

The regulation of human mesenchymal stem cell
chondrogenesis through multiaxial load

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"It's been emotional"

-Big Chris

Thesis Summary

The repair of damaged articular cartilage remains a clinical problem despite the development of numerous surgical approaches for cartilage regeneration. As result new options for therapeutic approaches are being sought. One of the candidate cell types for cartilage repair are mesenchymal stem cells (MSCs). These cells can be isolated from a number of different tissues and have the ability to differentiate down several different mesenchymal lineages. This thesis focused on the use of MSCs for repairing damaged articular cartilage. Specifically I investigated the effect of producing regenerative medicine type constructs containing different populations of MSCs on the induction of chondrogenesis in response to mechanical load, compared the induction of chondrogenesis in MSCs through the application of exogenous TGF- β 1 and multiaxial mechanical load and identified potentially novel markers of MSC chondrogenesis.

The results presented in this thesis show that the induction of chondrogenesis in MSCs can be manipulated by producing constructs that contain separate populations of MSCs. The work demonstrated that seeding a layer of MSCs on the loaded surface of a fibrin-poly(ester-urethane) scaffold could increase the deposition of histologically detectable matrix. However, it was not possible to determine the mechanism responsible for this.

Comparison of the secretomes of MSCs stimulated with TGF- β 1 and mechanical load showed that these two forms of chondrogenic stimulation are not analogous and that a number of markers, including GRO and MMP13 may be useful for monitoring the progression of MSCs through chondrogenesis and hypertrophy.

These data provide further insights into the effect of joint-like load on MSCs within tissue engineering/regenerative medicine style constructs, and the chondrogenic response of MSCs to this stimulation, which may prove to be useful for the development of constructs for cartilage repair.

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Abbreviations

2D – Two dimensional

3D – Three dimensional

ACI – Autologous chondrocyte implantation

ALCAM – Activated leukocyte cell adhesion molecule

ALK – Activin receptor like kinase

ALP – Alkaline phosphatase

α MEM – Alpha minimal essential medium

BCP – Bromochlorophenol

bFGF – Basic fibroblast growth factor

BLC – B lymphocyte chemoattractant

BMI – Body mass index

BMP – Bone morphogenic protein

BrdU – bromodeoxyuridine

CACI – Collagen-covered autologous chondrocyte implantation

cDNA – Complementary DNA

CXCL13 – B lymphocyte chemoattractant

DMEM – Dulbecco's modified Eagle's medium

DMMB – 1,9-dimethyl methylene blue

DNA – Deoxyribonucleic acid

DR6 – Death receptor 6

DSHB – Developmental studies Hybridoma bank

EACA – ϵ -aminocaproic acid

FAM – 6-carboxyfluorescein

FBS – Foetal bovine serum

FEM – Finite element modelling

GAG – Glycosaminoglycan

GDF – Growth and differentiation factor

GRO α – Growth related oncogene α

IHH – Indian hedgehog

IP3 – Inositol trisphosphate

LAP – Latency associated peptide

LTBP – Latent TGF- β binding protein

MACI – Matrix-induced autologous chondrocyte implantation

MCP3 – Monocyte specific chemokine 3

MDC – Macrophage derived chemokine

MIF – Macrophage migration inhibitory factor

MIP3 α – Macrophage inflammatory protein 3 α

MMP13 – Matrix metalloproteinase 13

MSC – Mesenchymal stem cell

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NO – Nitric oxide

NOS – Nitric oxide synthase

NSAID – Non-steroidal anti-inflammatory drug

OA – Osteoarthritis

OATS – Osteochondral autograft transfer system

OCT – Osteochondral transfer

OPG – Osteoprotegerin

PBS – Phosphate buffered saline

PBS-T – Phosphate buffered saline 0.1% Tween 20

PDGF – Platelet derived growth factor

PEEK – Polyether ether ketone

PGR4 – Proteoglycan 4

PKA – Protein kinase A

PKC – Protein kinase C

PK – Proteinase K

PMMA – poly(methyl-methacrylate)

PTHrP - Parathyroid hormone related protein

RNA – Ribonucleic acid

RS cells – Rapidly self-renewing cells

R-Smad – Receptor activated Smad

RT – Reverse transcription

RT-PCR – Real-time polymerise chain reaction

Runx2 – Runt-related transcription factor 2

SCID mice – Severe combined immune deficiency

TAMRA – tetramethylrhodamine

TAK1 – TGF- β activated kinase 1

TGF- β – Transforming growth factor β

TIMP – Tissue inhibitor of metalloproteinases

uPAR – Urokinase receptor

VEGF – Vascular endothelial growth factor

v/v – Volume per volume

w/v – Weight per volume

Chapter 1 General Introduction and Thesis Aims

Sections of this Chapter have been published in:

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1.1 Introduction

As the weight bearing material of diarthrodial joints, articular cartilage facilitates the almost frictionless movement of joint surfaces, despite being regularly exposed to mechanical forces in the mega Pascal range (Hodge et al., 1986). However, despite cartilage's capacity to dissipate and absorb load, it is susceptible to damage through trauma or disease, and once damaged it is all but incapable of affecting a repair. The susceptibility of cartilage to damage and its poor reparative response means that even small lesions can produce significant amounts of pain, joint stiffness, immobility and, over time, increases the risk of osteoarthritis (Brown et al., 2006). The lack of regeneration demonstrated by articular cartilage was first realised more than two hundred and fifty years ago by the anatomist and surgeon William Hunter (Hunter, 1743). For a long time, it has been the goal of surgeons to develop a reliable method to repair damaged articular cartilage. These techniques have ranged from debris removal techniques, such as debridement and lavage developed in the 1940's, to osteochondral transplant techniques, marrow stimulation techniques and the latest generation of cell based tissue engineering techniques such as autologous chondrocyte implantation (Insall, 1967, Brittberg et al., 1994, Redman et al., 2005). Each of these techniques has different advantages and disadvantages and improvements are still needed.

1.2 Cartilage, chondrocytes and extracellular matrix

Chondrocytes account for a small percentage of the total volume of human articular cartilage, perhaps as low as 1.65% (Hunziker et al., 2002); the rest of the tissue consists of a dense extracellular matrix consisting mainly of two macromolecules: type II collagen and the aggregating proteoglycan aggrecan. These two molecules account for 15-22% and 4-7% of the wet weight of cartilage respectively (Mow et al., 1992). The other substance found in large amounts in cartilage is water; this makes up approximately 80% of cartilage's wet weight (Sophia Fox et al., 2009). Cations such as sodium and calcium are attracted to the large amounts of negative charge found on the aggrecan molecule's glycosaminoglycan chains. This leads to an increase in osmotic potential that draws water into the tissue. It is the presence of large amounts of water within cartilage, combined with the organisation of the aggrecan and type II collagen that allow it to resist the large loads that it is exposed to.

Mature articular cartilage is constructed of four layers; the surface zone, middle zone, deep zone and calcified zone (Figure 1.1). Each zone has a different collagen arrangement and the morphology of the chondrocytes in each zone reflects the different macromolecular structures surrounding them (Becerra et al., 2010). The type II collagen fibrils within articular cartilage form arch like structures (as demonstrated in Figure 1.2) referred to as the arcades of Benninghoff (Benninghoff, 1925). The fibres change from being vertically orientated in the deep zone to form a dense overlapping mesh in the mid zone, and then the fibres then curve to run parallel to the surface in the surface zone. The horizontal fibres in the superficial zone give the cartilage surface tensile strength to resist the shear forces exerted upon it during joint articulation. Correspondingly the surface zone contains flattened chondrocytes that express a natural lubricating molecule called lubricin or proteoglycan 4 (PRG4), which helps produce and protect articular cartilage's almost frictionless surface (Schumacher et al., 1994). In the middle zone, chondrocytes become more rounded, and are dispersed unevenly throughout the matrix, whereas the chondrocytes of the deep zone are spherical and organised into clear columns. The calcified layer acts as a transitional layer between the articular cartilage and the bone beneath it; the chondrocytes of the calcified layer contain little cytoplasm or cellular machinery.

