycan (sGAG) content was quantified using the dimethylmethylene blue dye-binding assay, with a shark chondroitin sulphate standard. Total collagen content was determined by measuring orthohydroxyproline via the dimethylaminobenzaldehyde and chloramine T assay (Kafienah and Sims 2004). A hydroxyproline-to-collagen ratio of 1:7.69 was used (Ignat'eva et al. 2007). Cell culture media was analysed for sGAG and collagen secreted.

5.3.6 Histology and immunohistochemistry

Constructs (n = 2) were fixed in 4% paraformaldehyde, wax embedded and sectioned at 5 μm to produce a cross section perpendicular to the disc face. Sections were stained for sGAG with 1% alcian blue 8GX in 0.1 M HCl, and for collagen with picro-sirius red. The deposition of collagen type I and type II was identified through immunohistochemistry (Thorpe et al. 2010). Negative and positive controls of porcine ligament and cartilage were included for each batch.

5.3.7 Theoretical prediction of mechanical environment within agarose constructs

This portion of the study was contributed as a personal communication from Mr Thomas Nagel. To estimate the effect of semi-confinement on the mechanical environment within the construct, pore pressure and maximum principal strain were predicted for day 0 constructs. The loading protocol of the bioreactor was simulated with material properties derived from a sample specific fit. Cell-seeded constructs were modelled as fluid saturated porous media using a finite strain formulation applied previously to agarose in compression (Görke et al. 2010). Surfaces in contact with media were modelled as free draining, while fluid flow was prohibited across surfaces in contact with the loading platens or the confinement well. Nonlinear permeability was modelled following Gu *et al.* (2003). Briefly, the solid matrix was modelled as nonlinearly viscoelastic based on a multiplicative decomposition of the deformation gradient into elastic and viscous parts $F = F_e F_v$. An evolution equation was defined for the viscous right Cauchy-Green tensor C_v:

$$\dot{C}_v = \frac{1}{\eta_v} C_v T_{ov}$$

where η_v is the viscosity function (Lion 1997; Görke et al. 2010). While the equilibrium free Helmholtz energy potentials where based on a Neo-Hookean formulation

$$\psi_{eq} = C_1\left[I_1(C) - \ln\left(I_3(C)\right) - 3\right] + D_2(\ln I_3(C))^2$$

the viscoelastic overstresses where derived from the exponential potential

$$\psi_{ov} = \frac{C_{1v}}{\alpha_v}\left[e^{\alpha_v\left[I_1(CC_v^{-1}) - \ln\left(I_3(CC_v^{-1})\right) - 3\right]} - 1\right] + D_{2v}(\ln I_3(CC_v^{-1}))^2$$

The equilibrium properties C_1 and D_2 were determined analytically from the relaxed part of the ramp and hold test. D_{2v} was set to $C_{1v}D_2/C_1$. The remaining parameters in the viscoelastic potential and the viscosity were fit to unconfined ramp and hold force relaxation curves using a differential evolution algorithm developed by Storn and Price (Storn and Price 1997). For further details see Görke *et al.* (Görke et al. 2010).

5.3.8 *Theoretical prediction of oxygen concentration within agarose constructs*

This portion of the study was contributed as a personal communication from Mr Thomas Nagel. Oxygen concentration in the constructs was modelled using a diffusion-reaction type equation. The reaction term followed Michaelis-Menten kinetics:

$$\frac{\partial c}{\partial t} = D\nabla^2 c - n\frac{Q_m c}{K_m + c}$$

Here, c is the oxygen concentration, D the diffusion coefficient, n the cell density, Q_m the maximum consumption rate and K_m the concentration at half the maximum consumption rate. The diffusion coefficient in 2% agarose was determined using the Mackie and Meares relation

$$\frac{D_{ag}}{D_{H_2O}} = \frac{\phi_f^2}{\left(2 - \phi_f\right)^2}$$

so that $D_{ag} = 2.76\times10^{-3}$ mm²/s (Sengers et al. 2005). The cellular consumption value was set to $Q_m =$ 12.3 fmol/(cell×h), derived from measurements on hMSCs undergoing chondrogenic differentiation (Pattappa et al. 2010). For K_m, an average value from those reported for chondrocytes in agarose (Sengers et al. 2005; Guaccio et al. 2008; Demol et al. 2011) was chosen (1×10^{-7} µmol/mm³). The sensitivity of the simulation outcome to this value is very low. The construct was modelled as axisymmetric. At surfaces in contact with the culture medium, the oxygen concentration was prescribed as 200 µM, while at surfaces in contact with the bottom of the well, the confining chamber and the symmetry axis the flux was set to zero. The simulations were performed for cell concentrations of $n = 20\times10^6$ cells/mL and $n = 50\times10^6$ cells/mL.

5.3.9 *Statistical analysis*

Statistics were performed using MINITAB 15.1 software package (Minitab Ltd., Coventry, UK). Where necessary, a Box-Cox transformation was used to normalise data sets. Construct groups were analysed for significant differences using a general linear model for analysis of variance with factors of group, confinement, dynamic compression, construct region and interactions between these factors examined. Tukey's test for multiple comparisons was used to compare conditions.

Significance was accepted at a level of $p \leq 0.05$. Numerical and graphical results are presented as mean ± standard error.

5.4 Results

5.4.1 Confinement spatially alters pore pressure and tensile strain within agarose hydrogels during dynamic compression

This portion of the study was contributed as a personal communication from Mr Thomas Nagel. The mechanical environment within an agarose hydrogel during dynamic compression was predicted for both unconfined and confined configurations. A relatively homogenous strain environment is predicted within the unconfined construct while the confined constructs experience higher tensile strains in the top of the construct with predominantly compressive strains of lower magnitude present in the bottom (Figure 5.2). The model predicts pore pressures in the bottom of the confined constructs which are about an order of magnitude greater than that in the unconfined configuration where a relatively homogenous pressure environment exists.

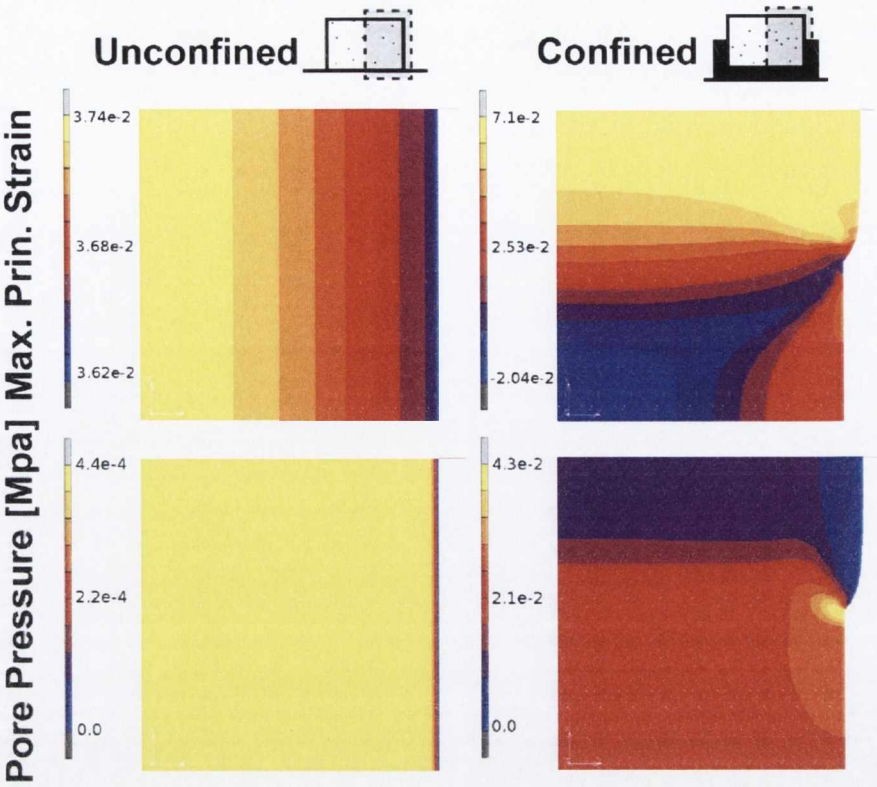

Figure 5.2: Theoretical predictions of the maximum principle strain and pore pressure [MPa] for both unconfined and confined configurations during steady state dynamic compression at day 0.

5.4.2 Oxygen tension within engineered tissues is modulated by seeding density and confinement

This portion of the study was contributed as a personal communication from Mr Thomas Nagel. The oxygen concentration within cell seeded agarose constructs after 24 hours was predicted for both unconfined and confined configurations at seeding densities of 20×10^6 cells/mL or 50×10^6 cells/mL (Figure 5.3). Due to cellular consumption, an oxygen gradient develops over time. For unconfined constructs sitting on the base of a dish, oxygen concentration decreases towards the bottom centre; from 2×10^{-4} μmol/mm^3 at the top surface to 1.49×10^{-4} μmol/mm^3 and 0.73×10^{-4} μmol/mm^3 for 20×10^6 and 50×10^6 cells/mL respectively at the bottom centre. For the confined configuration, this gradient was accentuated, with the development of a low oxygen region (0.99×10^{-4} μmol/mm^3) at 20×10^6 cells/mL, and an anoxic region (3×10^{-9} μmol/mm^3) predicted at 50×10^6 cells/mL across the bottom of the construct. Oxygen concentration was seen to be a function of cell density with increased consumption leading to predictions of lower oxygen concentrations within the construct; a trend which was exacerbated by confinement.

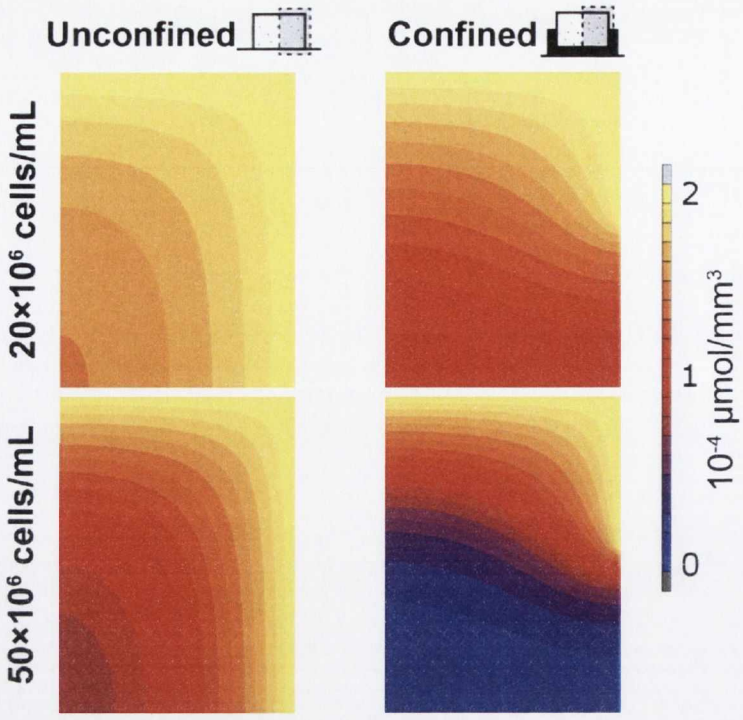

Figure 5.3: Theoretical predictions of the oxygen concentration [µmol/mm^3] after 24 hours within agarose constructs seeded with both 20×10^6 and 50×10^6 cells/mL under unconfined and confined conditions.

5.4.3 Confinement enhances sGAG accumulation in the bottom of engineered cartilaginous constructs

MSCs were encapsulated in agarose at 20×10^6 cells/mL, confined, and cultured in free-swelling (unloaded) conditions to day 21 at which point the biochemical content was independently assessed within the top and bottom regions of the construct (Figure 5.4A). No differences in DNA content were observed between the top and bottom of either confined or unconfined constructs. For unconfined constructs sGAG content did not change significantly with construct depth. However confinement led to an increase in sGAG in the construct bottom when compared to unconfined controls (0.470±0.009 %ww vs. 0.283±0.024 %ww; p=0.0002); producing a depth-dependent gradient in sGAG accumulation within these constructs (p<0.0001). When media was analysed, it was revealed that confinement served to decrease sGAG secretion to the media (p=0.004; Figure 5.4B); in spite of this, total sGAG produced (accumulated plus secreted to media) was still greatest in confined constructs (527.95±4.536 µg vs. 485.348±13.595 µg; p=0.0154). A gradient in

collagen content was present in unconfined constructs at day 21, with greater collagen accumulation in the bottom region (p=0.0247; Figure 5.4A). When constructs were confined there was no difference between the two regions. Analysis of media revealed that while confinement did not affect collagen accumulated, it reduced collagen secreted to the media, such that more collagen was produced in unconfined constructs (929.45±15.426 μg vs. 753.852±12.597 μg; p<0.0001; Figure 5.4B).

Histological staining confirmed that spatial variations exist in the distribution of sGAG and collagen within the constructs (Figure 5.4C). In confined constructs, intense alcian blue and collagen type II staining is evident towards the edge in the bottom (confined) region of the construct when compared to unconfined controls. A zone of lower intensity staining was apparent towards the bottom centre of confined constructs.

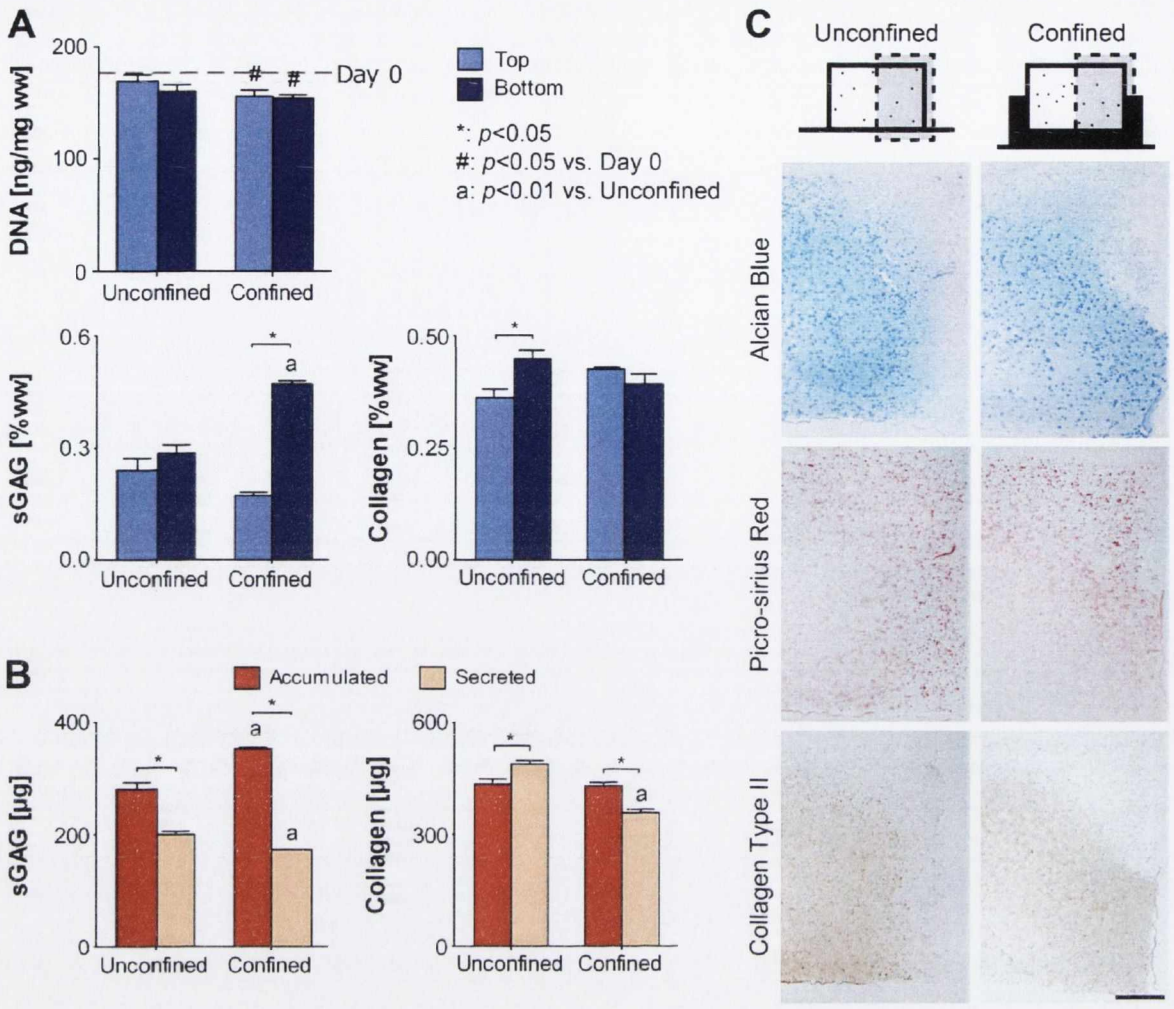

Figure 5.4: MSCs encapsulated in agarose at 20×10^6 cells/mL were cultured for 21 days in unconfined or confined conditions. (A) The top and bottom regions of unconfined and confined free-swelling constructs were analysed for DNA, sGAG and collagen contents. (B) Culture media was analysed for sGAG and collagen secreted. (C) Unconfined and confined constructs were stained with alcian blue for sulphated mucins, picro-sirius red for collagen and specifically for collagen type II. Representative full-depth half construct sections are shown as indicated. Scale bar 500 µm.

5.4.4 Radial confinement coupled with dynamic compression produces zonal gradients in biochemical composition mimicking articular cartilage

MSCs were encapsulated in agarose at 20×10^6 cells/mL and cultured in unconfined free-swelling (unloaded) conditions for 21 days, at which point constructs were confined and dynamic compression applied from day 21 to 42. This delayed application of dynamic compression was motivated by our previous findings that application of loading from day 0 inhibited chondrogenesis of MSCs (Thorpe et al. 2008; Thorpe et al. 2010). DNA content dropped for all day 42 groups

compared to day 0 values ($p \leq 0.0001$; Figure 5.5). At day 42 a gradient in DNA content was evident with greater cell viability in the bottom than the top ($p=0.0001$). Confinement of constructs from day 21 served to promote viability in the confined region such that the bottom regions of confined constructs contained a higher DNA content than the same region in unconfined controls ($p=0.0002$).

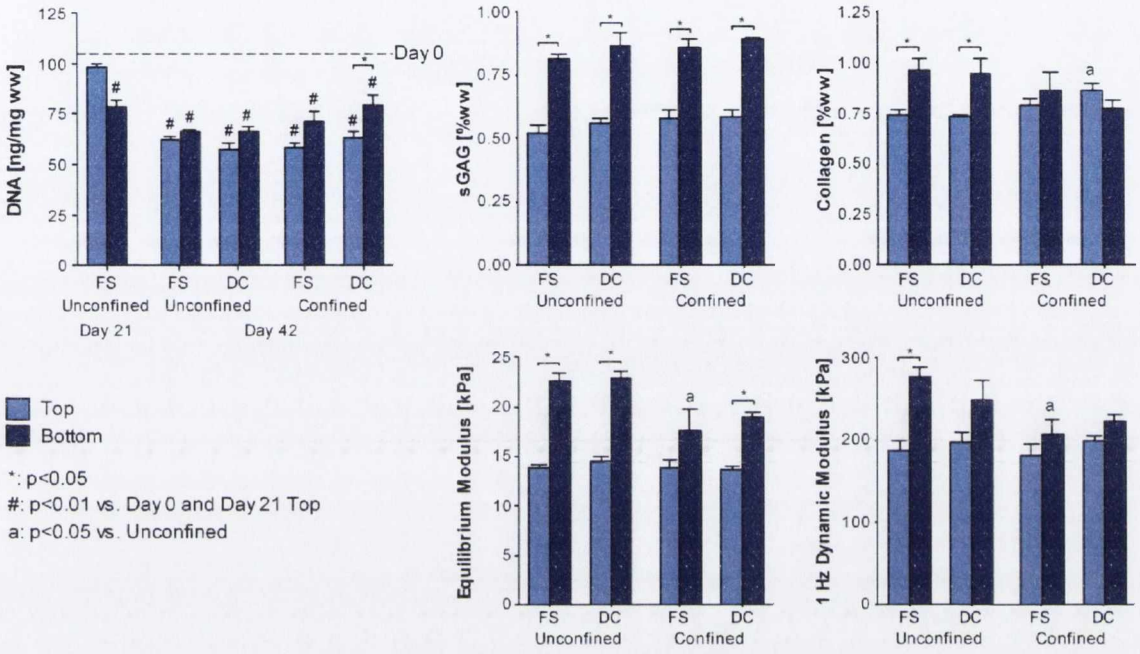

Figure 5.5: Agarose constructs containing MSCs at 20×10^6 cells/mL were confined from day 21 to day 42 of culture while 10% dynamic compression was applied. The top and bottom regions of constructs were analysed for DNA, sGAG and collagen contents. Top and bottom regions of constructs were also mechanically tested for both the equilibrium modulus and the dynamic modulus at 1 Hz. FS: free-swelling; DC: dynamic compression.