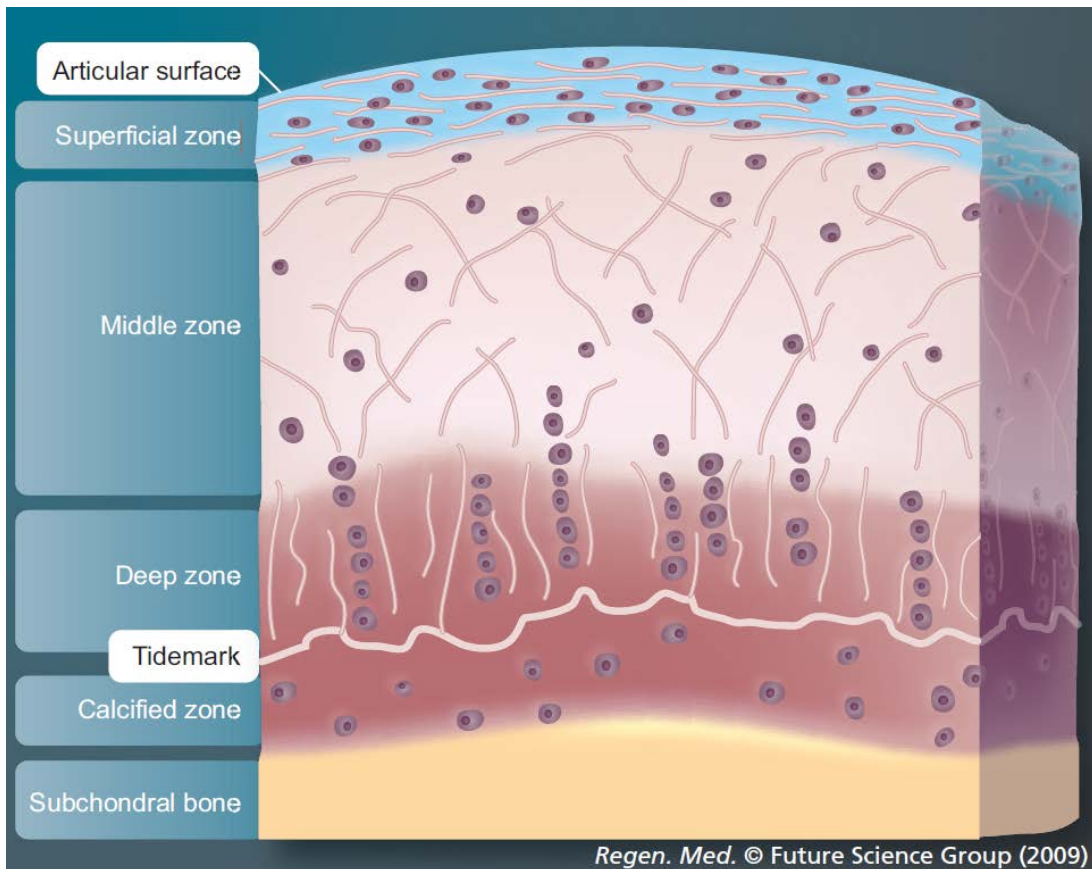


Figure 1.1

The structure of articular cartilage, this image shows the different zones of articular cartilage and the variation in cell distribution and collagen organisation within the different zones. Reproduced with kind permission from Regenerative Medicine as agreed by future medicine Ltd. (Stoddart MJ, Grad S, Eglin D, Alini M. Cells and biomaterials in cartilage tissue engineering. *Regen Med.* 2009 Jan;4(1):81-98.).

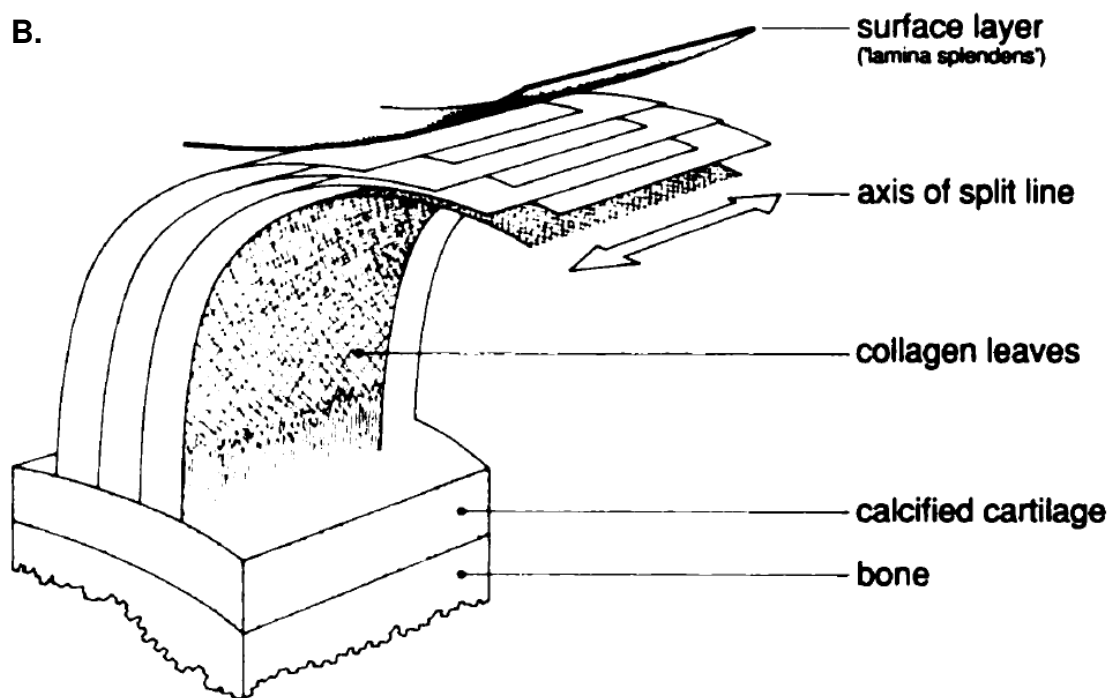


Figure 1.2

A Scanning electron microscopy image (80x) showing collagen leaves forming arcades in bovine articular cartilage (image A). AS denotes the articular surface (lamina splendens). Image B is a cartoon showing the arcade like organisation of collagen fibres within articular cartilage. Both reproduced with kind permission and copyright © of the British Editorial Society of Bone and Joint Surgery (Jeffery AK, Blunn GW, Archer CW, Bentley G. Three-dimensional collagen architecture in bovine articular cartilage. *J Bone Joint Surg [Br]* 1991;73-B:795-801.).

Aggrecan molecules are held in place by the dense network of type II collagen fibres. Their negative charge acts to draw in water causing the tissue to swell, as a result of the Donnan effect (Donnan, 1924), the resulting swelling pressure produces a tensile load in the collagen fibres. It is this balance between the swelling pressure caused by the influx of water and the tensile resistance generated in the collagen fibres that allow cartilage to resist compressive loads. When cartilage experiences a load greater than the force generated by the swelling pressure, water is forced out of the tissue. This increases the concentration of proteoglycans and their large negative charge draws water back into the tissue upon relaxation. This movement of water out of the tissue under load, and its return during unloading, allows cartilage to act like a shock absorber, dissipating the loads applied to it.

Cartilage's avascularity is a major contributor to its poor repair response as there is no supply of clotting agents or cells to produce repair material following damage or insult (Becerra et al., 2010). Although there is no known repair response within cartilage, damage that is severe enough to lead to the development of a full thickness defect (i.e. a defect that penetrates both the articular cartilage and the underlying subchondral bone) can result in the filling of the defect with blood from the underlying marrow cavity. The result of bleeding from the marrow cavity is the formation of a clot which can then lead to the formation of fibrocartilaginous repair tissue (Shapiro et al., 1993, Steadman et al., 2001).

1.3 Conservative and surgical options for the treatment of damaged cartilage

Among the most common causes of damage to cartilage are trauma, osteoarthritis and osteochondritis dissecans. Each of these three conditions can present in similar ways, with pain, swelling and impaired movement of the joint (Madry et al., 2011). Treatment for these conditions is aimed at reducing pain and improving joint mobility (Steinmeyer and Konttinen, 2006). As a result, the first line of conservative treatments are non-steroidal anti-inflammatory drugs (NSAIDs), and following these opiate pain killers may be used, with some of the final options including hyaluronic acid or corticosteroid injections given into the joint (Steinmeyer and Konttinen, 2006). Once conservative medical options have been exhausted, there are a variety of different surgical options that can be used to treat damaged cartilage. These techniques are chosen due to patient factors such as age, body mass index (BMI) and activity levels, and factors based on the nature of the lesion e.g. size, type as well as location of the lesion within the joint (Michael et al., 2010).

The earliest surgical techniques to deal with damaged cartilage were debridement and lavage; these were introduced in the 1940's and pioneered by surgeons such as Magnuson and Haggart (Insall, 1967). The purpose of these procedures was to remove any loose cartilage bodies or fragments from the joint capsule, and to smooth any rough areas of cartilage, as these were, at the time, believed to be the cause of symptoms associated with cartilage damage (Insall, 1967). Debridement and lavage were originally performed as open techniques, however they can now be performed arthroscopically (Bird and Ring, 1978). Various studies have investigated the benefit of these therapies, but the benefits that they provide appear to be short term and they do not alter the progression of osteoarthritis (OA) (Lutzner et al., 2009). Moseley et al. (2002) suggested that improvements in pain scoring post-procedure were simply a result of the placebo effect, as they found no difference between a sham surgery group and groups treated with arthroscopic debridement or lavage (Moseley et al., 2002).

Bone marrow stimulation techniques are based on the principle that when a full depth defect penetrates the subchondral bone, bleeding from the bone marrow leads to the production of repair tissue within the lesion, as described in the previous subsection. The earliest of these techniques to be introduced was Pridie drilling, developed in the 1950's by KH Pridie (Insall, 1967). This technique involves drilling holes into the subchondral plate with a drill (originally using a ¼ inch drill bit) in order to stimulate bleeding (Insall, 1967). Abrasion arthroplasty is a modification of the original 'housekeeping arthroplasty' technique and is similar to Pridie drilling, but instead of using a drill bit an automated arthroscopic burr is used to perforate the subchondral bone to stimulate bleeding (Johnson, 1986).

The third, and most common marrow stimulation technique is microfracture, introduced by JR Steadman. This technique simply uses a surgical awl to create holes in the subchondral plate approximately 3-4mm apart, which allows marrow blood to enter the defect from the bone marrow cavity below (Steadman et al., 2001).

The problem with these techniques is that although the clot produced fills the defect and produces repair tissue, this tissue is composed mostly of fibrous connective tissue, with a lower collagen type II content and higher collagen type I and GAG content than articular cartilage (LaPrade et al., 2008). This repair material also lacks the important organisational structure of articular cartilage and is a poor mechanical substitute for hyaline cartilage, which can lead to graft failure in response to joint loading (LaPrade et al., 2008). The integration of the repair tissue has also been shown to be poor, which can lead to degeneration of the repair tissue or even necrosis (Mobasheri et al., 2009). Despite these problems, there has been some considerable success with this technique, particularly in younger more active patients (Kreuz et al., 2006). In rabbit models, there is some evidence of the formation of more hyaline like repair tissue through the 'maturation' of a fibrocartilaginous tissue as a result of this technique (Steadman et al., 2001).

Osteochondral autograft transfer system (OATS) is a surgical repair procedure that involves the harvesting of osteochondral plugs from low weight-bearing regions of the joint, or from an allogeneic or even cadaveric donor and placing them into the cartilage defect being repaired (Meyers et al., 1989, Hangody et al., 2004). There are two common forms of this procedure; osteochondral transfer (OCT) which involves the transfer of one plug from a harvest site to a recipient site, and mosaicplasty which uses multiple plugs to fill larger recipient lesions (as large as 4cm in diameter) (Hangody et al., 2004). This technique has been successful, resulting in decreased pain and improved mechanical function of the joint (Hangody et al., 2004). However, there is evidence of cell death at the donor site in autologous donors, which poses the risk of donor site degeneration. Donor site degeneration can also occur as a result of repair tissue not forming naturally in response to bleeding from the subchondral plate triggered by plug removal, and may even require corrective surgery (LaPrade and Botker, 2004). The levels of integration seen in bony regions of the plug is good; however the cartilage regions have shown very little integration, which can cause degeneration of the implant site, leading to graft failure (Horas et al., 2003, Matricali et al., 2010).

Various other techniques have been used to repair damaged cartilage; osteotomies are used to correct malalignment of joints in order to prevent further degeneration due to abnormal loading (Wright et al., 2005). Soft tissue grafts of perichondrium or

periosteum have also been transplanted in to cartilage defects to stimulate a repair response (Rubak, 1982, Amiel et al., 1985).

The final solution for severe damage is a hemi arthroplasty or a total joint replacement. However, when joint replacement procedures are carried out in younger patients, revision surgery becomes necessary when the implant fails; this is much more difficult than the initial surgery and has a higher rate of complications (Hamilton et al., 2015).

1.4 Cell Based Therapies

The difficulties associated with producing hyaline cartilage-like repair tissue in defects using the surgical techniques described in the previous subsection, combined with the problems associated with revision surgery of total joint replacements in young active individuals (Hamilton et al., 2015), has led to the expansion of tissue engineering approaches to treat damaged cartilage.

Tissue engineering is a very broad term, and can cover a multitude of different approaches, strategies or mechanisms for the treatment of disease; however the classical view of tissue engineering is the use of scaffolds or matrices seeded *ex vivo* with cells to effect a repair in a target tissue (Langer and Vacanti, 1993, Bianco and Robey, 2001).

In order for cartilage therapy to be successful, either via surgical techniques or tissue engineering, the defect being repaired needs to be filled with a mechanically stable hyaline cartilage-like substance that will not deteriorate over time, and will integrate well with the surrounding tissue (Redman et al., 2005). Within the field of tissue engineering, there has been particular focus on the repair and regeneration of damaged cartilage. This wide-ranging interest has led to a number of different and varied approaches using different materials to fill cartilage lesions.

Currently, the only cell based tissue engineering approach that is licenced for use in patients is autologous chondrocyte implantation (ACI) and its modified form is matrix-induced autologous chondrocyte implantation (MACI). ACI was pioneered by Mats Brittberg and first used in 1987, and involves the use of autologous chondrocytes to produce repair tissue within a defect (Brittberg et al., 1994). ACI and MACI have been approved both by the Food and Drug Administration in the USA and as advanced-therapy medicinal products by the European Medicines Agency.

ACI involves two operations (Figure 1.3): in the first a small sample of cartilage (weighting between 200-300mg) is removed from the joint periphery (such as the medial femoral condyle, or intercondylar notch). Once harvested, the cartilage shavings are then enzymatically digested, first in pronase and then collagenase, to release the chondrocytes from the extra-cellular matrix (Brittberg et al., 1994, Brittberg, 2010). The chondrocytes are then expanded *in vitro*. Once the required numbers of chondrocytes have been produced they are reseeded back into the joint to effect a repair (Brittberg, 2010). The original ACI procedure involved harvesting a piece of perichondrium from the proximal medial tibia and suturing the explant over the debrided defect, then the flap was sealed with fibrin glue and the chondrocytes injected underneath the flap (Brittberg et al., 1994). This initial technique had several problems e.g. the periosteal membrane could undergo hypertrophy causing the need for further surgery, the

harvesting of the periosteum also introduced another site of morbidity alongside the cartilage harvest site and the defect itself. These problems led to the second generation ACI technique, also referred to as collagen-covered ACI (CACI) (Brittberg, 2010). The replacement of the periosteal flap used in the first generation with a bilayer collagen type I/III membrane helped to prevent hypertrophy, and removed the morbidity associated with periosteal harvesting, but the technique still requires an arthrotomy (Brittberg, 2010). The current third generation of ACI, called matrix-induced ACI (MACI) uses biodegradable collagen matrices seeded with the chondrocytes and anchored into the defect with fibrin glue (Cherubino et al., 2003). The advantage of this technique is that the first operation can be carried out with a miniarthrotomy, and the second arthroscopically, reducing the risk of complications as a result of or during surgery (Brittberg, 2010). ACI is most commonly used to treat larger defects (ranging from 3-10cm²), and has mainly been used within the knee joint (Madry et al., 2011).