In all groups a zonal gradient in sGAG was present with significantly more sGAG present in the bottom of constructs by day 42 ($p<0.0001$; Figure 5.5). These trends were also evident when sGAG accumulation was normalised to DNA content (Figure 5.6). Neither dynamic compression nor confinement between day 21 and day 42 had a significant effect on sGAG accumulation. Of the total sGAG produced, over half remained within the construct ($p=0.0343$; Figure 5.7), while confinement had no significant effect on sGAG release to the media. Overall, while dynamic

compression had no effect on sGAG accumulation, it did result in an increase in sGAG secreted to the media (p=0.0344).

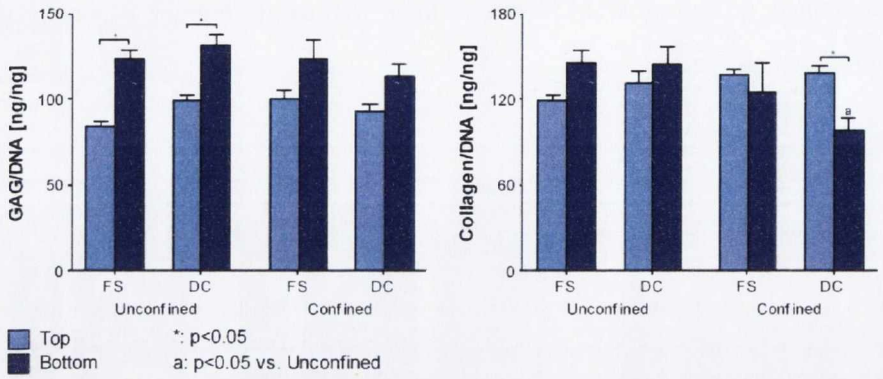

Figure 5.6: Agarose constructs containing MSCs at 20×10^6 cells/mL were confined from day 21 to day 42 of culture while 10% dynamic compression was applied. The top and bottom regions of constructs were analysed for sGAG and collagen contents which were normalised to DNA content. FS: free-swelling; DC: dynamic compression.

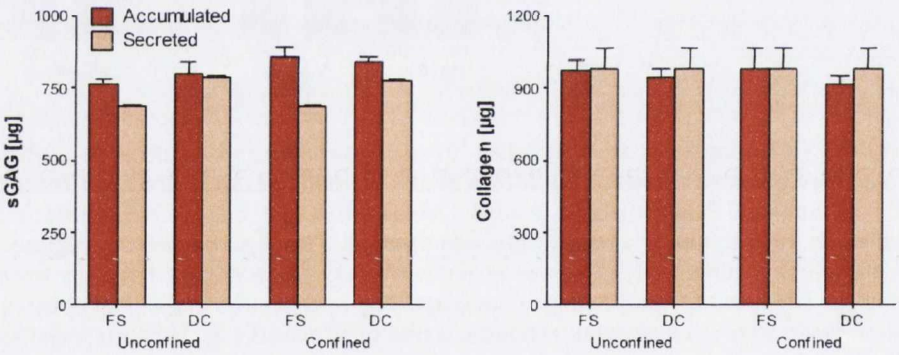

Figure 5.7: Agarose constructs containing MSCs at 20×10^6 cells/mL were confined from day 21 to day 42 of culture while 10% dynamic compression was applied. sGAG and collagen accumulated within the construct and secreted to culture media was measured. FS: free-swelling; DC: dynamic compression.

By day 42 unconfined constructs also exhibited a zonal gradient in collagen content with greater accumulation in the bottom of the construct (p=0.0016). However when constructs were confined from day 21-42, this zonal gradient was no longer significant. In fact, when confinement was combined with dynamic compression (DC), this zonal gradient was reversed such that collagen

content in the top of confined DC constructs was greater than that in unconfined controls (0.868±0.033 vs. 0.736±0.011; p=0.0252; Figure 5.5). When normalised to DNA content, collagen accumulation in the top of confined constructs was significantly higher than that in the bottom (p=0.0382; Figure 5.6). Approximately half the total collagen produced was secreted to the media, with neither confinement nor dynamic compression having any significant effect (Figure 5.7).

Staining of construct sections with alcian blue or picro-sirius red revealed positive staining within all groups (Figure 5.8). Confinement acted to increase the intensity of collagen staining in the top half of the construct when compared to unconfined controls; particularly in constructs subjected to dynamic compression. Similar staining patterns were evident for collagen type II. Pericellular collagen type I staining was evident around the edge of unconfined free-swelling constructs (Figure 5.8). When confined, staining for collagen type I was only evident in the top (unconfined) corner of the construct. Interestingly, staining for type I collagen was absent in confined constructs subjected to dynamic compression.

The equilibrium and dynamic modulus increased with time for all conditions (p<0.001), although there was no significant difference in bulk mechanical properties between the different groups by day 42 (data not shown). In agreement with sGAG zonal gradients, the equilibrium modulus was greater in the bottom of constructs (p<0.0001; Figure 5.5).

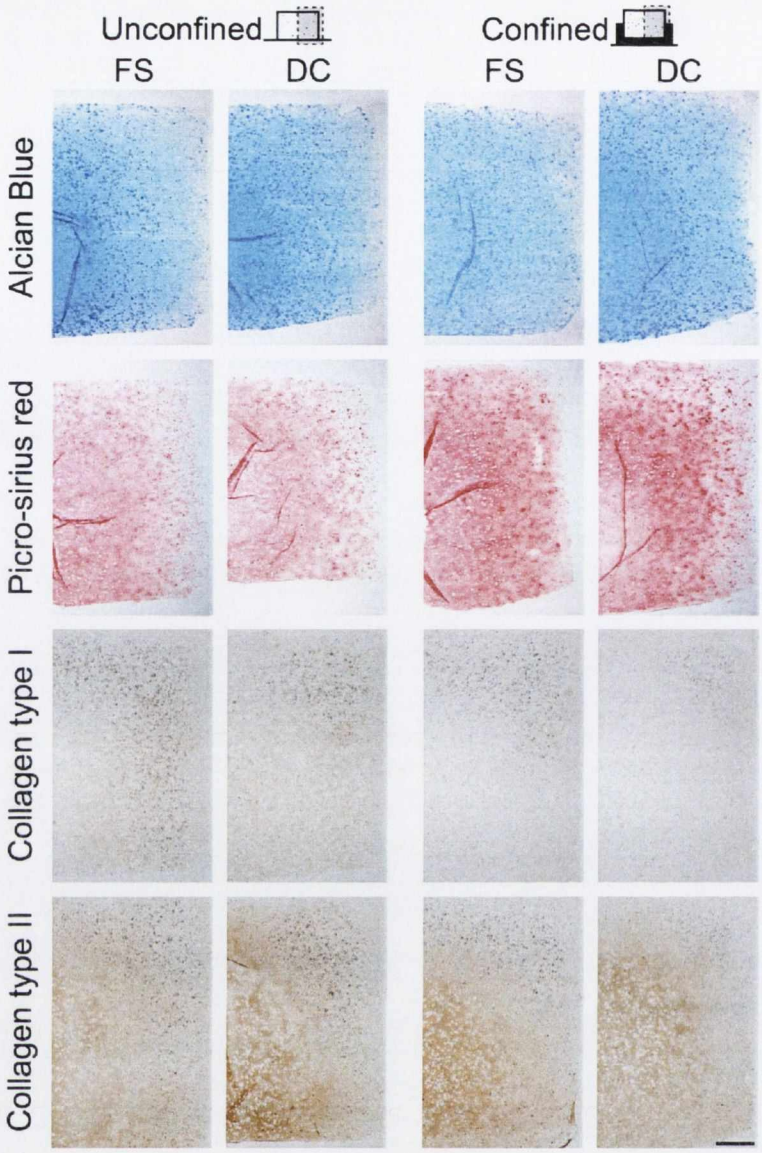

Figure 5.8: Agarose constructs containing MSCs at 20×10^6 cells/mL were confined from day 21 to day 42 of culture while 10% dynamic compression was applied. Constructs at day 42 were stained with alcian blue for sulphated mucins, picro-sirius red for total collagen, and specifically for collagen type I and type II. Representative full-depth half construct sections are shown. FS: free-swelling; DC: dynamic compression. Scale bar 500 μm.

5.4.5 MSC response to extrinsic signals is dependent on the cell seeding density

While cartilaginous constructs with gradients in zonal composition similar to that in normal articular cartilage can be engineered by controlling the environment through the depth of the developing construct, absolute levels of matrix accumulation were still lower than native values. In an attempt to address this issue, MSCs were encapsulated in agarose at a higher seeding density of

50×10^6 cells/mL and cultured in unconfined free-swelling (unloaded) conditions for 21 days, at which point constructs were confined and dynamic compression applied from day 21 to 42.

By day 42, dynamic compression led to an increase in sGAG content for both confined and unconfined constructs (p=0.0005; Figure 5.9). However this increase in sGAG occurred only in the top of the construct (p=0.0001); resulting in a zonal gradient with greater sGAG accumulation in the top of the construct for confined DC constructs (1.538±0.040 %ww vs. 1.170±0.092 %ww; p=0.0013). On analysis of culture media, confinement was seen to inhibit total sGAG produced (p=0.0094; Figure 5.11). Notably, more sGAG was secreted to the media than retained within the construct (p=0.0020). Confinement acted to increase collagen accumulation by day 42 in the construct top when compared with unconfined controls irrespective of the application of dynamic compression (p=0.0137; Figure 5.9). However, confinement led to an overall decrease in collagen content in the construct bottom in comparison to unconfined controls (p=0.0026; Figure 5.9, Figure 5.10). On inclusion of collagen secreted to the media, it was evident that confinement at 50×10^6 cells/mL inhibited total collagen production (p=0.0407; Figure 5.11). Staining of construct sections with alcian blue or picro-sirius red revealed a steady accretion of positive staining for all groups (Figure 5.12). Confinement acted to increase the intensity of both picro-sirius red and collagen type II staining in the top half of the construct when compared to unconfined controls. Collagen staining decreased toward the core region of all constructs and was more heterogeneous than alcian blue staining.

Dynamic compression acted to enhance both the equilibrium and dynamic modulus for the whole construct (p<0.05). However, this increase was only evident in the top of DC constructs, which were stiffer than the corresponding region in FS controls (p<0.05; Figure 5.9). Confinement had a negative effect on whole construct stiffness (p<0.05); attributed to a decrease in both equilibrium and dynamic moduli in the bottom of confined constructs corresponding to lower matrix accumulation in this region (p<0.01; Figure 5.9).

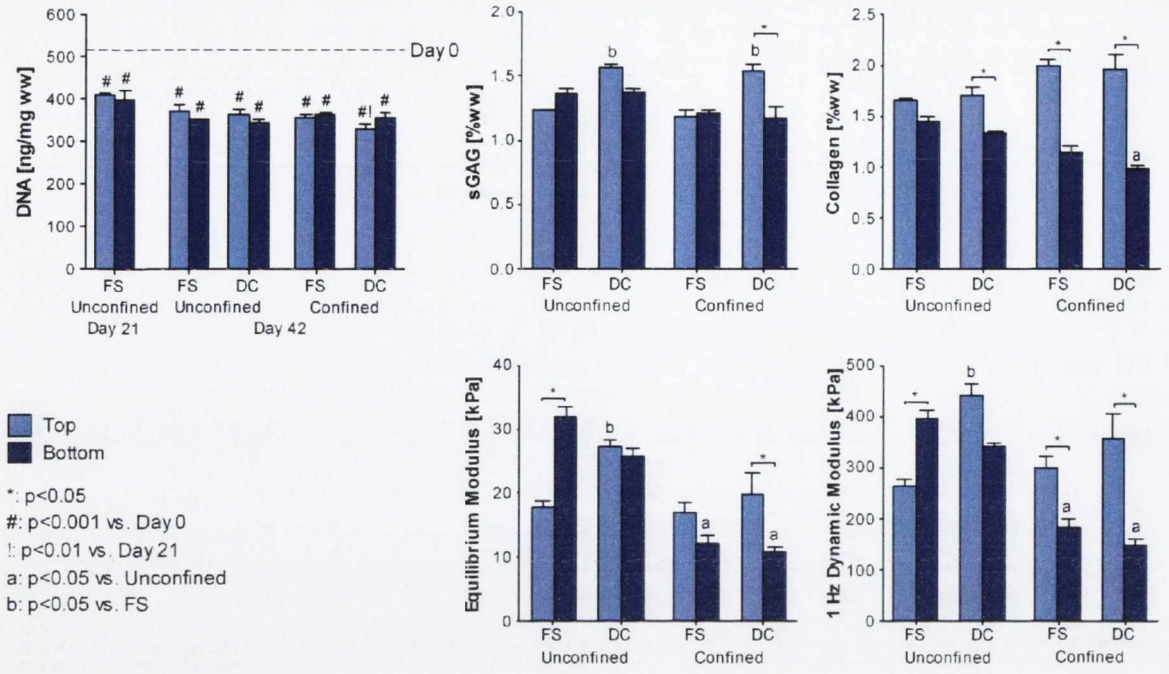

Figure 5.9: Agarose constructs containing MSCs at 50×10^6 cells/mL were confined from day 21 to day 42 of culture while 10% dynamic compression was applied. The top and bottom regions of constructs were analysed for DNA, sGAG and collagen contents. Top and bottom regions of constructs were also mechanically tested for both the equilibrium modulus and the dynamic modulus at 1 Hz. FS: free-swelling; DC: dynamic compression.

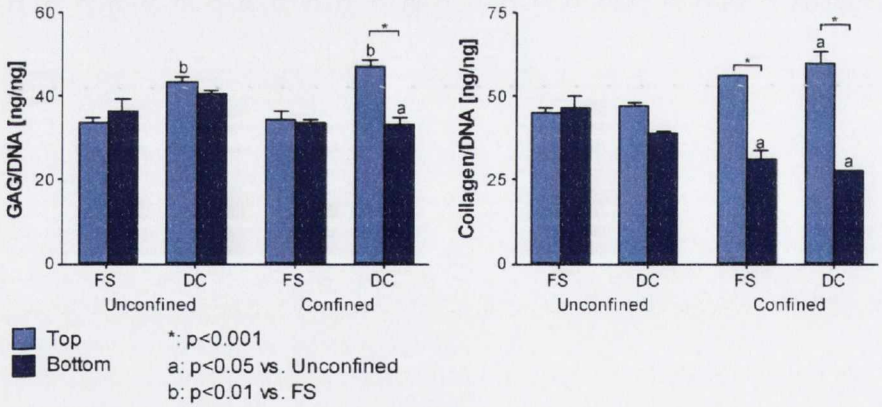

Figure 5.10: Agarose constructs containing MSCs at 50×10^6 cells/mL were confined from day 21 to day 42 of culture while 10% dynamic compression was applied. The top and bottom regions of constructs were analysed for sGAG and collagen contents which were normalised to DNA content. FS: free-swelling; DC: dynamic compression.

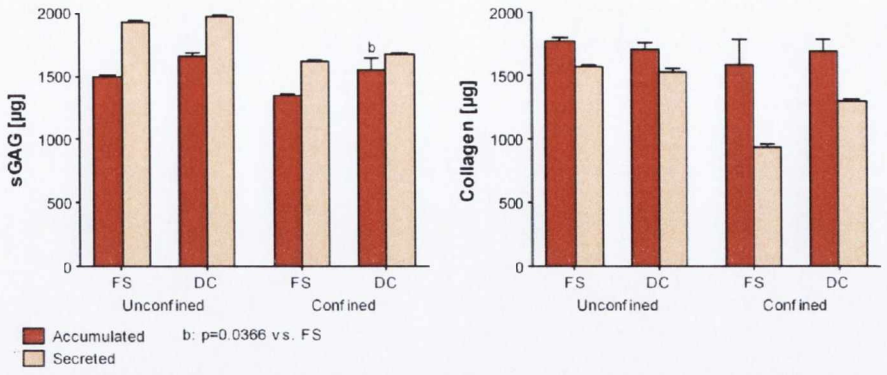

Figure 5.11: Agarose constructs containing MSCs at 50×10^6 cells/mL were confined from day 21 to day 42 of culture while 10% dynamic compression was applied. sGAG and collagen accumulated within the construct and secreted to culture media was measured. FS: free-swelling; DC: dynamic compression.

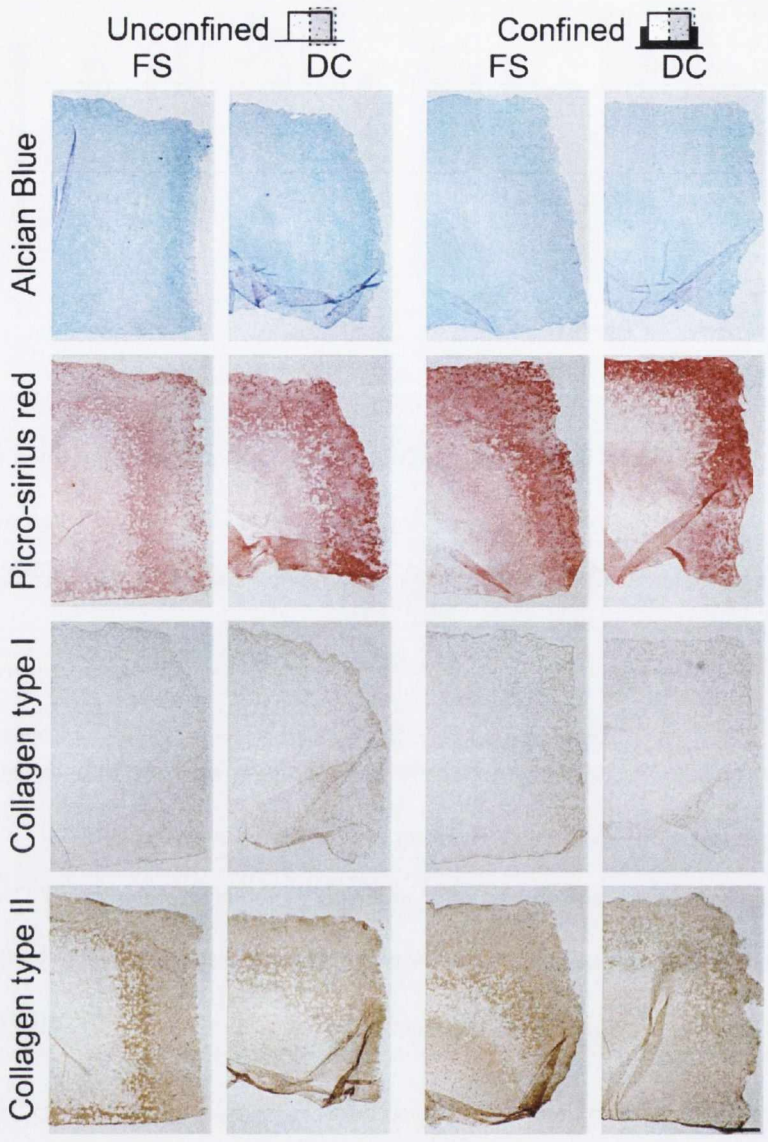

Figure 5.12: Agarose constructs containing MSCs at 50×10^6 cells/mL were confined from day 21 to day 42 of culture while 10% dynamic compression was applied. Constructs at day 42 were stained with alcian blue for sulphated mucins, picro-sirius red for total collagen, and specifically for collagen type I and type II. Representative full-depth half construct sections are shown. FS: free-swelling; DC: dynamic compression. Scale bar 500 µm.

5.5 Discussion

Significant developments have been made regarding the use of MSCs for functional cartilage tissue engineering (Vinatier et al. 2009a; Huang et al. 2010b; Khan et al. 2010). However, attempts to engineer a tissue with zonal organisation mimicking normal articular cartilage using MSCs have

been limited. Through incorporation of cartilage-extra cellular matrix (ECM) components into PEG hydrogels, Nguyen *et al.* (2011a) were able to direct MSC differentiation into zone-specific phenotypes. Subsequently, this model was used to create a zonally organised construct with matrix composition and mechanical properties which varied with depth (Nguyen et al. 2011b). This paper presents an alternative approach and has shown that the depth dependent properties of cartilage tissue engineered using MSCs can be modulated by controlling the local environment within the developing construct. Bone marrow derived mesenchymal stem cells (MSCs) were encapsulated in agarose hydrogel and confined up to half their thickness. This reduced oxygen levels in the bottom of the construct and when combined with dynamic compression, increased tensile strains across the top of the construct. These spatial changes in the local environment corresponded with increased sGAG accumulation in the bottom of constructs and increased collagen accumulation in the construct top. Consequently, a tissue with inhomogeneous distributions of sGAG, collagen and mechanical properties similar, but not identical to native cartilage was established. In an effort to increase extracellular matrix (ECM) concentration and mechanical properties, constructs were seeded at a higher cell density (50×10^6 cells/mL). This failed to instigate the creation of native-like zonal gradients in biochemical constituents and mechanical properties, demonstrating that the response of MSCs to extrinsic biochemical and biophysical cues is dependent on the local cell seeding density and subsequent levels of ECM accumulation.