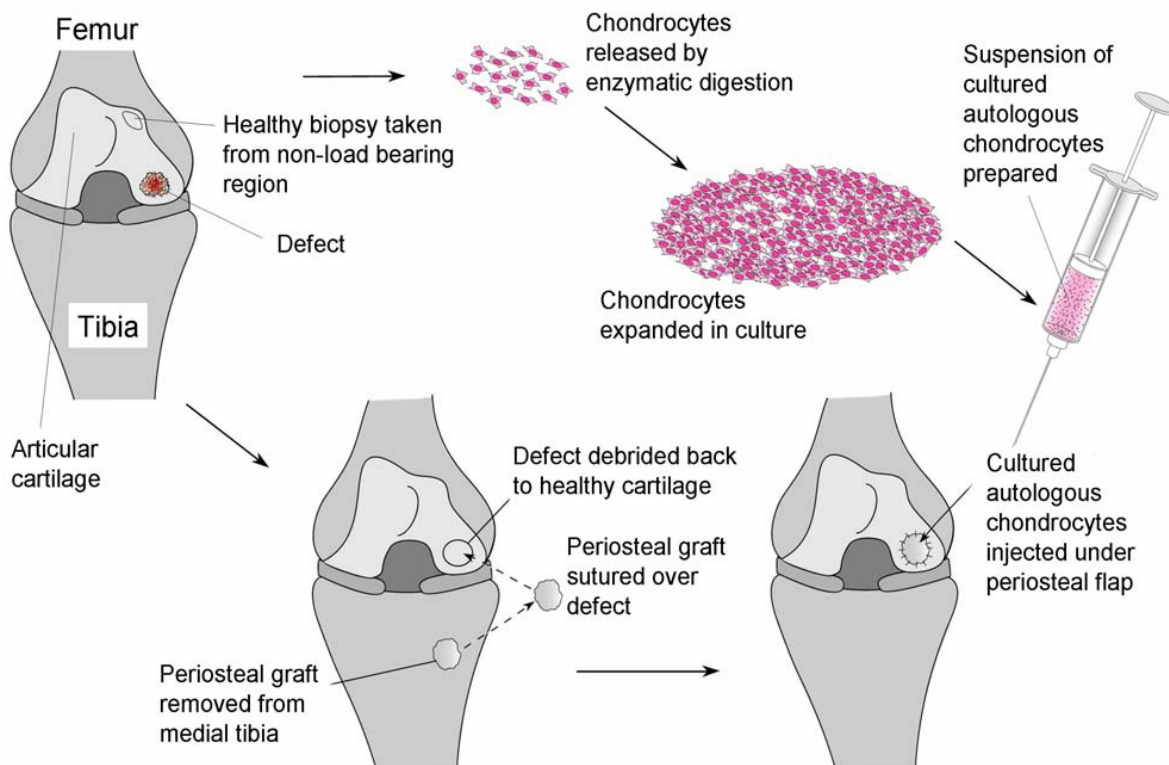


Figure 1.3

A schematic showing the constitutive steps of autologous chondrocyte implantation for the repair of cartilage defects. Reproduced with the kind permission from the authors (Redman SN, Oldfield SF, Archer CW. Current strategies for articular cartilage repair. Eur Cell Mater. 2005 Apr 14;9:23-32; discussion 23-32.).

ACI has been a successful technique; even the first generation used in the late 1980's produced improvements in joint function, reduced pain scores and in some cases generated hyaline-like repair tissue (Brittberg, 2010). A study by Micheli et al. into the outcome of ACI procedures, reported graft failure in only 6% of cases (Micheli et al., 2001). However, evidence produced by Knutsen et al. showed that when ACI is compared with microfracture surgery there is very little, if any, significant difference between the clinical outcomes of the two procedures (Knutsen et al., 2007). Despite this reported similarity in clinical outcome the histological quality of the repair tissue may be superior with ACI (Saris et al., 2008). MACI has been shown to produce 72% good or excellent results, but this was not significantly different from the 59% good or excellent results observed with the second generation CACI. However, the much improved surgical techniques used during the MACI make it a preferable approach (Brittberg, 2010).

Despite showing clinical efficacy, ACI has had, and continues to have some problems associated with it. The current method for chondrocyte collection in ACI involves the removal of slivers of cartilage, from which chondrocytes can be extracted; these slivers are collected from the joint during a miniarthrotomy which involves partial opening of the joint cavity (Brittberg, 2010). The collection of cartilage from the surface of the joint can also lead to donor site morbidity and the potential for further degeneration at the site of harvest (Matricali et al., 2010). In contrast, bone marrow derived mesenchymal stem cells (MSCs), which are another potential cell source for cartilage repair, can be harvested from the bone marrow (e.g. of the iliac crest) using a syringe, during a bone marrow aspiration. Although a bone marrow aspiration is painful for the patient it can be performed with a local anaesthetic, and involves far less risk than the miniarthrotomy used to collect chondrocytes for ACI, which may expose the joint cavity to infection, and carries the same risks as any other procedure that involves general anaesthesia.

Another major drawback with ACI is the *in vitro* cell culture stage. Culturing chondrocytes in monolayer causes them to undergo dedifferentiation and the adoption of a fibroblast like phenotype (Holtzer et al., 1960, von der Mark et al., 1977). This is due, in part, to the loss of the chondrocyte's natural spherical morphology, which cannot be maintained in monolayer culture, and results in the adoption of a flattened fibroblast-like morphology (Benya and Shaffer, 1982). These changes cause an increase in the expression of markers of dedifferentiation such as type I collagen and reduce the chondrocytes ability to be redifferentiated and undergo chondrogenesis in order to produce repair tissue (von der Mark et al., 1977, Benya and Shaffer, 1982). MSCs maintain their chondrogenic ability better than chondrocytes in monolayer culture, although their replicative capacity is not infinite. Pelttari et al. showed that after six passages chondrocytes were no longer able to deposit cartilage-like matrix during

redifferentiation (Pelttari et al., 2008). Banfi et al. (2000) estimated that the useful clinical limit for MSC expansion would be seventeen population doublings, more than twice as many as chondrocytes can be usefully expanded by (Banfi et al., 2000). As the number of population doublings that an MSC culture has been through rises, the proportion of larger cells in the population begins to increase. These cells are less active, and have less differentiation capacity than the smaller rapidly self-renewing cells (RS cells) in the population (Smith et al., 2004, Fehrer and Lepperdinger, 2005). Along with the increase in larger cells, individual cells lose their ability to differentiate in to adipocytes, chondrocytes and osteoblasts in a stochastic (hierarchical) manner (Muraglia et al., 2000). The first lineage that is closed to MSCs is adipogenesis and this occurs at approximately twenty two population doublings; the cells then retain the ability to form chondrocytes and osteoblasts for a considerable time in culture before chondrogenesis is lost and the cells can only become osteoblasts (Muraglia et al., 2000). As well as maintaining their ability to produce useful cartilage-like repair tissue longer than chondrocytes in culture, MSCs also have a higher rate of proliferation. The very low cell density in cartilage, combined with the small areas available for harvesting and the limited room for the expansion of chondrocytes *in vitro*, means that ACI can only utilise a very small number of cells, whereas a larger number may have more success in producing hyaline-like cartilage repair tissue.

Although ACI has been shown to be effective clinically by Brittberg et al. (Brittberg et al., 1994, Brittberg, 2010), some questions have been raised about the quality of ACI repair tissue, particularly with regards to the generation of mechanically inferior fibrocartilage (LaPrade et al., 2008). ACI is a multistep procedure, which increases the chances of an infection and other complications, as well as requiring a costly cell culture expansion stage which can lead to the dedifferentiation of the chondrocytes being prepared for implantation. As a result there are considerable efforts being made to develop improved cell based approaches for articular cartilage repair. One of the avenues being investigated is the use of mesenchymal stem cells (MSCs) rather than chondrocytes in tissue engineering applications.

1.5 Mesenchymal Stem Cells

Although interest in mesenchymal stem cells (MSCs) has increased exponentially over the last 15 years they have been known about (by different names) for over 130 years. The first description came in 1867 when Julius Cohnheim proposed the idea that fibroblasts involved in wound repair may originate in the bone marrow (Prockop, 1997). Later, in the 1970's and 1980's, Friedenstein and others, including Howlett and Owen demonstrated that these fibroblasts, isolated by Cohnheim, were multipotent cells capable of differentiating in to various forms of cells able to generate mesenchyme like tissues (Friedenstein et al., 1970, Friedenstein et al., 1974, Friedenstein et al., 1976, Howlett et al., 1986, Owen and Friedenstein, 1988). In 1999, Pittenger et al. were able to demonstrate the multipotent capabilities of these cells by differentiating cells from individual donors down the chondrogenic, osteogenic and adipogenic lineages.

Dubbed mesenchymal stem cells by Arnold Caplan, these cells go by a number of names; pericytes, colony forming unit fibroblasts, mesenchymal stromal cells, but despite having a myriad of names there is not an absolute definition of what an MSC actually is. The broad definition of an 'MSC' is often given as; a culture adherent multipotent progenitor cell that can differentiate down the adipogenic, chondrogenic and osteogenic lineages (Caplan, 2009). This definition sounds conclusive, but the adherent cell cultures produced from MSC sources such as bone marrow are heterogeneous, so a more stringent definition is required (Battula et al., 2009). In order to refine the definition, others have tried to use cell surface markers to provide a real distinction between MSCs and other adherent cells, however, the lack of a definitive MSC marker has made this very difficult (Battula et al., 2009). Currently, the most quoted definition of a mesenchymal stromal cell comes from the International Society for Cellular Therapy (ISCT); their definition comes in three parts: In order to be considered a MSC a cell must be; plastic adherent, 95% or more of the cells of a colony must express CD105, CD73 and CD90, and less than 2% of the cells can express CD45, CD434, CD14, CD11b, CD79 α , CD19 or HLA class II. Finally, they must be able to at least differentiate into adipocytes, chondrocytes and osteoblasts when stimulated *in vitro* (Dominici et al., 2006).