Computational modelling was used to assess the alterations in oxygen tension and the mechanical environment induced by confinement. Due to the uncertainty associated with the parameter identification for constructs later in culture where elaborated extracellular matrix may hamper nutrient transfer and inhomogeneously alter construct mechanics, the simulations were performed for the initial cell seeded agarose constructs only. Despite this limitation the simulations provided insight into the spatial gradients in oxygen tension throughout the construct and its dependence on cell density. While these predictions may not be quantitatively accurate, they qualitatively estimate the oxygen gradients throughout the construct and provide a valid comparison of such gradients for differing cell density and confinement conditions. It was also shown that pore pressures are

increased significantly in the bottom of confined constructs and that the pressure and strain fields become highly heterogeneous with a pronounced gradient throughout the construct depth.

Initially, the effect of confinement was examined over 21 days. Confinement enhanced sGAG accumulation in the bottom (confined) region of the construct. One explanation for this was that confinement was simply acting to reduce secretion of ECM components into the media. While less sGAG was secreted from confined constructs, the total produced remained significantly higher with confinement (Figure 5.4B), supporting the hypothesis that the low oxygen microenvironment predicted within the bottom of confined constructs does enhance chondrogenesis. Regions of weaker alcian blue and collagen type II staining (Fig 4C) in both confined and unconfined constructs also appeared to correlate with predicted regions of higher oxygen tension (Fig. 3). However, confinement from day 21 to day 42 did not lead to enhanced sGAG accumulation. In fact, sGAG accumulation was concentrated in the bottom of both confined and unconfined constructs at day 42. Given that care was taken to prevent constructs from flipping over during culture (thereby ensuring that the top surface of the construct remained face upwards for the culture duration), a low oxygen region will naturally develop in the bottom of the construct due to cellular consumption (Fig. 3), which may explain why sGAG accumulation in the long term is higher in the bottom of both confined and unconfined constructs.

When confinement was combined with dynamic compression, collagen accumulation increased in the top of the construct resulting in creation of a native-like zonal gradient in both the biochemical composition and compressive properties of the engineered tissue. Mechanics have long been thought to play a role in the differentiation and subsequent modelling and remodelling of mesenchymal tissues (Pauwels 1960; Carter et al. 1988; Prendergast et al. 1997). Intermittent cyclic tension has specifically been shown to increase collagen content within MSC seeded constructs (Connelly et al. 2010; Baker et al. 2011). In the present study, tensile strains across the top of dynamically compressed constructs were predicted to increase with confinement (Figure 5.2), implicating this stimulus in driving the higher levels of collagen synthesis in this region of the construct. Interestingly, the combination of confinement and dynamic compression also acted to inhibit collagen type I accumulation.

The effect of dynamic compression was also dependent on the cell seeding density. At 50×10^6 cells/mL dynamic compression acted to enhance sGAG accumulation and the overall mechanical properties in both unconfined and confined constructs. Long-term dynamic compression of MSC seeded agarose applied after 21 days of unloaded pre-culture has previously been shown to augment sGAG accumulation (Thorpe et al. 2010), and construct stiffness (Huang et al. 2010a). This finding that MSC response to dynamic compression is dependent on cell seeding density has not previously been reported. The mechanism through which dynamic compression augments chondrogenesis remains unclear. At the higher seeding density of 50×10^6 cells/mL, it is possible that greater cell-cell contact and signalling may enhance MSC response to dynamic compression. It is also possible that a threshold level of ECM accumulation is required for dynamic compression to stimulate additional synthesis. Démarteau *et al.* (2003) demonstrated that chondrocyte response to dynamic compression was positively correlated to the level of ECM. Increasing the cell density was seen to increase matrix accumulation which may modulate the response to dynamic compression. Additionally, dynamic compression may facilitate nutrient transport within the construct (Albro et al. 2008). Increasing cell density acts to increase the severity of nutrient gradients due to cellular consumption in the construct periphery, which may be at least partially overcome by the application of dynamic compression.

Although fluid pressures in the bottom of confined constructs were increased due to dynamic compression, this was not found to increase sGAG accumulation in this region of the construct at either cell seeding density. This may be due to the fact that the pressure generated remains over an order of magnitude less than that reported to elicit increases in gene expression in MSC aggregates (Angele et al. 2003; Miyanishi et al. 2006b).

While a construct with zonal gradients similar to articular cartilage was achieved with 20×10^6 cells/mL, increasing the cell density to 50×10^6 cells/mL failed to elicit the same zonal gradients. Increasing the cell seeding density acts to increase oxygen and nutrient consumption, resulting in a more acute decrease in oxygen concentration away from the periphery. Confinement of constructs added to the severity of this gradient, such that an anoxic region was predicted in the bottom of confined constructs at 50×10^6 cells/mL (Figure 5.3). This concentration was lower than that

predicted theoretically in the deep zone of native articular cartilage (Zhou et al. 2004). It has been shown that while MSCs can survive under conditions of severe continuous hypoxia provided sufficient glucose is available; their metabolic activity is compromised culminating in lower ECM production and possible arrest of differentiation (Deschepper et al. 2011). Vascularised cartilage canals are present in developing cartilage (Hall 2005; Lecocq et al. 2008) and may provide a route for oxygen and nutrient supply. Mimicking such nutrient paths in engineered constructs (Buckley et al. 2009a; Sheehy et al. 2011) may provide a means to overcome transport limitations in such tissues.

Engineering cartilaginous grafts with structural organisation is crucial to the long term repair of cartilage lesions. By controlling the oxygen tension and mechanical environment through the depth of the developing tissue, a construct with sGAG and collagen zonal gradients akin to that of articular cartilage can be engineered using MSCs.

Chapter 6 Cell-matrix interactions and the external mechanical environment modulate mesenchymal stem cell differentiation in response to TGF-β

For the purposes of this thesis, the study presented is adapted from the original article which is currently under review.

6.1 Abstract

In addition to soluble cues, mesenchymal stem cell (MSC) differentiation is known to be regulated by factors such as cell shape, cell adhesion and mechanical stimulation. The aim of this study was to explore how cell-matrix interactions and extrinsic mechanical signals interact to determine stem cell fate in response to transforming growth factor-β3 (TGF-β3). MSCs were seeded in agarose and fibrin hydrogels with dynamic compression of 10% strain at 1 Hz applied for 3 hours/day in the presence of either 1 ng/mL or 10 ng/mL TGF-β3. Markers associated with the chondrogenic, myogenic and endochondral phenotypes were assessed. Free-swelling unloaded agarose constructs stained positively for chondrogenic markers alcian blue and collagen type II, and appeared to be progressing toward terminal differentiation as indicated by mineral staining. MSC seeded fibrin constructs appeared to progress along an alternative pathway in free-swelling conditions with short-term evidence of both chondrogenic and myogenic differentiation, with stronger evidence of myogenic differentiation in long-term culture. Dynamic compression, initiated at the onset of TGF-β3 stimulation, appeared to suppress differentiation toward any investigated lineage in both fibrin and agarose. Given that fibrin clots have been shown to support a chondrogenic phenotype *in vivo* within mechanically loaded joint defect environments, we next explored the influence of long term dynamic compression on MSC differentiation in fibrin. Mechanical signals generated by this extrinsic loading ultimately governed MSC fate; directing MSCs along a chondrogenic phenotype

as opposed to the default myogenic route supported by the uncompressed fibrin clot. In conclusion, this study demonstrates how external cues such as the mechanical environment can override the influence that specific substrates, scaffolds or hydrogels have on determining stem cell fate.

6.2 Introduction

Bone marrow derived mesenchymal stem cells (MSCs) could potentially provide an effective cell source for tissue engineering and regenerative medicine applications. MSCs are highly expandable and can differentiate along several pathways including the chondrogenic, osteogenic, adipogenic and myogenic lineages (Caplan 1991). MSC differentiation into distinct lineages is dependent on cues present in the local microenvironment. While much attention has centred on the use of soluble factors to direct MSC differentiation (Pittenger et al. 1999; Minguell et al. 2001; Vinatier et al. 2009b), less focus had been placed on other potentially important insoluble signals such as cell shape, cell adhesion, substrate rigidity and mechanical cues from the external environment (Huang et al. 2004a; McBeath et al. 2004; Engler et al. 2006; Connelly et al. 2008; Kelly and Jacobs 2010).

It has long been proposed that mechanical signals can direct the differentiation of MSCs (Pauwels 1960; Carter et al. 1988; Prendergast et al. 1997). Using one such mechanoregulation model, Khayyeri *et al.* (2011) successfully predicted the spatial differentiation patterns of neo-tissue within an *in-vivo* bone chamber in response to an applied biophysical stimulus such that osteogenic differentiation occurred in regions with low distortional strain and interstitial fluid flow, chondrogenic differentiation at intermediate levels of distortional strain and fluid flow, and fibrous connective tissue where distortional strain was high. *In vitro* bioreactor studies also support the hypothesis that stem cell differentiation is mechano-regulated. In the absence of chondrogenic growth factors, dynamic compressive strain applied to hydrogel encapsulated MSCs has been shown to enhance chondrogenic gene expression and protein synthesis (Huang et al. 2004a; Campbell et al. 2006; Mauck et al. 2007; Kisiday et al. 2009; Pelaez et al. 2009; Li et al. 2010a). The response of MSCs to both biochemical and biophysical cues is more complex, with reports of the simultaneous application of TGF-β3 and dynamic compression supressing chondrogenic gene

expression and matrix accumulation in the early stages of differentiation (Campbell et al. 2006; Thorpe et al. 2008; Huang et al. 2010a; Haugh et al. 2011). When the initiation of dynamic compression is delayed, allowing cytokine driven differentiation to occur prior to its application, chondrogenic gene expression (Mouw et al. 2007; Haugh et al. 2011), matrix accumulation (Thorpe et al. 2010) and construct mechanical properties (Huang et al. 2010a) are increased.

Cell-matrix interactions also play a key role in regulating stem cell fate. Integrin mediated adhesion to the extracellular matrix has been shown to modulate both chondrogenic and osteogenic MSC differentiation (Connelly et al. 2008; Kundu et al. 2009; Steinmetz and Bryant 2011), and may act as a means for mechanotransduction (Kock et al. 2009; Wang et al. 2009b). A natural hydrogel which facilitates integrin binding to ligands present on the scaffold is fibrin (Janmey et al. 2009). Fibrin has been shown to support chondrogenic differentiation (Li et al. 2009; Pelaez et al. 2009), but it similarly facilitates osteogenic (Catelas et al. 2006; Weinand et al. 2006) and myogenic (Nieponice et al. 2007; O'Cearbhaill et al. 2010) differentiation. This material is reviewed in detail in Section 2.2.3.

We are only beginning to understand how biochemical and biophysical cues interact to determine stem cell fate within an environment that can potentially support differentiation along multiple pathways, such as a fibrin clot. In an *in vitro* setting, TGF-β has been shown to promote both chondrogenic differentiation (Johnstone et al. 1998; Mackay et al. 1998) and myogenic differentiation (Kinner et al. 2002; Jeon et al. 2006) of MSCs. This switch between chondrogenic and myogenic fates in the presence of TGF-β has been shown to depend on matrix stiffness (Park et al. 2011) and cell shape (Gao et al. 2010). Adherent and well spread MSCs were shown to undergo myogenic differentiation in response to TGF-β3, while cells that were prevented from spreading and flattening underwent chondrogenic differentiation (Gao et al. 2010). In an *in vivo* setting, insoluble cues will be provided not only by the substrate and associated changes in cell shape, but also from the external mechanical environment. The aim of this study was to explore how cell-matrix interactions and the extrinsic mechanical signals interact to determine stem cell fate in response to TGF-β. We first hypothesised that in response to TGF-β stimulation, a three dimensional hydrogel environment repressing cell adhesion and spreading (agarose) would

encourage the chondrogenic differentiation of MSCs, while a hydrogel promoting cell spreading and cell-cell interactions (fibrin) would ultimately support a more myogenic phenotype. We next explored how extrinsic mechanical signals, specifically dynamic compression of the hydrogel, would influence stem cell fate. We hypothesised that in a three dimensional hydrogel environment preferentially supporting myogenesis over chondrogenesis, that the long-term application of dynamic compression would override the effects of the local substrate, suppressing myogenesis and enhancing chondrogenesis of MSCs.

6.3 Methods

The commercial sources of reagents are listed in Appendix B.

6.3.1 Cell isolation and expansion

Porcine MSCs were isolated and maintained as previously described (Thorpe et al. 2010). Briefly, mononuclear cells were isolated from the femora of 4 month old porcine donors (~50kg) within 2 hours of sacrifice and plated at 10×10^6 cells per $75cm^2$ flask allowing colony formation. MSCs were maintained in high-glucose Dulbecco's modified eagles medium (4.5 mg/mL D-Glucose, 200mM L-Glutamine; hgDMEM) supplemented with 10% foetal bovine serum (FBS), penicillin (100 U/mL)-streptomycin (100 µg/mL) and amphotericin B (0.25µg/mL). Cultures were washed in Dulbecco's phosphate buffered saline (PBS) after 72 hrs. When passaged, MSCs were plated at 5×10^3 cells/cm^2 and expanded to passage two in a humidified atmosphere at 37°C and 5% CO_2.

6.3.2 Agarose and fibrin hydrogel encapsulation

For agarose encapsulation, MSCs were suspended in defined chondrogenic medium (CM) consisting of hgDMEM supplemented with penicillin (100 U/mL)-streptomycin (100 µg/mL), 0.25 µg/mL amphotericin B, 100 KIU/mL aprotinin, 100 µg/ml sodium pyruvate, 40 µg/mL L-proline, 1.5 mg/mL bovine serum albumin, 4.7 µg/mL linoleic acid, 1× insulin–transferrin–selenium, 50 µg/mL L-ascorbic acid-2-phosphate, 100 nM dexamethasone. This cell suspension was mixed with agarose (Type VII) in PBS at a ratio of 1:1 at approx. 40°C, to yield a final agarose concentration

of 2% and a cell density of 15×10^6 cells/mL. The agarose-cell suspension was cast between stainless steel plates, allowed cool to 21°C for 30 min., and cored to produce cylindrical constructs (Ø 5mm×3mm thickness).

For fibrin encapsulation, MSCs were suspended in 10,000 KIU/mL aprotinin solution containing 19 mg/mL sodium chloride and 100 mg/mL bovine fibrinogen type I-S (60-85% protein, ~10% sodium citrate and ~15% sodium chloride). This was combined 1:1 with 5 U/mL thrombin in 40 mM $CaCl_2$ (pH 7) and allowed to gel in an agarose mould for 45 min. at 37°C producing cylindrical constructs (Ø 5mm×3mm thickness) with final concentrations of 50 mg/mL fibrinogen, 2.5 U/mL thrombin, 5,000 KIU/mL aprotinin, 17 mg/mL sodium chloride, 20 mM $CaCl_2$ and 15×10^6 cells/mL. Constructs were maintained in 2.5 mL CM with the addition of either 1 ng/mL or 10 ng/mL TGF-β3. Media was exchanged every 3 or 4 days and sampled for biochemical analysis.

6.3.3 Dynamic compression application

Dynamic compressive loading was applied as described previously (Thorpe et al. 2010) to constructs throughout culture. Intermittent dynamic compression (DC) was carried out in an incubator-housed, dynamic compression bioreactor under strain control. The dynamic compression protocol consisted of 10% strain amplitude superimposed on a 1% pre-strain, with a 0.01 N per construct preload at a frequency of 1 Hz for 3 hours/day, 7 days/week for either 3 or 6 weeks.

6.3.4 Biochemical constituents

Constructs were analysed at day 0 (24 hours after cell encapsulation), day 21 and day 42. On removal from culture, construct diameter and wet weight (ww) were recorded and the construct frozen at -85°C for later analysis. The biochemical content of constructs (n = 3 or 4) was assessed at each time point as previously described (Thorpe et al. 2010). Samples were digested with papain (125μg/ml) in 0.1 M sodium acetate, 5 mM L-cysteine HCl, 0.05 M EDTA, pH 6.0 at 60°C under constant rotation for 18 hours. DNA content was quantified using the Hoechst Bisbenzimide 33258 dye assay as previously described (Kim et al. 1988). The sulphated glycosaminoglycan (sGAG) content was quantified using the dimethylmethylene blue dye-binding assay, with a shark chondroitin sulphate standard. Total collagen content was determined by measuring

orthohydroxyproline via the dimethylaminobenzaldehyde and chloramine T assay (Kafienah and Sims 2004). A hydroxyproline-to-collagen ratio of 1:7.69 was used (Ignat'eva et al. 2007). Cell culture media was analysed for sGAG, collagen (as above) and alkaline phosphatase using a commercially available assay kit as per manufacturer's instructions.

6.3.5 Cell viability and F-actin cytoskeleton fluorescent imaging

Cell viability was assessed using Calcein AM to stain live cell membranes and ethidium homodimer-1 to stain dead nuclei. MSC seeded constructs (n = 2) were sectioned to produce a cross section 0.5-1 mm thick perpendicular to the disc face. Sections were washed in PBS followed by incubation in Live/Dead solution containing 4μM calcein AM and 4μM ethidium homodimer-1. Sections were again washed in PBS and imaged with a Carl Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss Ltd., Hertfordshire, UK; calcein AM: 494 nm excitation, 517 nm emission; ethidium homodimer-1 in the presence of DNA: 528 nm excitation, 617 nm emission).

F-actin cytoskeletal filaments were visualised using rhodamine 110 conjugated phalloidin. Construct (n = 2) were sectioned as above, fixed in 4% paraformaldehyde overnight and washed in PBS. Cells were permeabilised in 0.5% Triton-X100, rinsed in PBS, and incubated for 1.5 hours in PBS with 1.5% BSA and rhodamine 110 conjugated phalloidin (1:40; 200U/mL). Scaffold slices were again rinsed in PBS and imaged with a Carl Zeizz LSM 510 confocal laser scanning microscope (rhodamine 110 conjugated phalloidin: 502 nm excitation, 524 nm emission).

6.3.6 Histology and immunohistochemistry

Constructs (n = 2) were fixed in 4% paraformaldehyde, wax embedded and sectioned at 5 μm to produce a cross section perpendicular to the disc face. Sections were cleared in xylene, rehydrated, and stained for sGAG with 1% alcian blue 8GX in 0.1M HCl, for collagen with picro-sirius red, and for calcific deposition with 1% alizarin red. Histochemical assessment of alkaline phosphatase (ALP) activity was performed as previously described (Miao and Scutt 2002). Deparaffinised hydrated tissue sections were incubated overnight in 1% magnesium chloride in 100mM tris-maleate buffer (pH 9.2) followed by incubation for 2 hours in ALP substrate solution consisting of

100mM tris-maleate buffer (pH 9.2) containing 0.2 mg/mL naphthol AS-MX phosphate and 0.4 mg/mL Fast Red TR. Sections were rinsed in distilled water and mounted in glycerol.

The deposition of collagen type I, collagen type II, collagen type X and α-smooth muscle actin was identified through immunohistochemistry. Sections were enzymatically treated with chondroitinase ABC in a humidified environment at 37°C. Cell membranes were permeabilised in 0.1% Triton-X100. Slides were blocked with goat serum and sections were incubated for 1 hour with the primary antibody diluted in blocking buffer as specified in

Table 6.1. After washing in PBS, sections were incubated for 1 hour in the secondary antibody, anti-mouse IgG biotin antibody produced in goat (1:133; 2 mg/mL) followed by incubation for 1 hour with ExtrAvidin-FITC (1:100). Sections were washed several times in PBS, nuclei were counterstained with DAPI (1:500; 1 mg/mL), and sections mounted using Vectashield. Sections were imaged with an Olympus IX51 inverted fluorescent microscope fitted with an Olympus DP70 camera (FITC: 495 nm excitation, 521 nm emission; DAPI: 358 nm excitation, 461 nm emission).

Table 6.1: Details of primary antibodies and controls used for immunohistochemistry.

Antigen	*Primary Antibody*	*Positive Control*	*Negative Control*
Collagen I	Monoclonal mouse (1:400)	Porcine ligament	Porcine articular cartilage
Collagen II	Monoclonal mouse (1:100; 1 mg/mL)	Porcine articular cartilage	Porcine ligament
Collagen X	Monoclonal mouse (1:200; 1.4 mg/mL)	Porcine growth plate	Porcine ligament
α-smooth muscle actin	Monoclonal mouse (1:75; 0.2 mg/mL)	Porcine femoral artery	Porcine cartilage

6.3.7 RNA isolation and real-time reverse transcriptase polymerase chain reaction

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was used to determine the relative gene expression changes in chondrogenic and myogenic specific genes subsequent to the application of dynamic compression. Total RNA was extracted from MSC seeded constructs following 21 days of culture. Constructs were harvested directly after the application of dynamic compression, snap frozen in liquid nitrogen, and stored at -85°C for later analysis. Total RNA was isolated from each construct via homogenisation using a rotor-stator homogeniser (Polytron PT 1200 E, Kinematica Inc, Switzerland) in 1 mL TRIzol® reagent followed by chloroform extraction with PureLink™ RNA Mini Kit as per manufacturer's instructions. RNA was re-suspended in RNase-free water prior to reverse transcription.