One of the roles of MSCs *in vivo* is the production of the bone marrow stroma (Panoskaltsis et al., 2005). The stroma is made up of the components of bone marrow that facilitate the haematopoietic process and the activity of haematopoietic stem cells and their progeny (Panoskaltsis et al., 2005). The stroma consists of an extracellular matrix which contains type I, III and IV collagen as well as proteoglycans (Panoskaltsis et al., 2005). The matrix contains adipocytes, osteocytes and other cell types such as

endothelial cells, which act together to create an environment suitable for haematopoiesis to occur (Panoskaltsis et al., 2005).

The original work on MSCs used cells isolated from bone marrow; we now know that MSCs can be found in a huge range of tissues including: fat, bone marrow, placenta, cartilage, foetal tissues (e.g. spleen and liver) and deciduous teeth (Bernardo et al., 2007).

As well as being found in many different tissue types, under the correct conditions MSCs are capable of producing a wide range of tissues *in vivo*. MSCs, by definition can be differentiated into cartilage, bone and fat producing cells. They can also be differentiated into muscle, marrow and tendon as well as other types of mesenchymal tissue producing cells including myeloid and lymphoid derived blood cells. There is even evidence of MSCs being used to produce non-mesenchymal cells such as hepatocytes and neurones, although these neuronal cells simply possessed a small number of neuronal cell markers (Mezey et al., 2000, Caplan, 2007); however, much more work is required to determine if MSCs can form functioning neurones. It is the fact that MSCs can be differentiated into a range of lineages *in vitro* that makes them such a potentially powerful tool within tissue engineering.

In order to differentiate MSCs down a desired lineage they must first be isolated from the original tissue sample. Within bone marrow there are only one to a hundred MSCs in every five thousand mononuclear cells, so selection is very important (Kastrinaki et al., 2008). The technique first used to isolate MSCs was adhesion (Friedenstein et al., 1974, Friedenstein et al., 1970). Adhesion to tissue culture plastic is still used to select for MSCs, however it is common for a density gradient centrifugation or red blood cell lysis step to be performed first in order to isolate the mononuclear cell population of the marrow before selecting for MSCs via adhesion (Gardner and Stoddart 2015). Once isolated, MSCs can be directed to differentiate down various cell lineages using a range of soluble factors or environmental/culture conditions.

Osteogenesis can be initiated in MSCs in monolayer by culturing them in a media containing ascorbic acid, dexamethasone and β -glycerophosphate (Pittenger et al., 1999). This causes an increase in osteogenic markers such as alkaline phosphatase and, over time, calcium deposits form as nodules which can be detected with alizarin red or von Kossa staining (Pittenger et al., 1999, Chamberlain et al., 2007).

In a similar technique, adipogenesis can be initiated in monolayer MSCs using dexamethasone, insulin, isobutyl methyl xanthine and indomethacin, leading to the formation of lipid filled vacuoles within the MSCs that can be stained using the oil red O technique, along with other adipogenic markers such as lipoprotein lipase (Pittenger et

al., 1999, Chamberlain et al., 2007). These lipid vacuoles coalesce and will ultimately fill the cell (Chamberlain et al., 2007).

In order to induce chondrogenesis in MSCs, the cells need to be in close contact, as well as being exposed to the correct soluble factors. In order to achieve this, MSCs are suspended in culture medium and spun in a centrifuge to produce a pellet culture (Johnstone et al., 1998). The pellet is then cultured in a growth medium containing TGF- β , dexamethasone, ascorbic acid, non-essential amino acids and ITS; this leads to the development of cartilaginous tissue that stains metachromatically with toluidine blue and contains type II collagen (Johnstone et al., 1998). A problem with this cartilage model, however, is that TGF- β induced chondrogenesis over time leads to hypertrophy of chondrogenic MSCs. This is associated with an increased expression of hypertrophic markers like matrix metalloproteinase 13 (MMP13) and type X collagen as well as an increase in osteogenic markers such as Runx2 and collagen type I (Pelttari et al., 2008, Mueller and Tuan, 2008). This process is similar to that observed during bone formation via endochondral ossification, in which chondrocytes undergo hypertrophy leading to apoptosis and calcification of the surrounding cartilaginous tissue. The development of hypertrophy in MSCs that are induced down the chondrogenic lineage could potentially affect native tissue surrounding an MSC implant (Mueller et al., 2010). As a result, techniques to prevent hypertrophy need to be developed before chondrogenically induced MSCs can be used clinically.

1.6 Endochondral ossification

There are just over two hundred bones in the human body; all of them develop in one of two ways: either by endochondral or intramembranous ossification. Endochondral ossification is the process by which the apical skeleton, vertebrae and base of the skull are formed. Intramembranous ossification is responsible for the formation of the facial bones, the pelvis and the skull cap (Buckwalter et al., 1996). Both of these processes begin with the formation of condensations of mesenchymal precursor cells, however, the rest of the two processes are very different. During intramembranous ossification, the mesenchymal progenitors form vascularised matrix rich membranes; the cells within these membranes then differentiate directly into osteoblasts and convert the soft tissue membrane into bone (Buckwalter et al., 1996). In order for bone to form through the process of endochondral ossification (shown in Figure 1.4), first a primordial cartilage template called the anlagen must form, providing the basis for the resulting bone (DeLise et al., 2000). The development of the anlagen occurs through cell-cell and cell-matrix interactions that trigger differentiation of mesenchymal precursors and the condensation into chondrocytes (Goldring et al., 2006). TGF- β is expressed soon after condensation begins, and acts to define the outline of the condensation and determine the shape of the developing bone (Goldring et al., 2006). The newly formed chondrocytes secrete matrix molecules such as type II collagen and aggrecan; the expression of these chondrogenic markers is triggered by SOX9 which is up-regulated in the cells within the condensation (Mackie et al., 2011). At this stage of the development the centre of the condensation contains proliferating chondrocytes, whilst the outer most layers of cells form a surrounding layer called the perichondrium (Lai and Mitchell, 2005). The first regions of calcified bone begin to form around the diaphysis of the condensate, leading to the formation of the 'bony collar'. This ring of bone provides access for developing blood vessels which bring with them osteoblast precursors (Mackie et al., 2011, Buckwalter et al., 1996). This neovascularisation leads to the development of the primary centre of ossification (Mau et al., 2007). At the epiphyses of the developing bones secondary centres of ossification also begin to form. The outwards spreading of calcification from the primary and secondary centres of ossification leads to the formation of the mineralised bone from the cartilage anlagen. The last remaining areas of cartilage can be found in the epiphyseal growth plates at either end of the bone. The growth plates will not mineralise until skeletal maturity and allow for the continued lengthening of the bone through childhood and adolescence (Mackie et al., 2011).

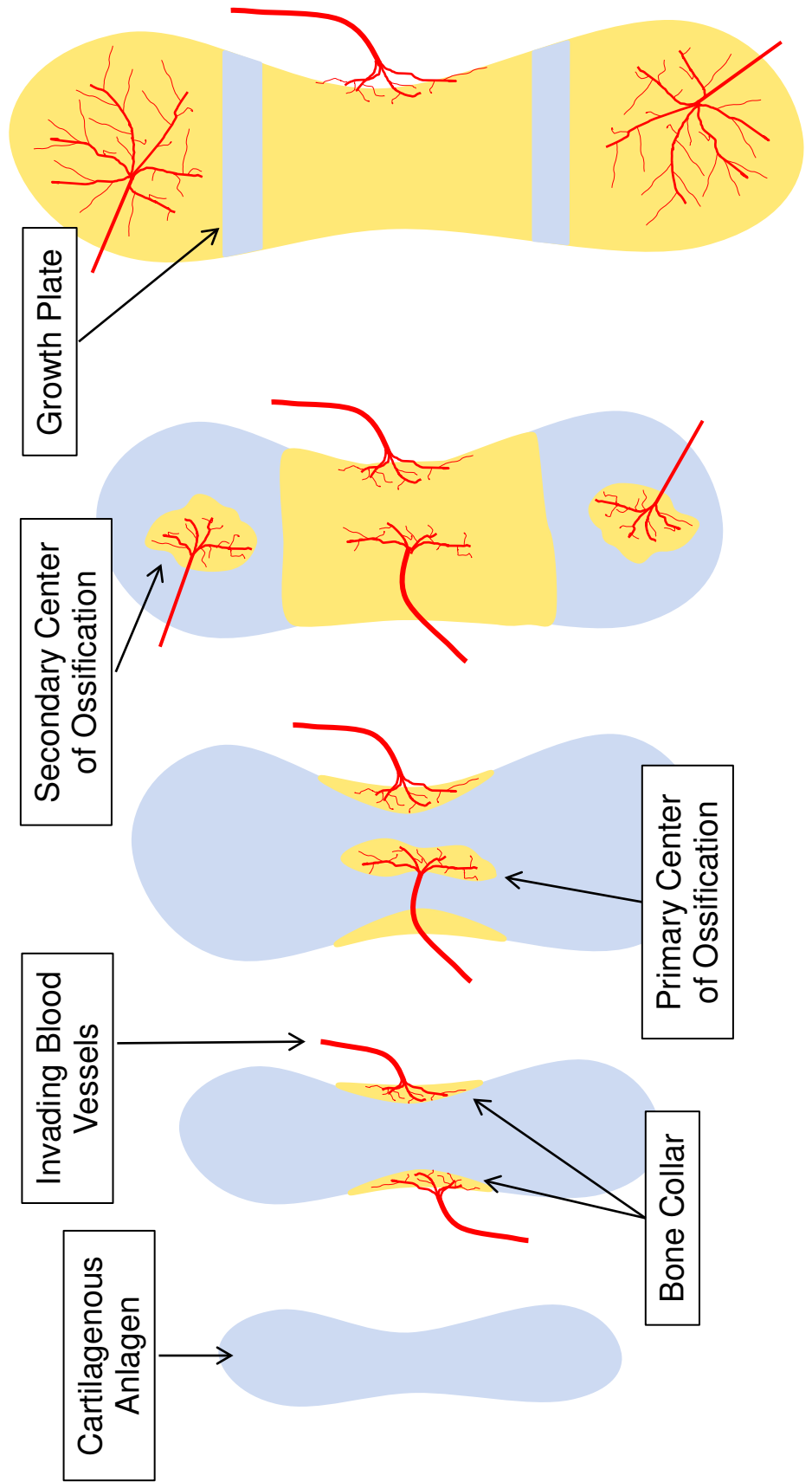


Figure 1.4

The formation of a long bone through the process of endochondral ossification. This diagram shows the progression of endochondral ossification from the formation of the cartilaginous anlagen followed by the invasion of blood vessels and initial calcification leading to the formation of the bony ring and the subsequent formation of primary and secondary centres of ossification resulting in the formation of a fully calcified tissue with the exceptions of the epiphyseal growth plates.

1.6 Hypertrophy

In order for the cartilage anlagen to be converted into bone, the proliferating chondrocytes that formed the initial template have to go through a process called hypertrophy (Goldring et al., 2006). Hypertrophy is the final stage of the terminal differentiation of chondrocytes during endochondral ossification, and allows for the conversion of the cartilage template into bone. This stage of differentiation gets its name from the hypertrophy that the chondrocytes undergo as they differentiate. The volume of the cells can increase by up to twenty times (Goldring et al., 2006). These hypertrophic chondrocytes begin to down-regulate the expression of chondrogenic markers such as type II collagen and aggrecan (Goldring et al., 2006, Mackie et al., 2011). As well as down-regulating chondrogenic markers, there is an up-regulation of hypertrophic and osteogenic markers in these cells which are not normally observed in chondrocytes such as type I and type X collagen, MMP13, runt-related transcription factor 2 (Runx2), and alkaline phosphatase (ALP) (Mueller and Tuan, 2008). The transcription factor Runx2 acts as a positive regulator for hypertrophic differentiation (Goldring et al., 2006). As the chondrocytes enter the later stages of hypertrophy, expression of the angiogenic vascular endothelial growth factor (VEGF) causes blood vessels to invade the hypertrophic zone (Goldring et al., 2006). The invasion of blood vessels brings with it osteoblast precursors, and leads to the calcification of the cartilaginous tissue. The expression of ALP by hypertrophic chondrocytes is key to the mineralisation of the hypertrophic zone, as the enzymatic activity of ALP clears the way for the process of calcification (Mackie et al., 2011). ALP encourages the formation of calcified tissue from the cartilaginous matrix by cleaving inorganic phosphates to form organic phosphates, and in doing so affects the balance of these two compounds in the developing bone (Orimo, 2010). The balance of these two forms of phosphate determines whether or not hydroxyapatite crystals can form, and by cleaving inorganic phosphate to produce organic phosphate, ALP helps to provide conditions suitable for calcification (Orimo, 2010).

1.6.1 Chondroptosis

Following the invasion of blood vessels and osteogenic precursors, hypertrophic chondrocytes are believed to undergo programmed cell death (Mackie et al., 2011). As the process of cell death progresses, cytomorphologic changes occur and two distinct populations begin to form; they are termed light and dark cells based on their differences when observed under a transmission electron microscope (Mackie et al., 2011). These two groups of chondrocytes undergo cell death in different ways (Mackie et al., 2011, Mueller and Tuan, 2008). Neither of the cells undergoes apoptosis; instead they have their own programmes of cell death which vary from the classical form of apoptosis: these processes are referred to as 'chondroptosis' (Roach and Clarke, 2000). Both the dark and light cells demonstrate chromatin condensation as part of the programme of cell death (Roach and Clarke, 2000). However, unlike the chromatin condensation observed at the periphery of the nucleus during the initial stages of apoptosis, the chromatin of chondroptotic cells form islands throughout the nucleus (Roach and Clarke, 2000). Dark cells then begin to develop endoplasmic compartments and autophagic vacuoles that digest the cells contents, and any remnants are then ejected into the cells lacunae (Roach et al., 2004). Light cells, however, undergo a process in which the expansion of the rough endoplasm leads to the formation of sacks around the organelles and other cell contents (Roach and Clarke, 2000). Lysosomes then release digestive enzymes that lead to the digestion of the cells contents within these cytoplasmic bodies (Roach and Clarke, 2000). The advantage of chondroptosis may be that autophagy removes the need for the inflammatory response required to remove the apoptotic bodies left behind by classical apoptosis (Roach et al., 2004). Such an inflammatory response cannot be mounted in cartilage, and would therefore potentially lead to secondary necrosis, if the debris was not removed by phagocytosis (Roach et al., 2004).

1.6.2 Molecular Mechanisms Regulating Hypertrophy

In vivo hypertrophy is regulated by a number of factors, most notably the parathyroid hormone related protein (PTHrP)/Indian hedgehog (IHH) axis within the growth plate (as described in the chapter introduction to Chapter 3, section 3.1 and Figure 3.1), however there are other regulators e.g. triiodothyramine, a thyroid hormone which enhances hypertrophy (Mueller and Tuan, 2008, Mackie et al., 2011). The slowing of bone elongation and abnormalities of the growth plate are signs of hypothyroidism in humans (Mackie et al., 2011). There is also some evidence for the involvement of WNT activity in relation to hypertrophy (Day and Yang, 2008). Activation of the canonical WNT pathway upregulates the expression of osteogenic genes by osteoblasts by increasing translocation of β catenin in to the nucleus of stimulated cells, and has also been shown to play a role in promoting chondrocyte differentiation and hypertrophy (Day and Yang, 2008, Mackie et al., 2011).

One of the major issues associated with using MSCs in cartilage therapy is their progression towards terminal differentiation and hypertrophy, in a manner reminiscent of endochondral ossification, when they are induced towards chondrogenesis (Johnstone et al., 1998, Mueller and Tuan, 2008). The phenotype of chondrogenically induced MSCs closely mimics the phenotype of hypertrophic chondrocytes in developing bones (Mueller and Tuan, 2008). The similarities between the two processes include the expression of certain genes including various isoforms of the FGF receptor and markers of hypertrophy including collagen type X and MMP13 (Mueller and Tuan, 2008).

In contrast, when chondrocytes are grown in high density pellet cultures they are more effective than MSCs at producing cartilage-like tissue; the tissue produced by chondrocytes is also mechanically superior and contains higher levels of aggrecan and type II collagen (Bernstein et al., 2010). When cultured in pellets, chondrocytes also maintain their phenotype, and unlike MSCs do not progress towards hypertrophy. When pellet cultures of chondrocytes are implanted into the subcutaneous pouch of severe combined immune deficiency (SCID) mice they remain as stable ectopic pieces of cartilage (Pelttari et al., 2006). However, when the same experiment is carried out with pellets of chondrogenically stimulated MSCs neovascularisation and calcification occur (Pelttari et al., 2006). These differences suggest that the differentiation that MSCs undergo when TGF- β is used to induce chondrogenesis is closer to the process of endochondral ossification than that observed in articular chondrocytes which produce stable articular cartilage (Mueller and Tuan, 2008). Identifying the differences between these two processes may allow for improvement of MSC differentiation protocols.