RNA concentrations were determined using the Quant-iT™ RNA assay and a Qubit® fluorometer. 100ng of RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit following manufacturer's instructions. Real-time PCR reactions were carried out in 20μL volumes containing 10μL 2× TaqMan® Universal PCR Master Mix, 5μL nuclease free water, 4μL cDNA template (1ng/μL) and 1μL 20× TaqMan® gene expression assay (primer/probes; FAM dye) as outlined in Table 6.2. Reactions were carried out on an ABI 7500 real-time PCR system with an amplification profile of 50°C for 2 min., 95°C for 10 min., followed by 40 cycles denaturation at 95°C for 15 sec. and annealing/amplification at 60°C for 1 min. Quantitative expression of target genes relative to the endogenous control reference gene (GAPDH) and the FS calibrator was carried out using the $2^{-\Delta\Delta C_T}$ method as previously described (Livak and Schmittgen 2001).

6.3.8 Statistical analysis

Statistics were performed using MINITAB 15.1 software (Minitab Ltd., Coventry, UK). Where necessary, a Box-Cox transformation was used to normalise data sets. Construct groups were analysed for significant differences using a general linear model for analysis of variance with factors of hydrogel, TGF-β3 concentration, dynamic compression and interactions between these factors examined. Tukey's test for multiple comparisons was used to compare conditions.

Significance was accepted at a level of $p \leq 0.05$. Numerical and graphical results are presented as mean ± standard error.

Table 6.2: TaqMan® primer/probes (Applied Biosystems, Life Technologies Ltd, Paisley, UK) used for real-time PCR.

Gene Name	*Gene Symbol*	*Assay ID (Applied Biosystems)*	*Amplicon Length*
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Ss03373286_u1	83
SRY (sex determining region Y)-box 9	SOX9	Ss03392406_m1	145
Aggrecan	ACAN	Ss03374822_m1	96
Collagen, type II, alpha 1	COL2A1	Ss03373344_g1	106
Actin, alpha 2, smooth muscle, aorta	ACTA2	Ss04245588_m1	84
Calponin 1, basic, smooth muscle	CNN1	Ss03392449_g1	83
Myosin, heavy chain 1, skeletal muscle, adult	MYH1	Ss03818758_s1	71
Collagen, type I, alpha 1	COL1A1	Ss03373340_m1	74
Collagen, type X, alpha 1	COL10A1	Ss03391766_m1	85

6.4 Results

6.4.1 Hydrogel type modulates MSC response to TGF-β3 stimulation

MSCs were encapsulated in agarose or fibrin hydrogels and cultured for 21 days in free-swelling unloaded conditions with the addition of either 1 ng/mL or 10 ng/mL TGF-β3. At day 21, F-actin cytoskeletal filaments were stained with phalloidin as an indication of stress fibre formation and cell morphology. MSCs seeded in agarose adopted spherical cell morphology with some processes evident at day 21, suggesting cell binding to elaborated pericellular matrix (Figure 6.1). In fibrin constructs, cells adhered to integrin binding sites present on the fibrin matrix and took on spread morphology clearly evident at day 21 (Figure 6.1). Evidence of cell-cell interactions were also observed in fibrin that were generally absent in agarose hydrogels. Cell viability staining did not

indicate any significant effect of hydrogel type or TGF-β3 concentration on live or dead cell numbers (not shown).

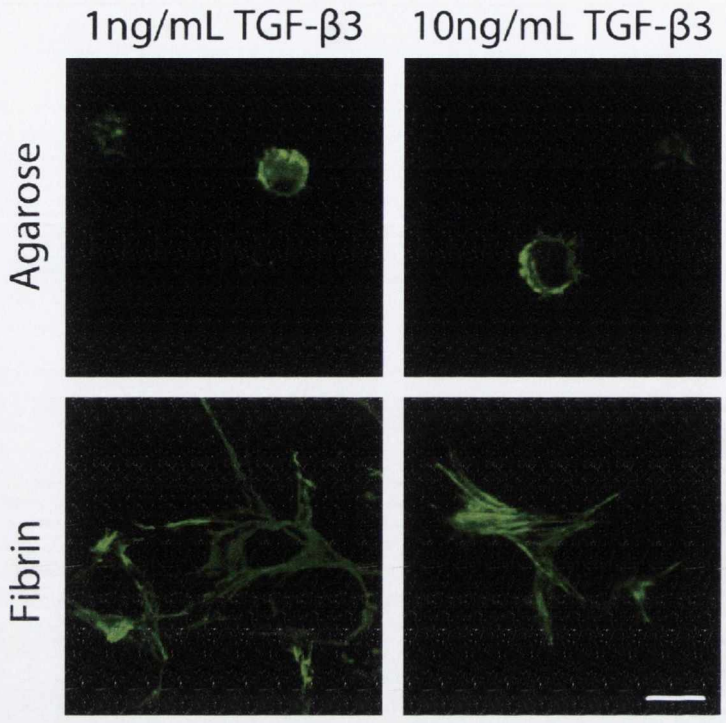

Figure 6.1: MSC seeded agarose and fibrin constructs were cultured for 21 days in FS (unloaded) conditions with the addition of either 1 ng/mL or 10 ng/mL TGF-β3. Cytoskeletal F-actin filaments in day 21 constructs were stained with rhodamine 110 conjugated phalloidin. Scale bar: 20 μm.

The influence of hydrogel type coupled with TGF-β3 concentration on markers of chondrogenic, osteogenic and myogenic differentiation was investigated. Agarose constructs supplemented with 10 ng/mL TGF-β3 accumulated the highest sGAG content at day 21 (Figure 6.2A). Alcian blue staining for sulphated mucins (Figure 6.2B) further confirmed this result. This coupled with intense collagen type II immunofluorescence (Figure 6.2C) indicated that agarose constructs supplemented with 10 ng/mL TGF-β3 achieved the most robust chondrogenic differentiation. Collagen type X staining was evident toward the centre of agarose constructs, and was reduced with increasing TGF-β3 concentration (Figure 6.2C). The media from the 10 ng/mL agarose constructs exhibited the highest alkaline phosphatase (ALP) content, significantly more than that at 1ng/mL TGF-β3 (Figure 6.2A; p=0.0001). Alizarin red staining was also evident at both 1 and 10 ng/mL TGF-β3 (Figure 6.2B); and corresponded with regions of intense collagen type I staining (Figure 6.2C).

Myogenic differentiation was assessed by immunofluorescent staining for α-smooth muscle actin. Some light staining was evident at 1 ng/mL TGF-β3 in agarose hydrogels; however this was not observed at the higher TGF-β3 concentration (Figure 6.2C).

sGAG accumulation in fibrin hydrogels also increased with TGF-β3 concentration (Figure 6.2A) and although sGAG in fibrin was comparable to agarose at 1 ng/mL TGF-β3, it was significantly less with 10 ng/mL supplementation (Figure 6.2A; $p.<0.0001$). Collagen type II immunofluorescence was negligible at 1 ng/mL TGF-β3, but stronger at the higher TGF-β3 concentration; although still markedly less than that in agarose constructs (Figure 6.2C). Total collagen accumulation however, was higher in fibrin than agarose (Figure 6.2A; $p<0.0001$); and was not significantly affected by TGF-β3 concentration. Fibrin constructs stained strongly for type 1 collagen at both TGF-β3 concentrations (Figure 6.2C) while collagen type X was absent from fibrin constructs (Figure 6.2C). ALP activity in fibrin hydrogels was significantly less than that in agarose ($p<0.0001$) and was unaffected by TGF-β3 concentration (Figure 6.2A & B). Alizarin red staining for calcific deposits was also absent in fibrin constructs (Figure 6.2B). A cell layer was seen to develop on the surface of fibrin constructs. This layer stained intensely for α-smooth muscle actin independent of TGF-β3 concentration (Figure 6.2C). If the culture period was extended to six weeks, the intensity of α-smooth muscle actin staining in FS fibrin hydrogels increased while the staining for type II collagen diminished, indicating that fibrin hydrogels preferentially support myogenic differentiation over chondrogenic differentiation (data presented as part of Figure 6.6). In addition, fibrin construct diameter reduced significantly with time for both TGF-β3 concentrations, (~6% diameter reduction; $p<0.001$) suggesting the adoption of a contractile MSC phenotype.

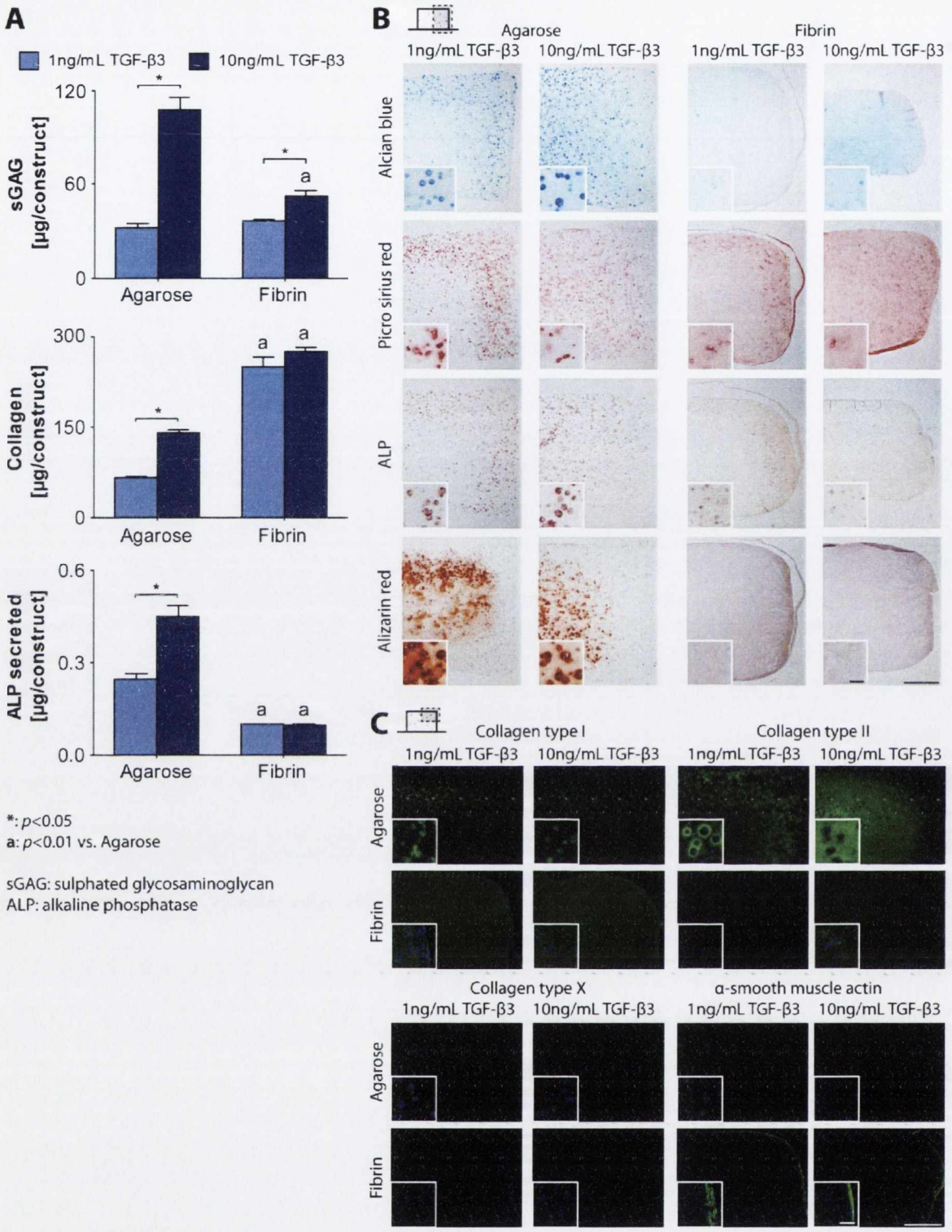

Figure 6.2: The influence of hydrogel type and TGF-β3 concentration on markers of chondrogenic, osteogenic and myogenic differentiation after 21 days FS culture. A: sGAG (top) and total collagen (middle) biochemical content of constructs and alkaline phosphatase (ALP) secreted to the media (bottom). B: Histological staining of constructs with alcian blue for sulphated mucins, picro sirius red for total collagen, alizarin red for calcific deposition, and naphthol phosphate with fast red for ALP enzyme activity. C: Immunofluorescent staining for collagen type I, collagen type II, collagen type X

and α-smooth muscle actin (green) with nucleus counterstained with DAPI (blue). Scale bar: 500 μm; inset scale bar: 50 μm.

6.4.2 Dynamic compression suppresses both chondrogenesis and myogenesis in agarose and fibrin hydrogels if applied at the onset of TGF-β3 induced differentiation

MSCs were encapsulated in both agarose and fibrin hydrogels and were cultured for 21 days in free swelling (FS) or dynamically compressed (DC) conditions in the presence of either 1 ng/mL or 10 ng/mL TGF-β3. Cell viability was unaffected by loading (data not shown). The application of dynamic compression (DC) inhibited sGAG accumulation in agarose constructs, though this effect was only significant at 10 ng/mL TGF-β3 (Figure 6.3A; $p<0.0001$). Collagen accumulation was also inhibited by DC in agarose (Figure 6.3A; $p<0.0001$), with a reduction in both collagen type I and collagen type II staining intensities (Figure 6.3C). Collagen type X staining was inhibited by DC in agarose (data not shown). In contrast, DC acted to increase ALP activity as indicated by enzyme secretion to the media (Figure 6.3A; $p=0.0001$) and staining for ALP activity (Figure 6.3B). In spite of this increased ALP activity, DC constructs were negative for calcific deposits as demonstrated by the absence of alizarin red staining (Figure 6.3B). Also, regions of intense ALP activity did not co-localise with alizarin red staining in unloaded constructs (Figure 6.3B). While some immunofluorescence for α-smooth muscle actin was observed in FS constructs supplemented with 1 ng/mL TGF-β3, DC acted to inhibit expression of this myogenic marker (Figure 6.3C).

As was the case with agarose, DC acted to inhibit sGAG ($p<0.0001$) and collagen ($p<0.0001$) accumulation in fibrin constructs (Figure 6.4A). Further evidence of suppressed chondrogenesis was seen in reduced alcian blue and collagen type II staining intensities (Figure 6.4B & C). As was the case with agarose, ALP activity increased in DC constructs as indicated by augmented ALP secretion to the media (Figure 6.4A; $p<0.0001$) and increased ALP staining (Figure 6.4B). Again, despite increases in ALP activity in DC fibrin constructs, no calcific deposition was observed through alizarin red staining (not shown). Though intense α-smooth muscle actin staining was present around the periphery of FS fibrin constructs, DC constructs stained negatively for this myogenic marker (Figure 6.4C).

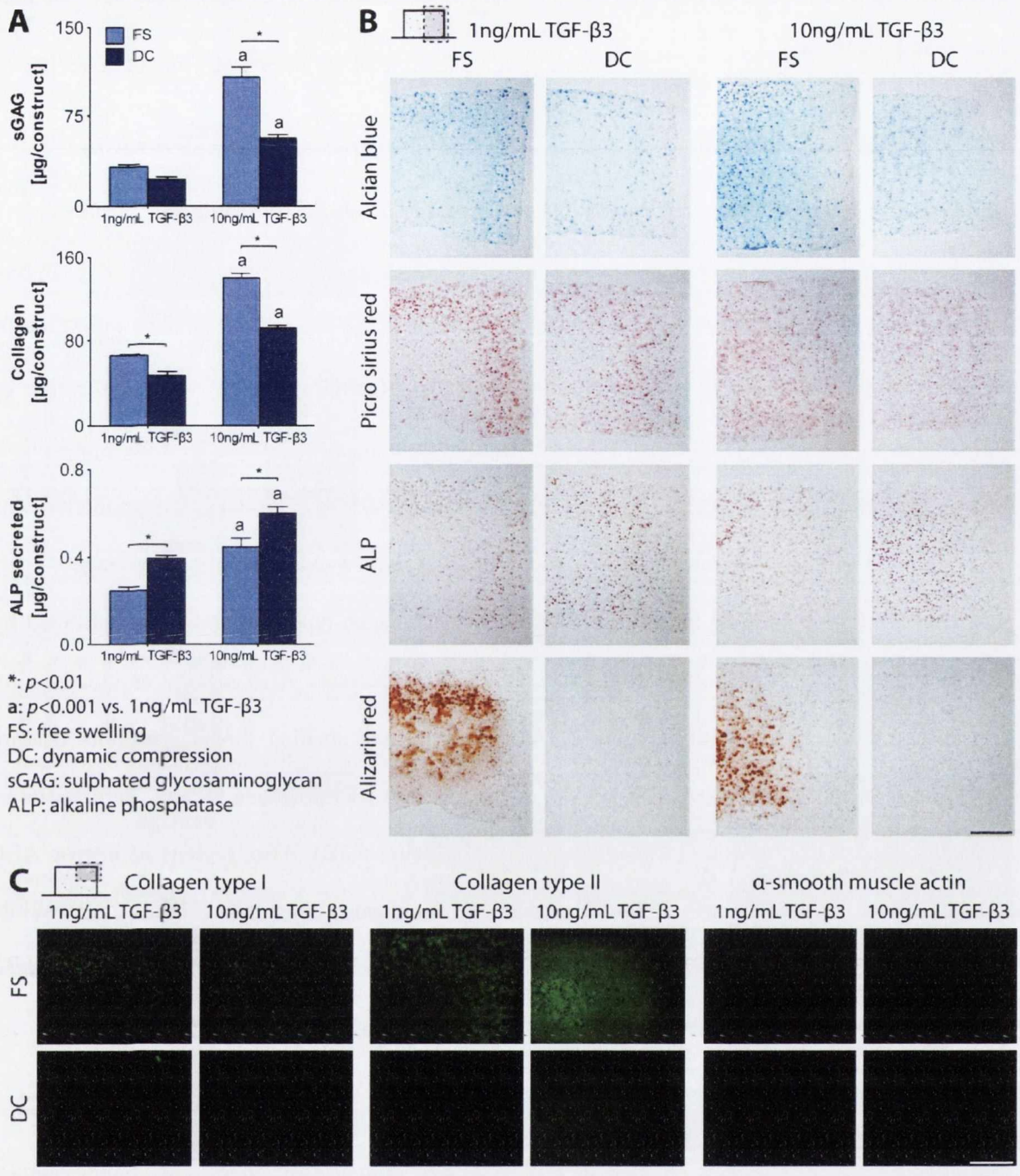

Figure 6.3: The influence of dynamic compression and TGF-β3 concentration on markers of MSC chondrogenic, osteogenic and myogenic differentiation after 21 days culture in agarose hydrogel. A: sGAG and total collagen biochemical content of constructs and alkaline phosphatase (ALP) secreted to the media. B: Histological staining of constructs with alcian blue for sulphated mucins, picro sirius red for total collagen, alizarin red for calcific deposition, and naphthol phosphate with fast red for ALP enzyme activity. C: Immunofluorescent staining for collagen type I, collagen type II and α-smooth muscle actin (green) with nucleus counterstained with DAPI (blue). Scale bar: 500 µm.

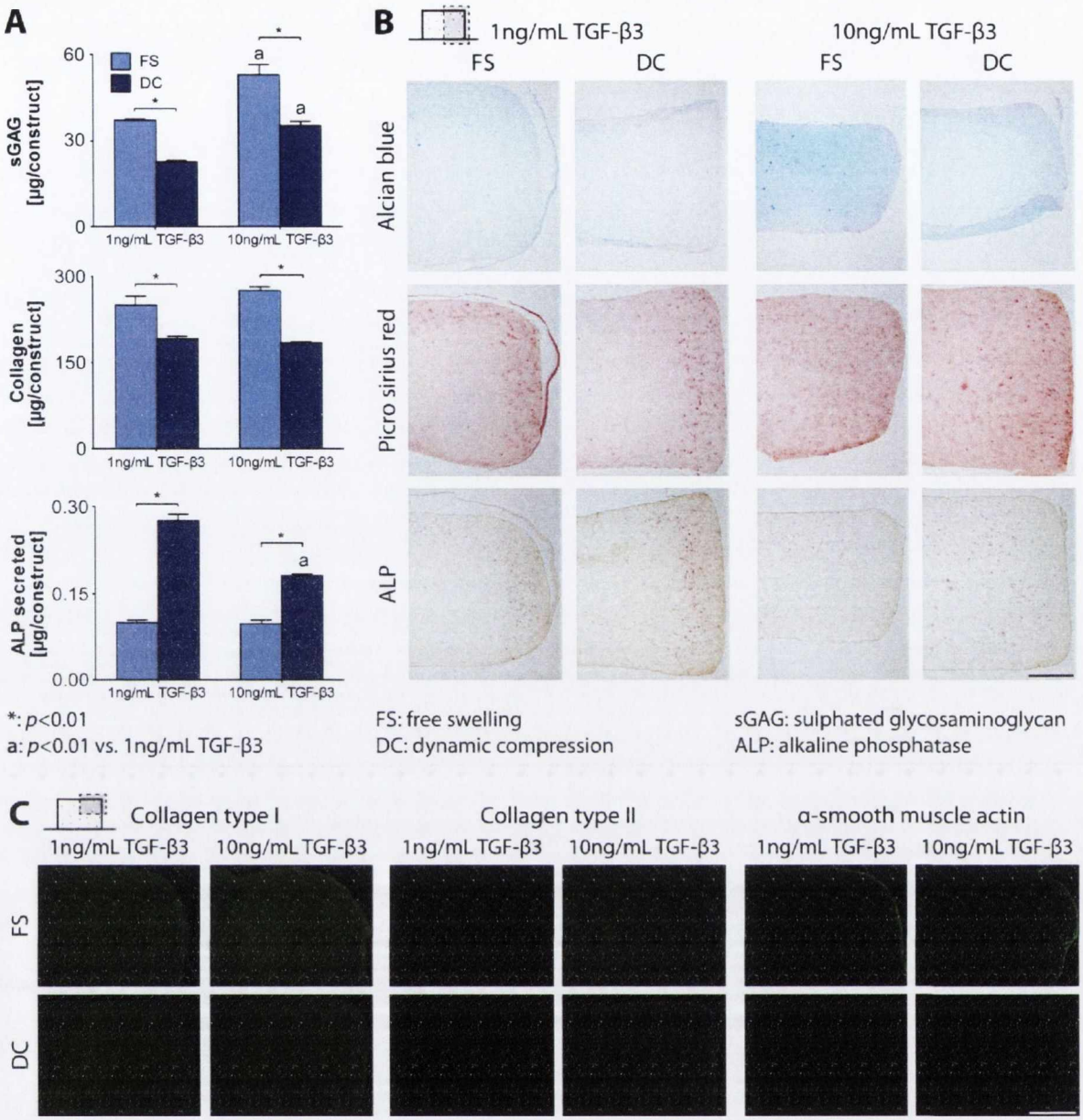

Figure 6.4: The influence of dynamic compression and TGF-β3 concentration on markers of MSC chondrogenic, osteogenic and myogenic differentiation after 21 days culture in fibrin hydrogel. A: sGAG and total collagen biochemical content of constructs and alkaline phosphatase (ALP) secreted to the media. B: Histological staining of constructs with alcian blue for sulphated mucins, picro sirius red for total collagen, and naphthol phosphate with fast red for ALP enzyme activity. C: Immunofluorescent staining for collagen type I, collagen type II and α-smooth muscle actin (green) with nucleus counterstained with DAPI (blue). Scale bar: 500 µm.

6.4.3 Extrinsic mechanical stimuli determine the eventual chondrogenic versus myogenic fate decision in MSC seeded fibrin constructs

Fibrin appeared to initially support both a chondrogenic and a myogenic phenotype. Given that fibrin clots within osteochondral defects, a mechanically loaded environment, often support a chondrogenic phenotype, it was hypothesised that in the long-term dynamic compression could direct MSCs toward a chondrogenic as opposed to a myogenic phenotype. This hypothesis was partially motivated by our previous studies in agarose hydrogels which demonstrated that the application of dynamic compression following 3 weeks of TGF-β3 induced differentiation enhances subsequent chondrogenesis (Haugh et al. 2011). To further probe the effect of dynamic compression on differentiation within fibrin hydrogels, constructs were harvested for analysis of gene expression using real time RT-PCR after 21 days in the presence of 10 ng/mL TGF-β3. DC had no effect on the chondrogenic genes Sry-related high mobility group box-9 (SOX9) and aggrecan (ACAN; Figure 6.5). However DC did up-regulate collagen type II (COL2A1) gene expression relative to FS controls (Figure 6.5; p=0.0011). Gene expression for collagen type X (COL10A1), an endochondral marker, was expressed at low levels and was not modulated by DC (Figure 6.5). This corroborated with negative immuno staining for collagen type X (not shown). Collagen type I (COL1A1), a marker of both osteogenic and myogenic differentiation was down regulated in response to dynamic compression (Figure 6.5; p=0.0004). Expression of myogenic markers, α-smooth muscle actin (ACTA2) and calponin-1 (CNN1) were strongly down-regulated in DC fibrin constructs (Figure 6.5; p=0.0001 & p=0.0286 respectively), suggesting that DC inhibits myogenic differentiation. Gene expression of heavy chain myosin-1, a late marker of myogenic differentiation, was expressed at low levels in both FS and DC groups.

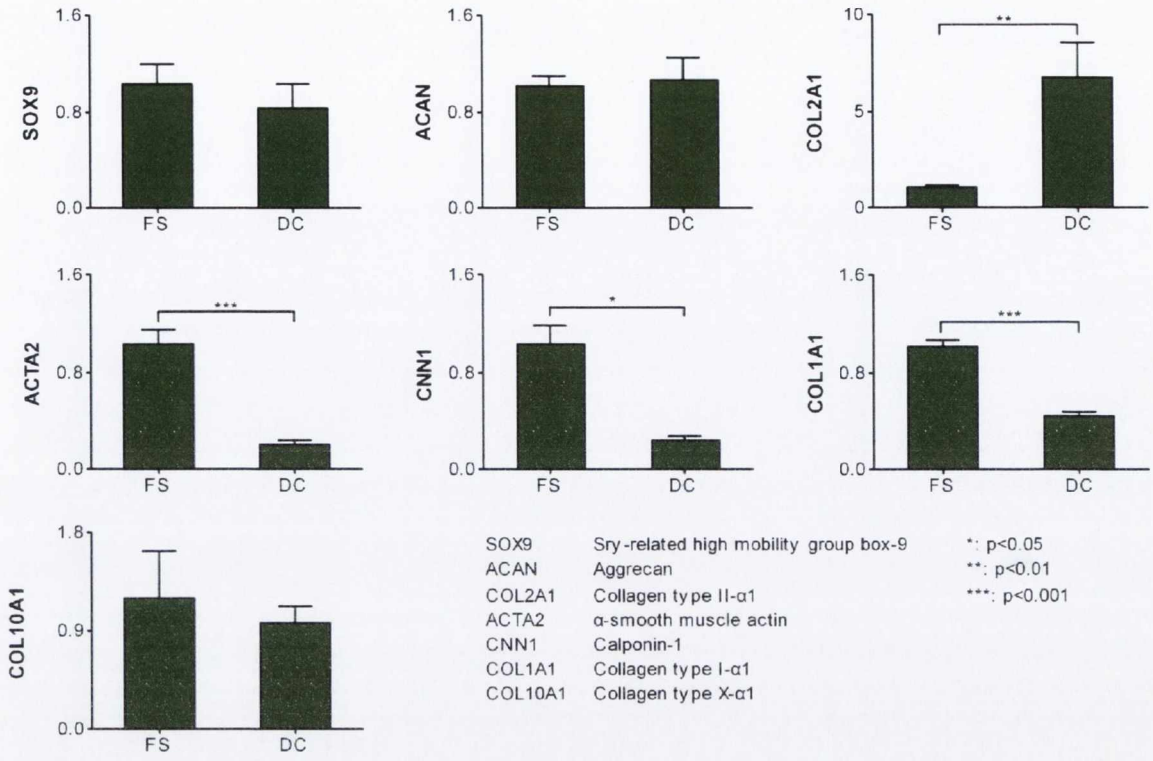

Figure 6.5: Dynamic compression was applied to fibrin encapsulated MSCs for 21 days with chondrogenic, myogenic and endochondral gene expression assessed at day 21. Dynamically compressed construct gene expression is presented relative to the free-swelling control. FS: Free-swelling. DC: Dynamic compression.

We next explored how such changes in gene expression would impact markers of differentiation in the long-term. MSCs encapsulated in fibrin hydrogels were cultured for 42 days with 10 ng/mL TGF-β3 in free swelling (FS), dynamically compressed (DC) or delayed dynamically compressed (DDC) conditions. Dynamically compressed (DC) constructs were subjected to dynamic compression for 3 hours daily from day 0 to day 42 while delayed dynamic compressed (DDC) constructs were cultured in FS conditions up to day 21 when dynamic compression for 3 hours daily was initiated and continued until day 42. As described previously, long-term FS culture in fibrin hydrogels appeared to be more conductive to a myogenic phenotype, as evident by increased collagen production (Figure 6.6A) and strong staining for both α-smooth muscle actin and type I collagen (Figure 6.6C). As might be expected with the suppression of myogenic differentiation, dynamic compression inhibited cell-mediated contraction, such that DC constructs had a significantly larger diameter than FS constructs (p=0.0208). Dynamic compression from day 0

(DC) led to an increase in the rate of sGAG and collagen accumulation from day 21 to day 42 compared to FS controls, such that total accumulation was comparable to FS controls by day 42 (Figure 6.6A). Initiation of dynamic compression at day 21 (DDC) had a positive effect on sGAG accumulation such that it was greater than both FS and DC constructs by day 42 (Figure 6.6A; p=0.0189 & p=0.0356 respectively). Although the total ALP secreted at day 42 did not differ across dynamic compression conditions, the temporal patterns of ALP secretion did. ALP secretion increased between days 21-42 compared with the first 21 days for FS and DDC constructs (Figure 6.6A; p=0.0002 & p=0.0004 respectively) while it decreased for DC constructs between days 21-42 (Figure 6.6A; p<0.0001). There were no discernible differences in ALP enzyme activity staining across dynamic compression conditions (Figure 6.6B) and all constructs stained negatively for calcific deposition with alizarin red (not shown).

Immunofluorescent staining for collagen type II at day 42 was markedly more intense for DC constructs than either FS or DDC constructs (Figure 6.6C), correlating with the up-regulation of COL2A1 gene expression in response to dynamic compression at day 21 (Figure 6.5). Collagen type I immunofluorescence was also reduced in DC constructs compared to both FS and DDC constructs at day 42 (Figure 6.6C). DC appeared to completely suppress α-smooth muscle actin staining, while DDC constructs exhibited some light staining close to the construct edge (Figure 6.6C).

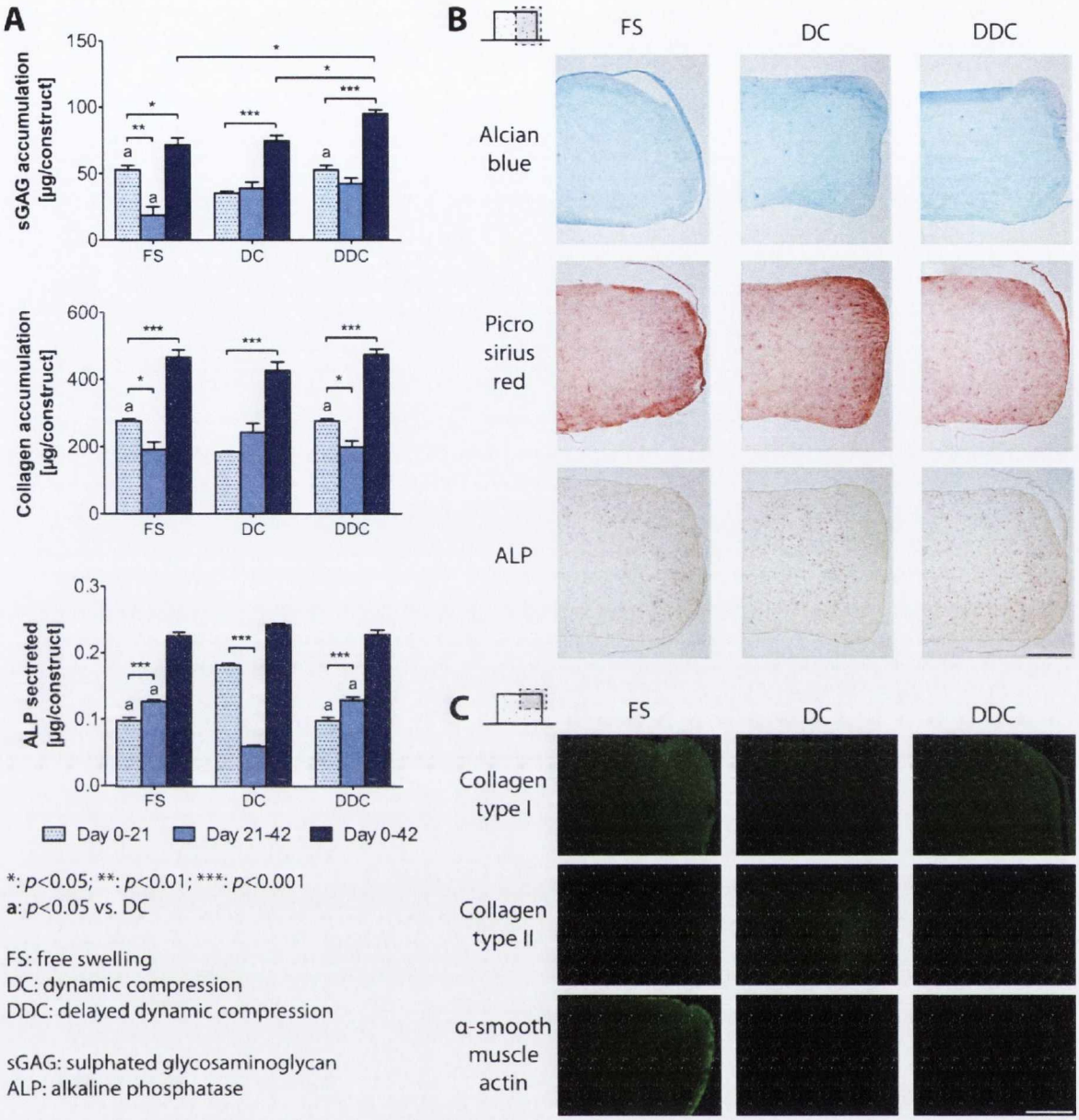

Figure 6.6: The influence of long-term dynamic compression on markers of MSC chondrogenic, osteogenic and myogenic differentiation after 42 days culture in fibrin hydrogel. A: sGAG accumulated (top), collagen accumulated (middle), and alkaline phosphatase (ALP) secreted to the media from days 0-21, days 21-42 and days 0-42 (bottom). B: Histological staining of constructs with alcian blue for sulphated mucins, picro sirius red for total collagen, and naphthol phosphate with fast red for ALP enzyme activity. C: Immunofluorescent staining for collagen type I, collagen type II and α-smooth muscle actin (green) with nucleus counterstained with DAPI (blue). Scale bar: 500 µm.

6.5 Discussion

The aim of the study was to explore how cell-matrix interactions and the external mechanical environment interact to regulate stem cell fate in response to TGF-β. Agarose was found to support a spherical cell morphology and a chondrogenic phenotype, while fibrin facilitated a spread cell morphology, stress fibre formation and cell-cell interactions. Initially fibrin constructs stained positive for both chondrogenic and myogenic markers, but in the long-term this substrate supported a more myogenic phenotype, as evident by strong staining for α-smooth muscle actin and weak staining for type II collagen after 6 weeks in free swelling culture. However, the ultimate fate decision of the MSCs was not determined by the specific characteristics of the local substrate, but rather by the external mechanical environment. Initially dynamic compression suppressed differentiation along either pathway, but in the long-term these mechanical signals directed the MSCs along a chondrogenic pathway as opposed to the default myogenic route supported by the uncompressed fibrin clot.

In a free swelling environment, the fate decision of MSCs was determined by the local substrate. Unlike agarose, fibrin facilitates integrin mediated cellular attachment through two pairs of RGD sites (Cheresh et al. 1989). Chondrogenic differentiation of MSCs has been shown to be repressed within RGD-modified agarose hydrogels (Connelly et al. 2008). Chondrogenic differentiation was most robust in agarose constructs where cells assumed a spherical morphology. Myogenesis was suppressed within agarose hydrogels. In contrast, myogenic differentiation was evident in fibrin constructs where MSCs took on a well spread morphology, with evidence of cell-cell interactions and the formation of F-actin stress fibres resulting in significant cell mediated hydrogel contraction. This is in agreement with Gao *et al.* (2010) who demonstrated that a rounded cell shape led to an up-regulation of chondrogenic genes while a spread cell shape led to an up-regulation of myogenic genes in the presence of TGF-β3. While fibrin does facilitate cell attachment, this may not be the only factor leading to myogenic differentiation in this hydrogel. Fibrin is a complex biological material with many growth factors and cytokines in addition to unknown ligands present, which may impact MSC differentiation within this scaffold (Breen et al. 2009).

Chondrogenically primed bone marrow MSCs have been shown to eventually undergo hypertrophy and endochondral ossification (Pelttari et al. 2006; Farrell et al. 2009; Hellingman et al. 2010). Agarose hydrogel appears to support an endochondral pathway for encapsulated MSCs, apparent in ALP activity and calcific deposits. No evidence of mineralisation or collagen type X was observed in fibrin constructs, suggesting that fibrin encapsulated MSCs may not have progressed sufficiently along the chondrogenic pathway to enable further differentiation toward an endochondral phenotype.

Dynamic compression initiated at the onset of differentiation suppressed markers associated with the chondrogenic, myogenic and endochondral phenotypes. Suppression of chondrogenesis with dynamic compression in agarose has been previously observed (Thorpe et al. 2008; Huang et al. 2010a). Both chondrogenic and osteogenic differentiation markers have been shown to be reduced in response to dynamic compression of MSCs encapsulated in RGD modified poly(ethylene glycol) hydrogel (Steinmetz and Bryant 2011). Furthermore, dynamic compression has been shown to inhibit cartilage specific matrix production in chondrocyte seeded fibrin (Hunter et al. 2004). The only marker increased by dynamic compression in the short-term was ALP enzyme activity as measured in the culture media. This has been proposed as an *in vitro* marker suggestive of subsequent calcification (Dickhut et al. 2009), however in this case, increased ALP activity did not translate to calcification. Loading may be suppressing mineralisation through inhibition of chondrogenesis and the subsequent progression along the endochondral pathway. Alternatively, dynamic compression could be preventing differentiation towards hypertrophy, helping to maintain a more stable chondrogenic phenotype.

While dynamic compression initially suppressed chondrogenesis in fibrin hydrogels, long-term dynamic compression was associated with increased expression of type II collagen. sGAG and collagen accumulation increased between day 21 and 42 in response to continued dynamic compression while a concurrent decrease in ALP activity was observed. Moreover, accumulated collagen was predominantly collagen type II, in agreement with increased gene expression at day 21. Most noticeable however was the complete suppression of α-smooth muscle actin and reduced

collagen type I expression, demonstrating that mechanical loading was driving the MSC fate decision in fibrin constructs.

At this stage the molecular mechanism through which dynamic compression regulates the myogenic versus chondrogenic fate decision in fibrin hydrogels is unclear. Cell shape has been shown to regulate this fate decision (Gao et al. 2010), although there was no strong evidence to suggest that dynamic compression is leading to a more spherical cell morphology in fibrin hydrogels. Gao *et al.* (2010) demonstrated that TGF-β3 activated Rac1 and increased N-cadherin expression in spread cells, leading to the up-regulation of myogenic genes. Dynamic compression could potentially impact at any point along this signalling cascade. For example, dynamic compression could potentially reduce the expression of N-cadherin or alter associations with N-cadherin at the cell membrane, for example N-cadherin and β-catenin association, which has previously be shown to be mechano-regulated (Arnsdorf et al. 2009). If this were indeed the case, it could potentially explain the temporal response of MSCs to loading. N-cadherin is required for the initial cellular condensation at the onset of chondrogenesis (Oberlender and Tuan 1994a), but its expression diminishes as differentiation along this pathway progresses (Oberlender and Tuan 1994b). If dynamic compression inhibits N-cadherin expression, it may explain the initial suppression of both myogenesis and chondrogenesis, but the long-term promotion of a chondrogenic phenotype. Further work is required to elucidate the molecular mechanism involved in this stem cell fate decision.

In conclusion, we have shown that MSC differentiation in response to TGF-β stimulation is dependent upon the hydrogel within which the cells are encapsulated. Perhaps more importantly, this study demonstrates that cues present in the *in vivo* environment, such as mechanical loading, can override the influence of specific substrates, scaffolds or hydrogels that have been shown to modulate stem fate in an *in vitro* context. In the context of stem cell based therapies for tissue regeneration, it may be that the site specific mechanical environment is the ultimate regulator of phenotype and function.

Chapter 7 Discussion

7.1 Overview

Tissue engineering aims to develop biological substitutes for the functional restoration of damaged or diseased tissue through the interdisciplinary application of principles of engineering and the life sciences (Langer and Vacanti 1993). The tissue engineering approach has gone through a recent change in focus with the identification of multipotent mesenchymal stem cells (MSCs) as a potential source for many applications (Caplan 1991). MSCs are responsive to a variety of external mechanical stimuli and biological factors, which can regulate their differentiation and function. The biochemical induction of MSCs is a powerful approach to achieve robust *in vitro* differentiation down many lineages, including the chondrogenic, myogenic, osteogenic and adipogenic pathways. However, as illustrated in Chapter 6, and elsewhere (Arnsdorf et al. 2010), biochemical stimulation alone may not be sufficient to ensure terminal MSC differentiation down the desired lineage.

It is widely accepted that MSCs respond to mechanical stimulation *in vitro* (Potier et al. 2010a) and considerable evidence exists to endorse the essential role of mechanical forces in healthy skeletal development (Nowlan et al. 2007). Biomechanical factors provide an ever present stimulus *in vivo* and must be considered as a vector with which to attain directed MSC differentiation. Through scaffold design, or as an intrinsic feature of most *in vivo* environments, mechanical stimulus can be maintained beyond *ex vivo* culture upon construct implantation. Additionally, mechanical signals can induce rapid (<300 ms) mechano-chemical conversion, resulting in responses 40 times faster than that induced by soluble growth factors (Wang et al. 2009a). Therefore the control of MSC chondrogenic differentiation through mechanical stimulation forms the motivation for this research.