It has been speculated that the rapid progression of MSCs towards hypertrophy during chondrogenic induction *in vitro* is caused by the loss of the important spatial and temporal signalling networks that exist for chondrogenically differentiating cells *in vivo* (Pelttari et al., 2006). Instead, taking a pellet culture as an example, all of the cells receive the same stimuli at the same time, resulting in relatively synchronous differentiation and removing any interactions between different populations of cells. This disorder may prevent MSCs from being able to maintain a stable chondrogenic phenotype in culture (Pelttari et al., 2006). As a result, rather than synchronously expressing type II collagen, aggrecan and other chondrogenic markers as a population in response to TGF- β induced chondrogenesis, MSCs begin to express both chondrogenic and hypertrophic markers within days of TGF- β exposure (Pelttari et al., 2006). Type X collagen production has even been detected before the full establishment of type II collagen production; this suggests either that MSCs that are chondrogenically differentiated in pellet culture are committed to terminal differentiation from an early stage of culture, or that a better marker of hypertrophy is required *in vitro* (Pelttari et al., 2006, Mwale et al., 2006).

Soluble factors play a major role in the chondrogenic differentiation of MSCs (Weiss et al., 2010, Fischer et al., 2010, Johnstone et al., 1998). When MSC pellets are cultured in medium conditioned by articular chondrocytes the expression of hypertrophic markers including type X collagen and IHH are lower than in MSC pellets cultured in unconditioned medium (Fischer et al., 2010). This shows experimentally the importance of soluble factors. This study also showed that when MSC pellets were cultured in a chondrogenic medium they only produced PTHrP for up to two weeks, after that, IHH signalling predominated (Fischer et al., 2010). PTHrP has been shown to be effective at down-regulating the expression of markers of hypertrophy in MSCs grown *in vitro* (Kim et al., 2008). Methods for maintaining PTHrP expression, such as DNA transfection, or ways of inducing natural expression may help to increase the chondrogenic stability of MSCs.

The apparent predisposition of bone marrow MSCs to progress towards hypertrophy may also be linked to their role within bone tissue. MSCs play an important role in bone healing after fracture (Dimitriou et al., 2005). These cells act as a key source of stem cells, which play a critical role in various stages of the fracture healing process (Dimitriou et al., 2005). This may mean that the cells harvested from bone marrow may have some underlying predisposition to osteogenesis rather than chondrogenesis.

Another potential contributing factor is the heterogeneous nature of MSC populations, which means that individual cells will have different capabilities to proliferate and

differentiate down different pathways, which may add to the disorganisation observed in pellet cultures (Mueller and Tuan, 2008).

Other causes of phenotypic instability in MSCs have been suggested e.g. the natural environment for chondrocytes is hypoxic, at approximately 2-5% oxygen. Lowering the oxygen tension that MSCs induced into chondrogenesis are exposed to leads to a down-regulation of markers of terminal differentiation (Lee et al., 2013). Mechanical load may also play a role in maintaining MSCs in a chondrogenic state; computer modelling techniques such as finite element modelling (FEM), as well as studies applying loads to chondrogenic cells *in vitro*, have shown that the application of low levels of compressive or shear load to chondrogenic cells will result in the production of cartilage (Huang et al., 2004, Lee et al., 2011, Schatti et al., 2011, Zahedmanesh et al., 2014).

There may also be other factors that are yet to be fully characterised or appreciated that are important to the successful maintenance of chondrogenesis in MSCs, e.g. the action of enzymes, like MMP13, that are produced during MSC differentiation and can play a role in the progression of cells towards hypertrophy (Wu et al., 2002).

In order for MSCs to be adopted clinically it is important for science to better understand their chondrogenic differentiation, and if possible determine the differential mechanism(s) whereby MSCs form transient cartilage, through a process reminiscent of endochondral ossification, whilst chondrocytes can form permanent articular cartilage. The majority of past and current work on the chondrogenic differentiation of MSCs is broadly focused on the response of cells to transforming growth factor β (TGF- β), which was first shown to have a chondrogenic effect on MSCs in the late 1990's (Johnstone et al., 1998).

1.7 Transforming Growth Factor β

The three isoforms of TGF- β (1, 2 and 3) are members of the TGF- β superfamily of proteins. This superfamily consists of more than 30 proteins and is split into two subfamilies: the TGF- β subfamily which includes TGF- β 1-3, activin A and B and Mullerian inhibiting substance, and the bone morphogenetic protein (BMP) subfamily containing BMP2 and BMP4-10 and the growth and differentiation factors (GDFs) (Wang et al., 2014a). These factors are involved in a plethora of processes including embryonic development, inflammation and tumorigenesis.

TGF- β is of particular interest for cartilage repair as it can be used to induce chondrogenesis in MSCs. This was first demonstrated by Johnstone et al. (1998), who were able to show the development of a chondrocyte-like phenotype and cartilage-like matrix deposition in MSCs cultured in pellets in the presence of dexamethasone and 10ng/ml TGF- β 1. Subsequent work by Barry et al. (2001) showed that all three TGF- β isoforms (1, 2 and 3) were capable of inducing chondrogenesis in MSCs, but that there were differences between the effects of the different isoforms. This was demonstrated by the increased induction of GAG synthesis and early induction of collagen type II production in pellets treated with TGF- β 2 and 3 rather than TGF- β 1 (Barry et al., 2001). Further investigation into the chondrogenic effects of the three TGF- β isoforms showed that no detectable difference in the induction of chondrogenesis could be observed between the three isoforms when used to chondrogenically stimulate human bone marrow derived MSCs in pellet cultures (Cals et al., 2012). However, greater mineralisation was observed in pellets exposed to TGF- β 2 or -3 than TGF- β 1 when the pellets were exposed to β -glycerophosphate, suggesting that the differential effects of the three isoforms may relate to terminal differentiation rather than chondrogenic induction (Cals et al., 2012).

The signalling induced by TGF- β superfamily members occurs through type I and II transmembrane receptors and is mediated by different classes of Smad proteins. Smads derive their name from a portmanteau of *Caenorhabditis elegans* SMA (small) and *Drosophilla* mothers against decapentaplegic (Macias et al., 2015).

TGF- β signalling is initiated by the formation of a heteromeric complex consisting of the TGF- β homodimer, a type I TGF- β receptor and a type II TGF- β receptor (Heldin et al., 2009) (Figure 1.5). Both of these receptors are transmembrane serine/threonine kinase receptors (Wang et al., 2014a).

The formation of this complex leads to activation of the type II receptor which in turn transphosphorylates the GS region (named for its high glycine and serine content (Massague, 1998)) of the type I receptor leading to type I receptor activation (van der Kraan et al., 2009, Wang et al., 2014a). Type I receptors are referred to as activin

receptor like kinases (ALK) of which seven have been discovered (ALK1-7) (van der Kraan et al., 2009). Five type II receptors have also been described: TGF β RII, ActRII, ActRIIb, BMPRII and MISRII (Wang et al., 2014a).

The activation of the type I receptor leads to the phosphorylation of receptor activated Smads (R- Smads) (Derynck and Zhang, 2003). There are five R- Smads, Smad1, 2, 3, 5 and 8. The receptor activated Smads are associated with and activated by different receptors, and therefore, with the different ligands specific for those receptors; ALK1, 2, 3 and 6 mediate BMP signalling through the Smad1/5/8 pathway whilst ALK 4, 5 and 7 bind to activins and TGF- β s and signal through Smad 2/3 (van der Kraan et al., 2009, Wang et al., 2014a). Evidence suggests TGF- β s can also bind to ALK1 and 2 activating signalling pathways previously associated with BMPs (van der Kraan et al., 2009). This interaction of TGF- β with the BMP pathway may be associated with the induction of hypertrophy in MSCs undergoing TGF- β driven chondrogenesis. In the case of TGF- β signalling, the type I receptor phosphorylates the two serine residues found in the – SXS C-terminal motif of Smad 2 and 3 (Heldin et al., 2009).

Two phosphorylated receptor Smads then form a heterotrimeric complex with Smad4 (common Smad), which is then translocated into the nucleus and can induce or suppress the expression of target genes at a transcriptional level (Derynck and Zhang, 2003). Smad4 is required for the majority of R-Smad activity but is not essential for all Smad activity (Heldin et al., 2009).

The transcriptional activity of Smads is then switched off by several mechanisms including Smad dephosphorylation, and the binding of SnoN and Ski proteins to the Smad complex which prevents its transcriptional activity, and targets the complex for ubiquitination and proteasome degradation (Heldin et al., 2009). SnoN and Ski are themselves Smad targets, so the transcriptional activity of the Smad complex is regulated by a negative feedback loop (Heldin et al., 2009). Smad mediated signalling is also regulated by the inhibitory Smads (6 and 7). These block type I receptor phosphorylation of R-Smads, therefore blocking the transduction of receptor activation to transcriptional regulation. Smad 6 preferentially inhibits Smad1/5/8 signalling (BMPs) whilst Smad 7 blocks both Smad 2/3 (TGF- β) and Smad 1/5/8 signalling (van der Kraan et al., 2009).