The primary objective of this thesis was to investigate the chondrogenic differentiation of mesenchymal stem cells (MSCs) in a 3-dimensional environment in response to mechanical stimuli. This was achieved through the encapsulation of bone marrow derived MSCs in three-

dimensional hydrogel constructs, which were subjected to intermittent dynamic compression. The studies which comprise this thesis have demonstrated that hydrogel encapsulated MSCs are responsive to mechanical stimulation. Furthermore, this response is dependent on factors such as the differentiation state of the cell, nutrient availability, oxygen tension, cell seeding density and cell-matrix interactions.

It was demonstrated that dynamic compression of agarose encapsulated MSCs inhibited their chondrogenic differentiation in the presence of TGF-β3 (Chapter 3). When agarose encapsulated MSCs were pre-differentiated in the presence of TGF-β3 in unloaded conditions, the subsequent application of dynamic compression following TGF-β3 withdrawal led to an increase in matrix accumulation (Chapter 4). Leading on from these results, a construct with zonal gradients in matrix constituents and mechanical properties mimicking aspects of articular cartilage was obtained through the application of dynamic compression in combination with partial confinement of the MSC seeded agarose hydrogel (Chapter 5). The density of MSC encapsulation in agarose was seen to modulate the response to mechanical stimulation with increased cell seeding density leading to an increase in cartilage specific matrix production in response to dynamic compression (Chapter 5). Finally cell-matrix interactions were investigated and it was demonstrated that dynamic compression regulated the chondrogenic-myogenic fate decision of MSCs seeded within fibrin hydrogel (Chapter 6).

Throughout this thesis many factors relating to the chondrogenic differentiation of MSCs and subsequent chondrogenesis of the engineered tissue were directly or indirectly investigated. While a number of these factors such as the differentiation state of the cell, nutrient availability, oxygen tension and cell seeding density warrant a more detailed discussion which can be found below, other factors such as TGF-β3 concentration varied across experiments. At the outset, unconfined dynamic compression initiated on MSC encapsulation inhibited construct chondrogenesis when cultured in the presence of 10 ng/mL TGF-β3 (Chapter 3 and Chapter 4). The TGF-β3 concentration of 10 ng/mL typically used to induce chondrogenic differentiation of MSCs is supra-physiological. Further studies were conducted in the absence of TGF-β3 and while dynamic compression did lead to small increases in sGAG accumulation, the absolute levels of matrix

accumulation were an order of magnitude lower than those induced through TGF-β3 supplementation alone (Appendix A). In the final study presented in Chapter 6, a lower TGF-β3 concentration of 1 ng/mL was investigated. Dynamic compression at this concentration similarly inhibited chondrogenesis. Not only did dynamic compression inhibit chondrogenesis, but it inhibited osteogenesis and myogenesis, suggesting that this mechanical stimulus is inhibiting extracellular matrix production (Chapter 6). Fibrin encapsulation of MSCs did not alter the cellular response with regard to matrix accumulation over 21 days (Chapter 6). However when dynamic compression was continued past day 21, pro-chondrogenic effects were observed with increased collagen type II deposition and sGAG synthesis in the second period of culture (day 21-day 42) when compared to free-swelling controls. This was a result not observed in Chapter 4 where MSCs were encapsulated in agarose; with matrix production remaining low throughout the 42 day culture period. Another difference between the studies in Chapter 6 and Chapter 4 was the duration of dynamic compression which was for 3 hours/day, 7 days/week to fibrin constructs in Chapter 6 while it was applied for 1 hour/day, 5 days/week for to agarose constructs in Chapter 4. Dynamic compression for 5 days/week appeared to have a negative effect on fibrin-MSC constructs leading to a large amount of cell-mediated contraction over the 2 unloaded days. This indicates that even early on in culture, MSCs are responsive to dynamic compression in fibrin. The differential effect of long-term dynamic compression in fibrin compared to agarose may be due to this daily dynamic compression, or to the increased daily duration of loading stimulus.

7.2 Modulators of MSC response to dynamic compression

7.2.1 Differentiation state of the cell

One of the principal findings of this thesis is that the MSC response to dynamic compression is modulated by the differentiation state of the cell and/or the development of a pericellular matrix (PCM). It is widely accepted that the application of appropriate levels of dynamic compression can enhance the biosynthetic activity of chondrocytes (Palmoski and Brandt 1984; Sah et al. 1989; Kim et al. 1994; Buschmann et al. 1995; Mauck et al. 2000). Therefore, the influence of long term

dynamic compression on MSC chondrogenesis in agarose hydrogel was investigated in the presence of TGF-β3. Contrary to expectation, it was found that dynamic compression inhibited functional MSC chondrogenesis as measured by ECM accumulation (Chapter 3). Though short term (≤7 days) dynamic compression of hydrogel encapsulated MSCs has been shown to up-regulate chondrogenic gene expression (Huang et al. 2004a; Huang et al. 2005; Terraciano et al. 2007), others have demonstrated a down regulation of gene expression when dynamic compression and TGF-β1 were applied in parallel (Campbell et al. 2006; Mouw et al. 2007). More recently this result was corroborated by Huang *et al.* (2010a) who despite seeing an increase in chondrogenic gene expression with dynamic compression in the presence of TGF-β3, found that dynamic compression significantly inhibited chondrogenic matrix accumulation by day 21.

Mouw *et al.* (2007) observed that the gene expression response of agarose encapsulated MSCs to dynamic compression was dependent on the chondrogenic state of the cell, with an enhanced response to dynamic compression seen only after 16 days of free-swelling pre-culture in the presence of TGF-β1. To investigate whether matrix accumulation would be similarly modulated, dynamic compression was applied to MSCs after 21 days pre-culture in the presence of TGF-β3 (Chapter 4). This delayed application of dynamic compression did not inhibit chondrogenesis as assessed by matrix accumulation and construct mechanical properties. 21 days of TGF-β3 supplementation induced a chondrogenic phenotype in agarose seeded MSCs. This was evident through the formation of a dense collagen type II and sulphated glycosaminoglycan rich pericellular matrix by day 21 (Figure 4.3); and may result in a chondrocyte-like response to dynamic compression. Interestingly, a beneficial effect of dynamic compression was only seen on removal of TGF-β3; a result mirrored by Lima *et al.* (2007) where chondrocyte matrix accumulation was increased in response to dynamic compression post 14 days pre-culture in the presence of TGF-β3. In contrast to the aforementioned chondrocyte study, continued TGF-β3 culture of MSCs remained a more potent pro-chondrogenic stimulus than dynamic compression; suggesting that while these cells will respond to dynamic compression in a manner similar to chondrocytes, they lack the matrix forming capacity of chondrocytes in the absence of growth factor stimulation.

Huang *et al.* (2010a) have also demonstrated a beneficial effect of dynamic compression post TGF-β3 induced differentiation on MSC chondrogenesis, however they did not observe an increase in matrix production, but an increase in construct mechanical properties, and only with continued TGF-β3 supplementation. A recent investigation by Haugh *et al.* (2011) into the spatial and temporal effects of dynamic compression on the chondrogenic gene expression of agarose encapsulated MSCs demonstrated that dynamic compression applied at day 21 enhanced expression of aggrecan and collagen type II genes, while initiation of dynamic compression at day 14 inhibited the expression of these genes (Figure 7.1).

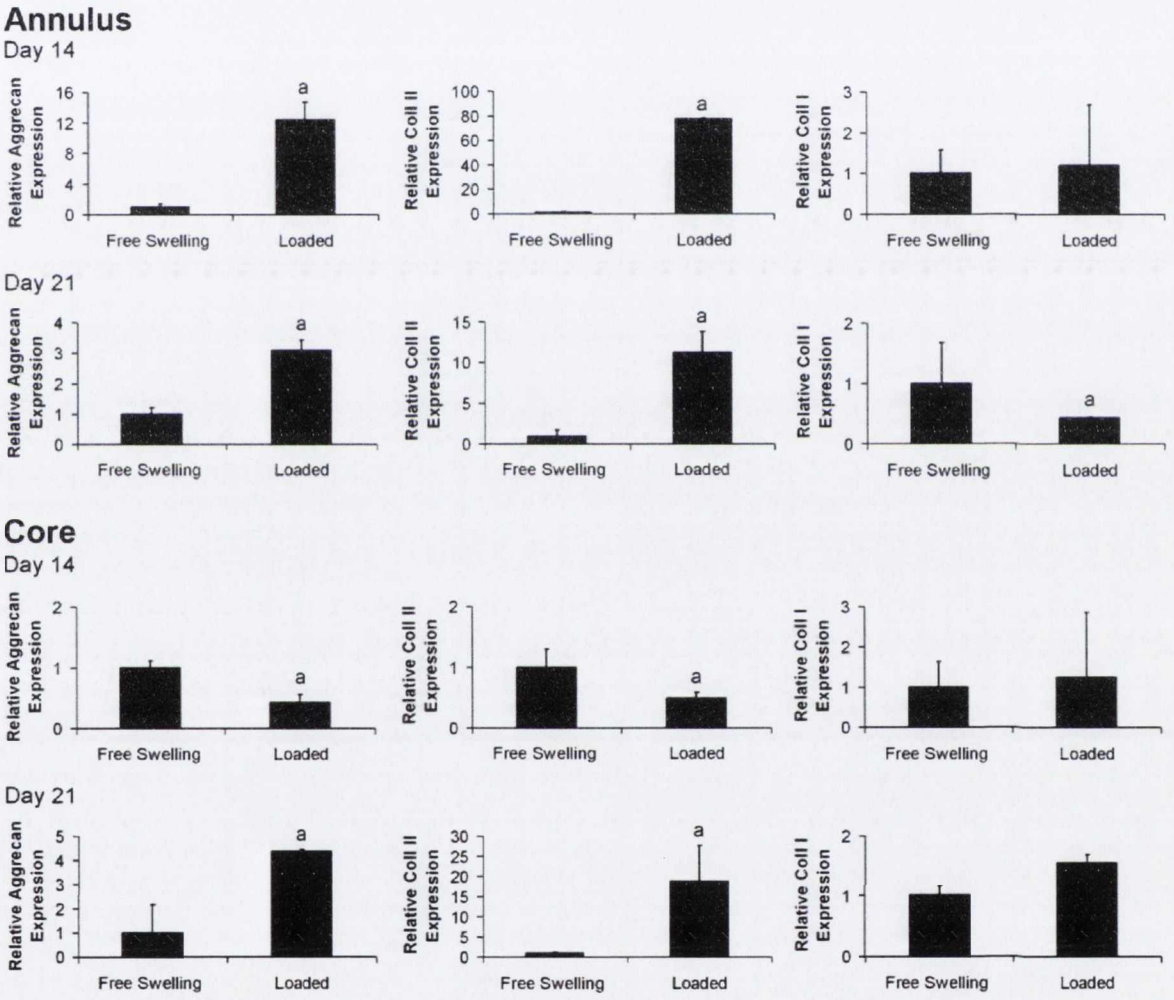

Figure 7.1: Changes in cartilage matrix specific gene expression in response to dynamic compression at days 14 and 21. Relative gene expression is normalized to expression in free swelling core and annulus samples at each time point. n = 3 samples per group per time point, a: $p<0.05$ versus free swelling group. Adapted from Haugh *et al.* (2011).

In an attempt to develop a cell which would react positively to dynamic compression immediately on encapsulation, chondrogenic priming of MSCs during monolayer expansion was investigated. The details of this study are presented in Appendix A. Monolayer MSCs were expanded in the presence of fibroblast growth factor-2 (FGF-2) and TGF-β3, and dynamic compression applied for 21 days after agarose encapsulation. Chondrogenic pre-culture in monolayer did not lead to enhanced matrix accumulation, suggesting that priming needs to occur in a three-dimensional environment. Thus, the studies herein suggest that a chondrogenic phenotype and/or a well-developed pericellular matrix must first be established for dynamic compression to have a positive effect on cartilage matrix specific gene expression.

7.2.2 Nutrient availability and oxygen tension

One consistent finding of this thesis was the spatial distribution of accumulated extracellular matrix (ECM) in constructs, implying that nutrient availability and/or oxygen tension are regulators of MSC chondrogenic differentiation. Separate analysis of core and annulus hydrogel regions identified that extracellular matrix accumulation was consistently superior in the central region for cell seeding densities of 15×10^6 or 20×10^6 cells/mL. One explanation may be that a large proportion of the extracellular matrix produced in the annulus is secreted to the culture media. Indeed, significant quantities of sGAG and collagen were observed in the culture media. Evidence suggests a role for TGF-β3 supplementation in the release of sGAG to the media.

An alternative explanation for this spatial difference in matrix deposition may be reduced oxygen tension in the construct core. Oxygen tension has been shown to play a role in the chondrogenic differentiation of MSCs with hypoxia promoting MSC chondrogenesis in the presence of chondrogenic growth factors (Kanichai et al. 2008). Meyer *et al.* (2010) recently investigated the interaction between low oxygen culture and dynamic compression, and found that low oxygen tension is a more potent promoter of chondrogenic differentiation than dynamic compression. Culture in 5% O_2 led to homogeneous matrix distribution in contrast to the core-annulus effects seen with culture at 21% O_2 (Figure 7.2), suggesting oxygen tension may play more of a role than

loss of extracellular components to the media. This is further supported by Haugh *et al.* (2011) who observed increased aggrecan and collagen type II gene expression in the construct core.

When the cell seeding density was increased from 20×10^6 to 50×10^6 cells/mL, the core region was relatively deficient in matrix while the periphery stained intensely for cartilage specific extracellular matrix (Figure 5.12). This shift in the region of intense matrix accumulation may be due to increased nutrient and oxygen consumption in the periphery such that cells residing in the centre of the construct are deprived. It has previously been shown that diffusional limitations in oxygen supply can result in the development of anaerobic conditions resulting in reduced matrix synthesis rates in chondrocyte seeded polymer scaffolds (Obradovic et al. 1999).

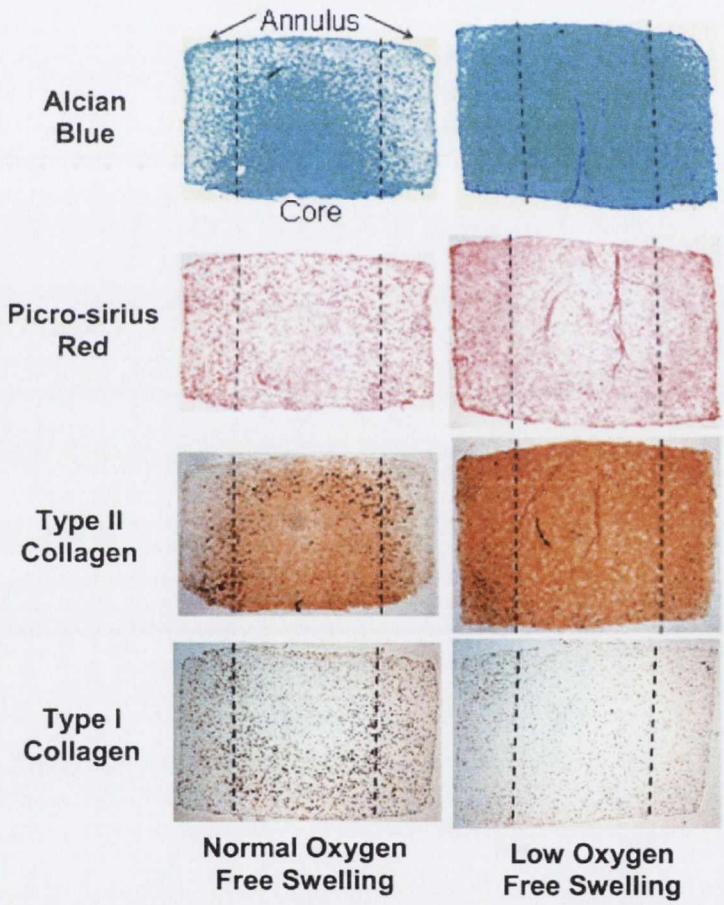

Figure 7.2: Histological full cross section (4×) for normal (21%) and low (5%) oxygen constructs at day 42. Alcian blue staining for sulphated proteoglycan, picro-sirius red for collagen and collagen types II and I immunohistochemistry. Adapted from Meyer *et al.* (2010).

Dynamic compression has been shown to increase solute transport within hydrogel constructs (Mauck et al. 2003a; Albro et al. 2008). One mechanism through which dynamic compression may modulate differentiation is through enhanced nutrient transport. The inhibition of chondrogenesis observed when dynamic compression is applied to constructs following encapsulation in the presence of TGF-β3 (Chapter 3) may be due to elevated oxygen levels in the construct core. Additionally, the enhanced sGAG accumulation detected as a result of dynamic compression in constructs seeded with 50×10^6 cells/mL (Chapter 5) may be due to enhanced solute transport into the centre of the construct, allowing a greater number of the cells present to actively produce extracellular matrix.

7.3 Experimental considerations

7.3.1 Cell source

The ultimate goal of tissue engineering is to develop techniques to aid the repair of human tissue. While no animal model permits direct application to humans, the principles and knowledge gained may translate in due course to human application (Reinholz et al. 2004). Animal models are critical to testing new techniques and products prior to clinical trials. Porcine MSCs have been successfully used by other researchers to investigate MSC differentiation (Ringe et al. 2002; Abukawa et al. 2003; Ando et al. 2007; Pei et al. 2008; Elder et al. 2009).

The minimum criteria to define human MSCs as outlined by the International Society for Cellular Therapy (Dominici et al. 2006) are as follows:

1. MSCs must be plastic-adherent when maintained in standard culture conditions.
2. MSCs must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR surface molecules.
3. MSCs must differentiate to osteoblasts, adipocytes and chondroblasts *in vitro*.

While surface marker expression of the MSCs used in these experiments was not performed, cells were plastic adherent and positive for the colony forming unit-fibroblast (CFU-F) assay, in addition to the consistent demonstration of tri-potentiality with multiple porcine donors. Others using similar isolation protocols have shown that porcine bone marrow MSCs isolated using the plastic adherence technique are positive for CD105 and CD90, and negative for CD11b, CD31, CD45 and CD133 (Vacanti et al. 2005; Bosch et al. 2006).

7.3.2 Multiple modes of mechano-stimulation

Dynamic compression is a combination of hydrostatic pressure, fluid flow and distortional strain. Each of these stimuli may differentially modulate MSC differentiation (Carter et al. 1988; Prendergast et al. 1997), and may explain why matrix accumulation varies spatially throughout hydrogel constructs. In Chapter 5, the heterogeneous mechanical environment under dynamic compression was exploited to develop a construct with depth varying properties. Others have additionally investigated the effect of shear in combination with dynamic compression in an attempt to better approximate the *in vivo* joint environment (Li et al. 2010a). To ascertain the effect of a specific stimulus such as hydrostatic pressure, distortional strain, or fluid flow, the stimulus in question would need to be applied alone.

Finite element modelling has provided a powerful tool for the analysis of the changing mechanical environment within a hydrogel undergoing dynamic compression. A number of studies have examined the spatial distribution of strain, fluid flow, and pore pressure within agarose (Section 5.4.1) (Huang et al. 2004a; Mauck et al. 2007) and fibrin (Noailly et al. 2008) constructs.

7.3.3 Strain transfer through hydrogel matrix

Though a specific strain regime can be accurately applied to a hydrogel construct, due to differences in stiffness between the hydrogel and cell it cannot be assumed that the cellular strain is the same as that applied, for example 10%. This strain transfer through the hydrogel to the construct is further compounded by the temporal development of pericellular matrix (PCM) surrounding the cell and the elaboration of extracellular matrix (ECM) throughout the construct. This phenomenon has been examined both experimentally and computationally for chondrocyte

seeded constructs (Knight et al. 1998; Knight et al. 2002; Appelman et al. 2011). The presence of PCM significantly reduces strain transfer to the cell, effectively shielding the cell from the applied stress (Knight et al. 1998). Appelman *et al.* (2011) in a computational study of cells within a hydrogel construct found that not only did PCM exhibit a stress shielding effect, leading to reduced peak stresses within the cell, but PCM modulated the stress distribution within and around encapsulated cells by redirecting maximum stresses from the cell periphery to the nucleus (Figure 7.3). These changes in strain transfer may mediate the MSC response to dynamic compression with time. It should be noted that these studies only relate to spherical shaped cells as transpire when encapsulated in agarose. For the spread cells observed in fibrin, the cellular deformation is likely to be very different. To the author's knowledge, cellular deformation in fibrin hydrogel in response to dynamic compression has not been investigated.

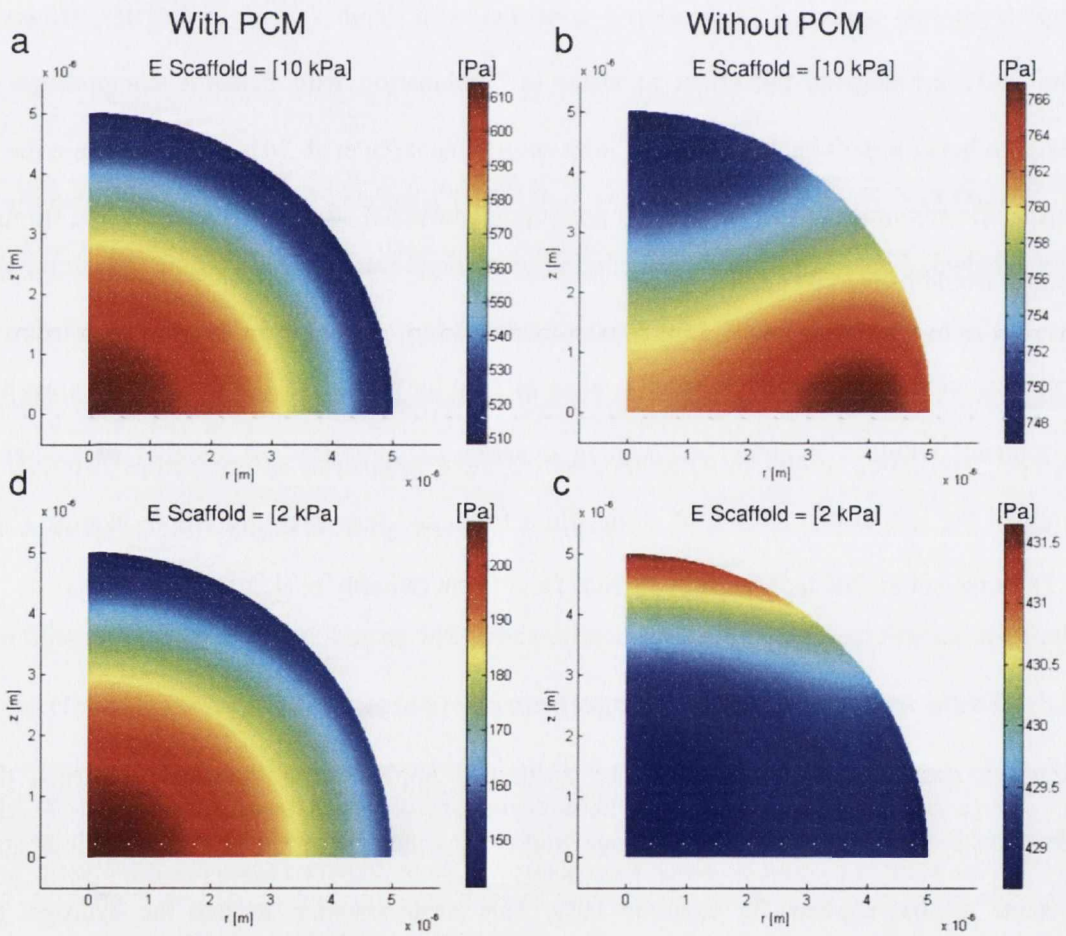

Figure 7.3: Von Mises stresses within the cell space ((a) and (d)) with and ((b) and (c)) without PCM for the cases that the scaffold modulus was ((a) and (b)) 10 kPa and ((c) and (d)) 2 kPa. Changes can be noted in both spatial distribution and magnitude. Adapted from Appelman *et al.* (2011).

7.3.4 Dynamic compression regime

The dynamic compression protocol employed changed over the course of this thesis. In Chapter 3, dynamic compression was applied at a frequency of 0.5 Hz, while in all other studies it was applied at 1 Hz. The duration of dynamic compression also changed with 1 hour applied in Chapter's 3 and 4, while dynamic compression was applied for 4 hours in Chapter 5, and 3 hours in Chapter 6. A dynamic compression frequency of 0.5 Hz was chosen in Chapter 3 as this falls within the physiological range. Given the observed inhibition of chondrogenesis as a result of dynamic compression, and evidence that 1 Hz dynamic compression enhances sGAG synthesis in chondrocyte seeded agarose constructs when compared to 0.3 Hz, it was decided to increase the loading frequency to 1 Hz.

Much evidence has since emerged indicating that 1 Hz may be the optimum dynamic compression frequency for chondrogenic differentiation of MSCs. Kock *et al.* (2009) demonstrated enhanced chondrogenic gene expression in agarose encapsulated MSC for 1 Hz dynamic compression when compared to 0.33 Hz. A similar study, comparing 0.1 Hz and 1 Hz dynamic compression after pre-differentiation in TGF-β3 found dynamic compression at 1 Hz increased construct stiffness when compared to 0.1 Hz (Huang et al. 2010a). Comparable results have been observed in fibrin constructs with one study demonstrating increased viability and chondrogenic gene expression in fibrin encapsulated MSCs with a dynamic frequency of 1 Hz compared to either 0.5 Hz or 0.1 Hz (Pelaez et al. 2009), while others attained increased sGAG accumulation and chondrogenic gene expression in MSC seeded fibrin-polyurethane composites with 1 Hz compared to 0.1 Hz dynamic compression.

In an effort to establish if chondrogenic differentiation of agarose encapsulated MSCs through dynamic compression in the absence of chondrogenic cytokines was possible, the duration of dynamic compression application and the applied strain were investigated. The details of this experiment are presented in Appendix A. Both 1 and 3 hours of dynamic compression to 5% or 10% strain led to increased sGAG content when compared to free-swelling (FS) controls (Figure A.1). However, 12 hours of dynamic compression inhibited sGAG accumulation. It was recently demonstrated that 4 hours of dynamic compression per day applied post free-swelling TGF-β3

induced differentiation is required to obtain an increase in the mechanical properties of MSC seeded agarose constructs compared to free-swelling (FS) controls (Huang et al. 2010a). In chapter 5, dynamic compression was to be used to modulate both matrix accumulation and the mechanical properties of MSC seeded agarose construct, and therefore dynamic compression was applied for 4 hours daily.

7.3.5 Biomaterial choice

The studies in this thesis utilised agarose and fibrin hydrogels for three-dimensional MSC culture. Hydrogels have many advantages over other scaffold types which include uniform cell seeding throughout the construct, and shape stability. Additionally, both the stiffness and permeability of these gels can be controlled through alterations in their constituent concentrations. Agarose, a thermoreversible polysaccharide hydrogel has been used extensively for long-term chondrocyte cultures (Buschmann et al. 1992) and more recently, chondrogenic culture of MSCs (Awad et al. 2004; Huang et al. 2004b). Fibrin, a natural biopolymer differs to agarose in that it is a bioactive scaffold (Breen et al. 2009). Fibrin is biodegradable, and its rate of degradation, in addition to its stiffness, and micro-structure can be extensively altered with variations in the concentrations of its components including fibrinogen, thrombin, calcium chloride, sodium chloride and factor XII (Janmey et al. 2009; Potier et al. 2010b). Fibrin component concentrations were chosen to produce a long-term stable hydrogel which has been shown to support the chondrogenic phenotype (Eyrich et al. 2007). Importantly, the properties of both hydrogels were tailored to permit application of physiological deformation loading (10% peak-to-peak at 0.5 Hz or 1 Hz) immediately on encapsulation.

Chapter 8 Conclusion

8.1 Main results

The overall objective of this thesis was to investigate the chondrogenic differentiation of mesenchymal stem cells (MSCs) in a three dimensional environment in response to mechanical stimulus; namely dynamic compression. The differentiation of MSCs in response to dynamic compression was explored in three dimensional agarose and fibrin hydrogels. The main conclusions of this thesis are as follows:

- The chondrogenic differentiation of agarose encapsulated MSCs is regulated by the mechanical environment. Dynamic compression of MSC seeded agarose constructs initiated at the onset of TGF-β3 stimulation inhibited chondrogenic differentiation.
- MSC response to dynamic compression is modulated by the differentiation state of the cell and/or the development of a pericellular matrix. Allowing TGF-β3 mediated differentiation to occur for 21 days prior to the initiation of dynamic compression resulted in a response which was no longer inhibitory of chondrogenesis.
- By controlling the environment through the depth of developing constructs, tissues with depth-dependent gradients in biochemical constituents and mechanical properties similar, but not identical to normal articular cartilage can be engineered using MSCs. Modulation of both oxygen tension and the mechanical environment resulted in region specific matrix synthesis which can be tailored to produce constructs with specific zonal compositions.
- Cell seeding density modulates the MSC response to dynamic compression in agarose hydrogel. Increasing MSC seeding density resulted in a more robust chondrogenic response to dynamic compression which enhanced cartilage specific matrix accumulation and overall construct mechanical properties.

- MSCs would appear to proceed towards terminal (endochondral) differentiation in agarose hydrogel in the presence of TGF-β3.
- *In vivo* cues such as the mechanical environment can override the influence of specific substrates, scaffolds or hydrogels that have been shown to modulate stem cell fate *in vitro*. Long term dynamic compression regulates MSC differentiation fate in a fibrin hydrogel; directing MSCs along a chondrogenic phenotype as opposed to the default myogenic route supported by the uncompressed fibrin clot.

This thesis demonstrates that the chondrogenic differentiation of MSCs is mechano-regulated. Knowledge of the response of hydrogel encapsulated MSCs to dynamic compressive strain will improve our capacity to engineer a functional cartilage replacement tissue through the development of optimum MSC conditioning regimes prior to implantation. Additionally, this work contributes to our general understanding of MSC mechanobiology and may impact a wide range of applications in stem cell related regenerative medicine.

8.2 Future directions

This thesis has provided significant insights into the mechanoregulation of MSCs. However, at least as many questions remain to be answered as when the work was initiated, largely due to the development of new insights as the work progressed. The following recommendations are made for future work on the subject of this thesis.

- MSC response to dynamic compression has been shown to be modulated by the differentiation state of the cell. The positive reaction to MSC differentiation seen after chondrogenic induction with TGF-β3 may be due to changes within the cell, or due to the stress shielding effects of the elaborated pericellular matrix. After chondrogenic induction, pericellular matrix could be digested away before initiation of dynamic compression. This would further elucidate the role of the pericellular matrix in mechanotransduction.

- While a construct with depth varying composition and mechanical properties analogous to articular cartilage was achieved, concentrations of cartilage specific matrix components and functional mechanical properties of the developed constructs were substantially inferior to that of articular cartilage. Cell source and cell seeding density are both variables which could have a powerful effect on the success of this technique. For example infrapatellar fat pad derived MSCs may exhibit superior functional chondrogenesis than bone marrow derived MSCs (Buckley et al. 2010), and their response to dynamic compression remains largely unknown.
- *In vivo* mechanical stimulation also incorporates shear loading across the articular surface. This stimulus may be necessary for the development of key matrix constituents such as proteoglycan-4 (lubricin) and for the development of Benninghoff collagen fibre architecture. This stimulus could be incorporated into a bioreactor design to better represent *in vivo* joint loading and possibly enhance the functional properties of engineered tissues.
- Bone marrow derived MSCs precede toward endochondral differentiation even in the presence of chondrogenic growth factors. The role that mechanical factors play in the maintenance of a stable chondrocyte phenotype needs to be further elucidated.
- While dynamic compression governs the chondrogenic versus myogenic switch for fibrin encapsulated MSCs, the mechanism through which this occurs remains to be identified. Rac1 and N-cadherin has been shown to regulate the switch in MSC fate due to cell shape. Forced expression of Rac1 and N-cadherin in dynamically compressed constructs may elucidate the potential role of this pathway in mechano-regulated MSC differentiation.

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Appendix A Can dynamic compression in the absence of growth factors induce chondrogenic differentiation of bone marrow derived MSCs in agarose hydrogels?

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For the purposes of this thesis, the study presented is adapted from the original article published in the International Federation for Medical and Biological Engineering (IFMBE) Proceedings, 2011, Vol. 30, pp. 43-46.

A.1 Abstract

The objectives of this study were twofold; to determine if cartilage specific matrix synthesis by mesenchymal stem cells (MSCs) is regulated by the magnitude and/or duration of dynamic compression in the absence of growth factors, and to investigate if expanding MSCs in the presence of both fibroblast growth factor-2 (FGF-2) and transforming growth factor β-3 (TGF-β3) would influence their subsequent response to dynamic compression following encapsulation in agarose hydrogels. Porcine bone marrow derived MSCs were suspended in agarose and cast to produce cylinders (Ø5 × 3 mm). Constructs were maintained in a chemically defined medium. Dynamic compression was applied at 1 Hz with strain amplitudes of 5%, 10%, and 5% superimposed upon a 5% pre-strain for durations of 1, 3 and 12 hours. MSCs were also expanded in the presence of FGF-2 and TGF-β3. The biochemical constituents of constructs were analysed. Under strain magnitudes of 5% and 10% and durations of 1 and 3 hours small increases in sGAG accumulation relative to unloaded controls were observed. However this was orders of magnitude lower than that induced

by TGF-β3 stimulation. Expansion in FGF-2 and TGF-β3 did not positively modulate chondrogenesis of MSCs in either unloaded or loaded culture.

A.2 Introduction

Chondrogenic differentiation of MSCs can be induced by members of the transforming growth factor-β (TGF-β) superfamily (Johnstone et al. 1998; Mackay et al. 1998; Yoo et al. 1998). The differentiation pathway and biosynthetic activity of MSCs is also somewhat regulated by their biophysical environment (Knothe Tate et al. 2008; Kelly and Jacobs 2010). MSCs have been cultured three-dimensionally in vitro in bioreactors designed to imitate joint loading, most commonly hydrostatic pressure and dynamic compression (Angele et al. 2003; Huang et al. 2004a; Mauck et al. 2007). It has been demonstrated that dynamic compressive loading in the absence of TGF-β family members can increase chondrogenic gene expression (Huang et al. 2004a; Huang et al. 2005; Campbell et al. 2006; Park et al. 2006; Mauck et al. 2007; Terraciano et al. 2007; Huang et al. 2010a) and accretion of cartilage specific ECM constituents (Park et al. 2006; Mauck et al. 2007; Kisiday et al. 2009). Nevertheless some uncertainty remains as despite increases in gene expression, a number of studies have also demonstrated no positive effect of loading on glycosamino-glycan accumulation in the absence of chondrogenic cytokines (Huang et al. 2010a; Meyer et al. 2010).

Expansion conditions can influence the subsequent chondrogenic differentiation of MSCs. Monolayer expansion in the presence of fibroblast growth factor-2 (FGF-2) has been shown to enhance proliferation during expansion and the subsequent chondrogenic potential of MSCs (Solchaga et al. 2005; Stewart et al. 2007; Ito et al. 2008; Solchaga et al. 2010). The addition of growth factors including FGF-2 and TGF-β1 to chondrocyte expansion media has been shown to modulate the cells potential to re-differentiate and respond to regulatory molecules on transfer to a 3D environment (Jakob et al. 2001; Capito and Spector 2006). Similarly, media including these growth factors added to fat pad and synovial cells during expansion resulted in enhanced sulphated glycosaminoglycan (sGAG) deposition and collagen type II gene expression (Marsano et al. 2007).

It remains unclear what role MSC expansion conditions will have on the cells subsequent response to biophysical stimulation.

As outlined, there is significant variability in the reported responses of MSCs to mechanical loading. This can potentially be attributed to numerous factors, one of which could be that MSCs are sensitive to the magnitude and duration of loading they experience, as suggested by a number of computational studies (Prendergast et al. 1997; Kelly and Prendergast 2005). It has previously been demonstrated that the frequency of dynamic compression modulates MSC viability and chondrogenic differentiation in the absence of growth factors (Pelaez et al. 2009). The first objective of this study was to determine if cartilage specific matrix synthesis by MSCs is also regulated by the magnitude and/or duration of dynamic compression in the absence of growth factors. Related to this question, it is unclear what effect MSC expansion conditions will have on their subsequent response to dynamic compression. The second objective of this study is therefore to investigate if expanding MSCs in the presence of both FGF-2 and TGF-β3 will influence their response to dynamic compression following encapsulation in agarose hydrogels.

A.3 Methods

The commercial sources of reagents are listed in Appendix B (Table B.1).

A.3.1 Experimental Design

This study comprised of two parts. The first involved MSC expansion in normal conditions (Norm) with constructs undergoing various magnitudes and daily durations of dynamic compression (DC) for 6 days in the absence of TGF-β3 as outlined in Table A.1. Unloaded free-swelling controls (FS) were kept both in the absence and presence of TGF-β3. Constructs were taken for analysis at day 7 and day 28. The second study involved MSC expansion either in normal conditions (Norm) or with the addition of FGF-2 and TGF-β3 (FGF/TGF). Constructs from both conditions were kept in the absence or presence of 10 ng/mL TGF-β3 (CM- or CM+), and loaded to 10% strain for 2

hours/day, 5 days/week for 3 weeks. Each part of the study was performed independently with separate porcine donors; $n = 4 - 6$ constructs per condition.

Table A.1: Loading conditions investigated in Study A.

Strain amplitude	*Duration*	*TGF-β3*
0%	0 hours	+/-
5%	1, 3 hours	-
10%	1, 3, 12 hours	-
5% (pre-strain) + 5%	1, 3, 12 hours	-

A.3.2 Cell isolation and expansion

MSCs were isolated from the femora of two 4 month old porcine donors (~50 kg) within 3 hours of sacrifice as de-scribed (Thorpe et al. 2008). Mono-nuclear cells were plated at a seeding density of 2.5×10^6 cells / 10 cm dish for colony forming unit fibroblast assay, or 10×10^6 cells / 75cm^2 flask for expansion in high-glucose Dulbecco's modified eagles medium (4.5 mg/mL D-Glucose, 200 mM L-Glutamine; hgDMEM) supplemented with 10% foetal bovine serum (FBS) and penicillin (100 U/mL)-streptomycin (100 µg/mL) (Norm). FGF-2 (5 ng/mL) and TGF-β3 (0.5 ng/mL) were added to a number of flasks from each animal (FGF/TGF) as described above. At the first passage colonies were stained with crystal violet and counted to obtain the colony-forming cell fraction. Cells were subsequently plated at 5×10^3 cells/cm^2 and expanded to passage two (18.5 – 20 population doublings) in a humidified atmosphere at 37 °C and 5% CO_2.

A.3.3 Agarose encapsulation and dynamic compression

MSCs from 2 donors were pooled, suspended in hgDMEM and mixed with 4% agarose (Type VII) in phosphate buffered saline (PBS) at a ratio of 1:1 at ~40 °C, to yield a final gel concentration of 2% and a cell density of either 30×10^6 cells/mL (Study A) or 15×10^6 cells/mL (Study B). The agarose-cell suspension was cast between two plates and cored to produce cylindrical constructs (Ø5 mm × 3 mm thickness). Intermittent dynamic compression was carried out as described

previously (Thorpe et al. 2010). The dynamic compression protocol consisted of strain amplitude and duration as described above at 1 Hz.

A.3.4 Biochemical analysis

The biochemical content of constructs (n = 3 – 4) was assessed at each time point as described previously (Thorpe et al. 2010). Constructs were cored using a 3 mm biopsy punch and digested with papain (125 μg/mL) at 60 °C for 18 hours. This enabled spatial variations in biochemical content of the core and annulus of the construct to be determined. DNA content was quantified using the Hoechst Bisbenzimide 33258 dye assay (Kim et al. 1988). Sulphated glycosaminoglycan (sGAG) was quantified using the dimethylmethylene blue dye-binding assay. Total collagen content was determined through measurement of the hydroxyproline content (Kafienah and Sims 2004) with a hydroxyproline-to-collagen ratio of 1:7.69 (Ignat'eva et al. 2007). Samples of cell culture medium taken for analysis at each media exchange (n = 3) were analysed for sGAG and hydroxyproline secreted to the media.

A.3.5 Statistical analysis

Statistics were performed using MINITAB 15.1 software package (Minitab Ltd., Coventry, UK). Groups were analysed for significant differences using a general linear model for analysis of variance. Tukey's test for multiple comparisons was used to compare conditions. Significance was accepted at a level of $p \leq 0.05$. Numerical and graphical results are presented as mean ± standard error.

A.4 Results

A.4.1 Study A: The influence of compression magnitude and duration

In the absence of TGF-β3 construct DNA content dropped with time in FS culture and was significantly less at day 28 than at either day 0 or day 7 ($p<0.05$). Conversely, with TGF-β3 addition, DNA content increased with time ($p<0.05$). FS controls in the presence of TGF-β3 demonstrated robust chondrogenesis, with sGAG accumulation of 1.03±0.05 %ww with TGF-β3

supplementation as opposed to 0.02±0.0003 %ww in its absence. While increases in sGAG were slight, statistically significant increases were seen above FS controls in construct annuli for both 5% and 10% strain for durations of 1 and 3 hours ($p<0.05$; Figure A.1). Dynamic compression periods of 12 hours were detrimental to accumulation of both sGAG and collagen in construct cores. In the absence of TGF-β3, ECM accumulation was greatest in the annulus region ($p<0.0001$), while in its presence ECM accumulation was greatest in the core ($p=0.0003$).

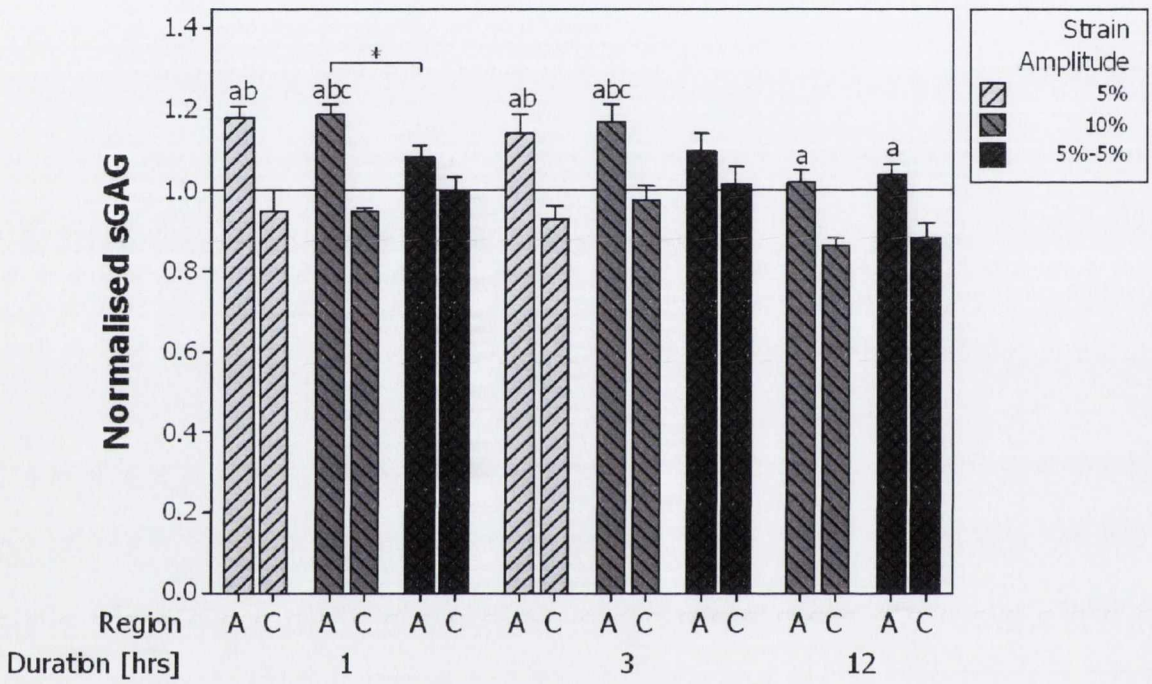

Figure A.1: Day 28 construct sGAG [%wet-weight] normalised to FS control. A: annulus, C: core. A: $p<0.05$ vs. core; b: $p<0.05$ vs. FS control; c: $p<0.05$ vs. 12 hrs.

A.4.2 Study B: Expansion conditions

Greater consistency in colony forming units between donors was observed for cells expanded in the presence of FGF-2 and TGF-β3 (Figure A.2). Colonies tended to be smaller and stained more intensely under FGF/TGF expansion than Norm conditions (Figure A.2). On analysis of expansion media, FGF/TGF expanded MSCs were found to have secreted substantial quantities of sGAG while non-measurable levels were found in the Norm expansion media group.

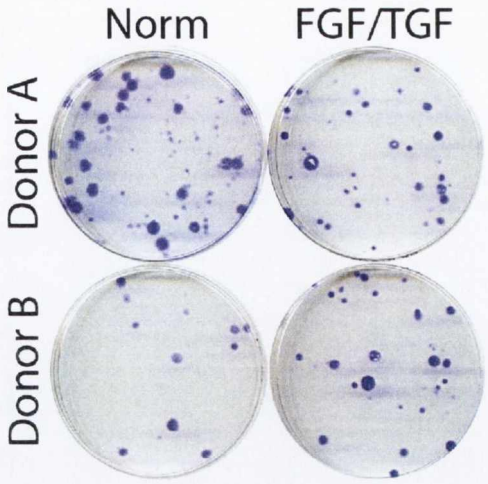

Figure A.2: Representative CFU-F assay plates for 2 porcine donors with Norm or FGF/TGF expansion.

Dynamic compression in CM+ conditions inhibited matrix accumulation, although increases were seen over day 0 sGAG and collagen levels. In the absence of TGF-β3, loading had no effect on sGAG or collagen accumulation (Figure A.3). Overall, sGAG accumulation was greatest in the Norm expansion FS constructs; however this was not significantly different than under FGF/TGF expansion (Figure A.3A). These same trends were also apparent for collagen accumulation (Figure A.3B). Despite low collagen accumulation levels under CM- conditions, a considerable quantity was secreted to the media (2.45±0.29 μg/construct accumulated vs. 56.79±2.62 μg/construct secreted; not shown). Significantly more collagen was secreted to the media than remained in the construct for all CM+ groups bar FS Norm expansion. A greater proportion of total collagen production was secreted to media for loaded constructs in comparison to FS (p=0.003), although levels remained lower than FS groups.

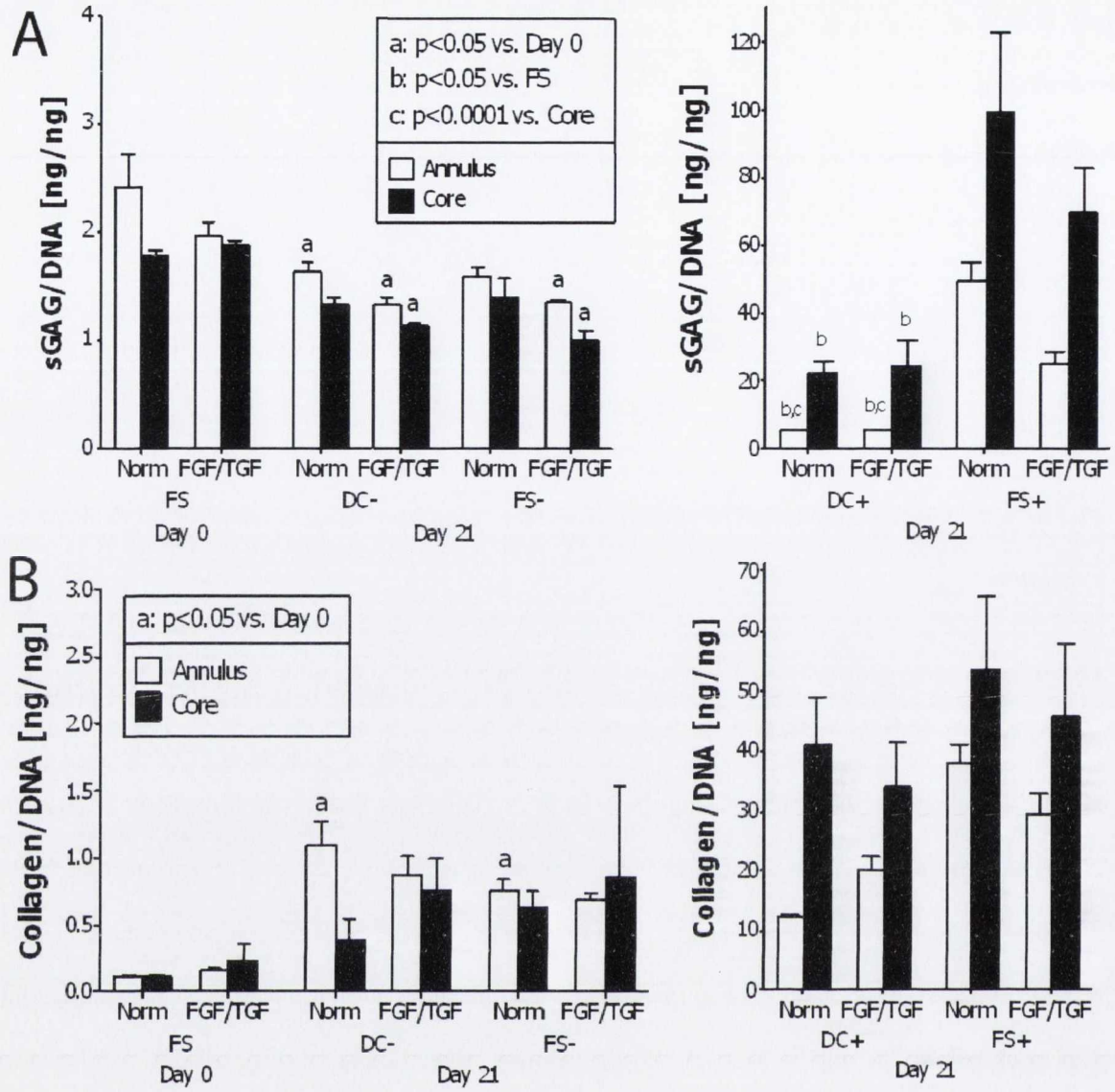

Figure A.3: Biochemical composition of annulus and core regions at day 0 and day 21 for Norm and FGF/TGF expansion. A: sGAG/DNA [ng/ng]. B: Collagen/DNA. Left: Agarose construct culture in absence of TGF-β3 (CM-), Right: Agarose construct culture in presence of TGF-β3 (CM+).

A.5 Discussion

Previously, Huang *et al.* (2004a) showed that dynamic compression alone applied to rabbit MSCs in agarose induced similar levels of aggrecan and collagen type II gene expression as TGF-β1 stimulation. Under certain magnitudes and durations we saw an increase in sGAG accumulation relative to FS controls. However this was orders of magnitude lower than that affected through

TGF-β3 stimulation as seen here and elsewhere (Mauck et al. 2007; Kisiday et al. 2009). In the absence of growth factors, dynamic compression has been shown to increase chondrogenic gene expression without a consequent increase in accumulated extra cellular matrix (ECM) (Huang et al. 2010a). This may suggest a disparity between gene expression results and accumulated ECM.

MSC expansion conditions did not affect the subsequent response to loading. Expansion with FGF-2 and TGF-β3 reduced sGAG and collagen accumulation in unloaded culture in the presence of TGF-β3, although this was not significant. This result is in contrast to previous work where fat pad and synovium cells were treated with FGF-2 and TGF-β1 (Marsano et al. 2007). This may be due to different concentrations of TGF-β used, the fact that they also added platelet-derived growth factor-bb, or simply variation between cell types. FGF-2 and TGF-β3 supplementation during expansion failed to illicit a more positive response to dynamic compression than found in cells expanded under Norm conditions. Future work will examine other scaffold systems where cells adhere to the substrate. This may alter the response of MSCs to mechanical stimuli.

To conclude, dynamic compression in the absence of growth factors can augment sGAG accumulation by bone marrow MSCs in agarose hydrogel in comparison to unloaded controls; however this remains orders of magnitude lower than that induced due to TGF-β3 stimulation. Expansion in FGF-2 and TGF-β3 did not positively modulate chondrogenesis of MSCs in either loaded or unloaded culture.

Appendix B Commercial sources of materials

Table B.1: Commercial sources of materials.

Cell isolation and expansion and culture	
Porcine donors	BioResources Unit, Trinity College Dublin, Ireland
Cell culture plastic	Nunc, Thermo scientific, Dublin, Ireland
Phosphate buffered saline (PBS)	Sigma-Aldrich, Arklow, Ireland
hgDMEM GlutaMAX™	Gibco, Invitrogen, Dublin, Ireland
Foetal Bovine Serum (FBS)	Gibco, Invitrogen, Dublin, Ireland
Penicillin-Streptomycin	Gibco, Invitrogen, Dublin, Ireland
Amphotericin B	Sigma-Aldrich, Arklow, Ireland
Sodium pyruvate	Sigma-Aldrich, Arklow, Ireland
L-proline	Sigma-Aldrich, Arklow, Ireland
Bovine serum albumin	Sigma-Aldrich, Arklow, Ireland
Linoleic acid	Sigma-Aldrich, Arklow, Ireland
Insulin-transferrin-selenium 100x (ITS)	Sigma-Aldrich, Arklow, Ireland
L-ascorbic acid-2-phosphate	Sigma-Aldrich, Arklow, Ireland
Dexamethasone	Sigma-Aldrich, Arklow, Ireland
Transforming growth factor-β3 (TGF-β3) (Chapters 3 & 4)	R&D Systems, Abingdon, UK
Transforming growth factor-β3 (TGF-β3) (Chapters 5 & 6 and Appendix A1)	ProSpec-Tany TechnoGene Ltd., Rehovot, Israel
Fibroblast Growth Factor-Basic Human Recombinant (FGF-2)	ProSpec-Tany TechnoGene Ltd., Rehovot, Israel
Crystal violet	Sigma-Aldrich, Arklow, Ireland

Agarose hydrogel encapsulation	
Agarose type VII	Sigma-Aldrich, Arklow, Ireland
Biochemical constituents	
Papain	Sigma-Aldrich, Arklow, Ireland
L-cysteine	Sigma-Aldrich, Arklow, Ireland
Sodium acetate	Sigma-Aldrich, Arklow, Ireland
EDTA (ethylenediaminetetraacetic acid)	Sigma-Aldrich, Arklow, Ireland
DNA assay kit	Sigma-Aldrich, Arklow, Ireland
Blyscan dimethylmethylene blue dye-binding assay kit	Biocolor, Carrickfergus, UK
Histology and immunohistochemistry	
Paraformaldehyde	Sigma-Aldrich, Arklow, Ireland
Alcian blue 8GX	Sigma-Aldrich, Arklow, Ireland
Picro-sirius red (Direct red 80)	Sigma-Aldrich, Arklow, Ireland
Hydrogen peroxidase	Sigma-Aldrich, Arklow, Ireland
Chondroitinase ABC	Sigma-Aldrich, Arklow, Ireland
Goat serum	Sigma-Aldrich, Arklow, Ireland
Rabbit polyclonal type II collagen (Chapter 3)	MD Biosciences, Zurich, Switzerland
Mouse monoclonal type I collagen	Abcam, Cambridge, UK
Mouse monoclonal type II collagen	Abcam, Cambridge, UK
Anti-rabbit biotin conjugate	Sigma-Aldrich, Arklow, Ireland
Anti-Mouse biotin conjugate	Sigma-Aldrich, Arklow, Ireland
VECTASTAIN Elite ABC kit	Vector Laboratories Ltd., Peterborough, UK
DAB Peroxidase Substrate Kit, 3,3'-diaminobenzidine	Vector Laboratories Ltd., Peterborough, UK

Appendix C Publications and conference contributions

C.1 Peer-reviewed publications

Thorpe, S.D., Buckley, C.T., Steward, A.J., Kelly, D.J. *"The external mechanical environment can override the influence of local substrate in determining stem cell fate"* In review

Thorpe, S.D., Nagel, T., Kelly, D.J. *"Engineering zonal cartilage using mesenchymal stem cells by modulating the local environment through the depth of the developing tissue"* In review

Thorpe, S.D., Buckley, C.T., Kelly, D.J. *"Can dynamic compression in the absence of growth factors induce chondrogenic differentiation of bone marrow derived MSCs encapsulated in agarose hydrogels?"* IFMBE Proceedings 30 43-46 2011

Thorpe, S.D., Buckley, C.T., Vinardell, T., O'Brien, F.J., Campbell, V.A., Kelly, D.J. *"The response of bone marrow derived mesenchymal stem cells to dynamic compression following TGF-β3 induced chondrogenic differentiation"* Annals of Biomedical Engineering 38(9) 2896-909 2010

Thorpe, S.D., Buckley, C.T., Vinardell, T., O'Brien, F.J., Campbell, V.A., Kelly, D.J. *"Dynamic compression can inhibit chondrogenesis of mesenchymal stem cells"* Biochemical and Biophysical Research Communications 377(2) 458-62 2008

C.2 Conference contributions

Thorpe, S.D., Buckley, C.T., Steward, A.J., Kelly, D.J. *"External mechanical stimulus can override the influence of local substrate in determining mesenchymal stem cell fate"* First Prize – Established Student Researcher; Bioengineering in Ireland[18], Belfast, Northern Ireland; January 2012 Oral Presentation

Thorpe, S.D., Buckley, C.T., Steward, A.J., Kelly, D.J. *"Cell-matrix interactions modulate mesenchymal stem cell response to dynamic compression"* Proceedings of the ASME 2011 Summer Bioengineering Conference, Farmington, Pennsylvania, USA; June 2011 Poster Presentation

Thorpe, S.D., Nagel, T., Kelly, D.J. *"Engineering Zonal Cartilaginous Tissues by Modulating the Local Environment Through the Depth of the Developing Construct"* Finalist - Engineers Ireland Biomedical Research Medal, Bioengineering in Ireland[17], Galway, Ireland; January 2011 Oral Presentation

Thorpe, S.D., Kelly, D.J. *"Engineering Zonal Cartilaginous Tissues Using Mesenchymal Stem Cells by Controlling the Local Environment Through the Depth of the Developing Construct"* 57th Annual Meeting of the Orthopaedic Research Society, Long Beach, California, USA; January 2011 Oral Presentation (Spotlight)

Thorpe, S.D., Buckley, C.T., Kelly, D.J. *"Can robust chondrogenesis of MSCs be initiated in absence of growth factors by modifying the magnitude or duration of cyclic compression?"* 17th Congress of the European Society of Biomechanics, Edinburgh, Scotland; July 2010 Oral Presentation

Thorpe, S.D., Buckley, C.T., Vinardel, T., O'Brien, F.J., Campbell, V.A., Kelly, D.J. *"Chondrogenesis of mesenchymal stem cells is regulated by dynamic compression post TGF-β3 induced differentiation"* TERMIS-EU 2010, Galway, Ireland; June 2010 Rapid-fire (Oral & Poster Presentations)

Thorpe, S.D., Buckley, C.T., Kelly, D.J. *"The influence of the magnitude and duration of dynamic compression on mesenchymal stem cells in the absence of chondrogenic growth factors"* 8th International Conference on Cell Engineering (ICCE), Trinity College Dublin, Dublin, Ireland; June 2010 Oral Presentation

Thorpe, S.D., Buckley, C.T., Vinardel, T., O'Brien, F.J., Campbell, V.A., Kelly, D.J. *"Dynamic compression of mesenchymal stem cells following cytokine induced differentiation augments glycosaminoglycan synthesis"* 4th UK Mesenchymal Stem Cell Meeting, University of Leeds, Leeds, UK; April 2010 Poster Presentation

Thorpe, S.D., Buckley, C.T., Vinardel, T., O'Brien, F.J., Campbell, V.A., Kelly, D.J. *"Dynamic compression post TGF-β3 induced differentiation augments chondrogenesis of mesenchymal stem cells"* 56th Annual Meeting of the Orthopaedic Research Society, New Orleans, Louisiana, USA; March 2010 Poster Presentation

Thorpe, S.D., Buckley, C.T., Vinardel, T., O'Brien, F.J., Campbell, V.A., Kelly, D.J. *"Dynamic compression post cytokine induced differentiation augments mesenchymal stem cell chondrogenesis"* Bioengineering in Ireland[16]; Dublin, Ireland; January 2010 Oral Presentation

Thorpe, S.D., Buckley, C.T., Kelly, D.J. *"Chondrogenesis of mesenchymal stem cells is differentially regulated by temporal application of dynamic compression"* eCM X Stem Cells for Musculoskeletal Regeneration; Davos, Switzerland; June-July 2009 Poster Presentation

Thorpe, S.D., Buckley, C.T., Vinardel, T., Haugh, M.G., O'Brien, F.J., Campbell, V.A., Kelly, D.J. *"Mesenchymal progenitor cells facilitate development of a mechano-responsive cartilage replacement tissue"* 12th Annual Sir Bernard Crossland Symposium; Queen's University, Belfast, Northern Ireland; April 2009 Oral and Poster Presentations

Thorpe, S.D., Buckley, C.T., Vinardel, T., O'Brien, F.J., Campbell, V.A., Kelly, D.J. *"Dynamic compressive loading can inhibit chondrogenesis of bone marrow derived mesenchymal progenitor cells"* 55th Annual Meeting of the Orthopaedic Research Society, Las Vegas, Nevada, USA; February 2009 Poster Presentation

Thorpe, S.D., Buckley, C.T., Vinardel, T., O'Brien, F.J., Campbell, V.A., Kelly, D.J. *"Could dynamic compressive loading inhibit chondrogenesis of mesenchymal stem cells?"* Bioengineering in Ireland[15]; Limerick, Ireland; January 2009 Oral Presentation

Thorpe, S.D., Buckley, C.T., Vinardel, T., O'Brien, F.J., Campbell, V.A., Kelly, D.J. *"Dynamic compression can inhibit chondrogenesis of mesenchymal stem cells"* 3rd UK Mesenchymal Stem Cell Meeting; University of Sheffield, Sheffield, UK; January 2009 Poster Presentation

Thorpe, S.D., Buckley, C.T., O'Brien, F.J., Robinson, A.J., Kelly, D.J. *"Fabrication methodologies regulate the initial mechanical properties of cell seeded hydrogels"* 16th Congress of the European Society of Biomechanics; Lucerne, Switzerland; July 2008 Poster Presentation

Thorpe, S.D., Buckley, C.T., O'Brien, F.J., Robinson, A.J., Kelly, D.J. *"Controlling the mechanical properties of cell seeded hydrogels"* Bioengineering in Ireland[14]; Sligo, Ireland; January 2008 Oral Presentation