As well as acting through the Smad pathway, TGF- β signalling is also mediated by non-canonical pathways such as MAPK pathways (e.g. TGF- β activated kinase 1 (TAK), Rho-like GTPase pathways and the phosphatidylinositol-3-kinase pathway (Derynck and Zhang, 2003, Wang et al., 2014a). The effects of these pathways on the chondrogenesis of MSCs has not been widely studied; however, evidence suggests that these pathways, TAK1 signalling in particular, may play a role in cartilage

development and the progression of chondrocytes towards hypertrophy (Wang et al., 2014a).

TGF- β plays a key role at all stages of cartilage development as well as in adult cartilage. TGF- β upregulated N-cadherin and fibronectin expression is key in the formation of the mesenchymal condensations that then go on to form the cartilaginous anlagen (Wang et al., 2014a). The proliferation and synthetic activity of proliferating and pre-hypertrophic chondrocytes is also driven in part by TGF- β signalling (van der Kraan et al., 2009). The process of terminal differentiation is not only affected by TGF- β associated Smad2/3 mediated signalling but also by BMP associated Smad1/5/8 signalling. TGF- β signalling through the Smad 2/3 pathway has been shown to be protective of the chondrogenic phenotype in growth plate chondrocytes (Wang et al., 2014a, Ballock et al., 1993) whilst Smad 1/5/8 signalling is involved in the induction of terminal differentiation both *in vivo* and *in vitro* (van der Kraan et al., 2009, Hellingman et al., 2011). A link has even been suggested between Smad mediated signalling and osteoarthritis, with a shift from predominantly Smad 2/3 signalling to increased Smad1/5/8 signalling during the development of arthritis (van der Kraan et al., 2009).

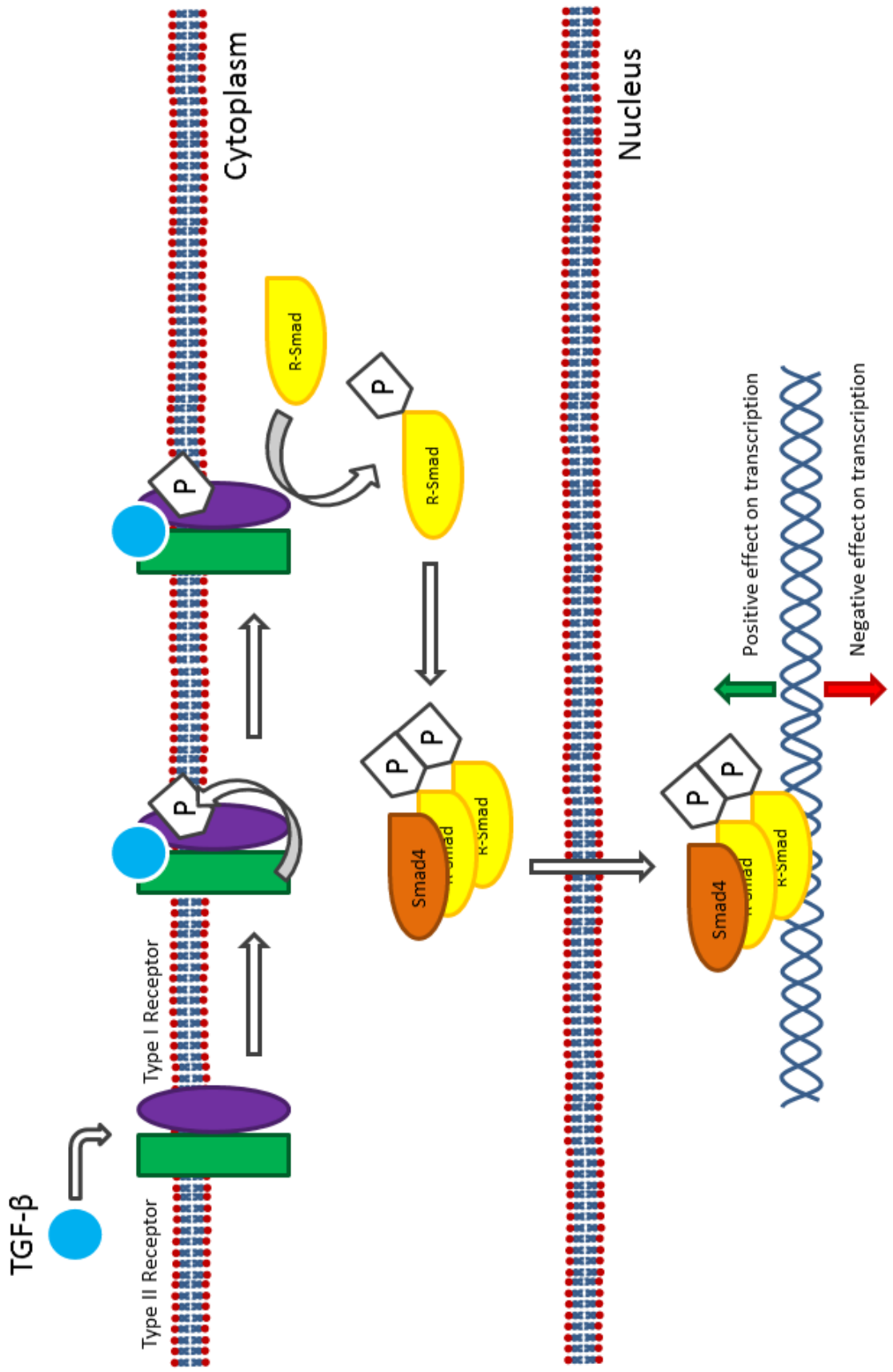


Figure 1.5

The TGF- β signalling pathway from transmembrane receptor binding to transcriptional regulation. Ligand binding leads to type II receptor activation, leading to phosphorylation and activation of the type I receptor. The active type I receptor phosphorylates R-Smads. Phosphorylated R-Smads form a complex with Smad4 which translocates to the nucleus where it can increase or decrease the expression of Smad (and therefore ligand) target genes.

1.8 Mechanostimulation

The effect of mechanical load on the development of skeletal tissue has been recognised for a long time. Wilhelm Roux suggested in the 1800's that different mechanical forces played a role in the development of connective tissue, and in 1922 Carey noted that the initial areas of ossification in the developing porcine femur occurred where the highest tensile loads existed, and suggested that the load must therefore affect the development of the skeleton (Carey, 1922). The importance of load in the development of the skeleton is also demonstrated by the failure of joint cavities and cartilage to form, as well as a large number of other skeletal abnormalities, in embryos in which the skeletal muscle is paralysed with botulinum toxin (Murray and Drachman, 1969). Mechanical loading not only affects the development of the musculoskeletal system, but also plays a role in the development of the heart and the lateral-asymmetry of the organs found in vertebrates (Kartagener and Stucki, 1962).

Loading remains important after development and seems to be essential to the maintenance of healthy articular cartilage (Palmoski et al., 1980, Palmoski and Brandt, 1981). As an example, the loading that chondrocytes experience *in vitro* can affect their synthetic activity e.g. sub-physiological loads have been shown to cause translational arrest (Lomas et al., 2011, Lee and Bader, 1997), whilst physiological loads have been linked to ECM production (Mauck et al., 2000, Lee and Bader, 1997).

The importance of load on the development of musculoskeletal tissues and the cells within these tissues is clear. As a result there has been a considerable effort in recent times to produce bioreactors and loading devices that can expose cells, with or without a supporting scaffold structure, to different forms of mechanical load, to either develop tissue engineering implants, or to model the effects of physiological loads on different preparations of cells and scaffolds (Grad et al., 2011). Bioreactors and loading devices have been used to apply a range of forces in a static and dynamic manner to both MSCs and chondrocytes; this introduction will now focus on the effects of: hydrostatic pressure, compression, shear, and multi-axial load on MSCs.

1.8.1 Hydrostatic pressure

Hydrostatic pressure is generated by the compression of a liquid within a confined space (Elder and Athanasiou, 2009). As cartilage is loaded, the fluid within it becomes pressurised and this generates hydrostatic pressure, which is key to the normal functioning of articular cartilage (Elder and Athanasiou, 2009). In order to mimic this in a loading device, the hydrostatic pressure experienced by cells in monolayer or in a scaffold can be increased by compressing the gas phase above the level of the medium, or by directly compressing the medium itself, causing an increase in hydrostatic pressure (Elder and Athanasiou, 2009). As with other forms of loading, cyclic hydrostatic pressure has been shown to be beneficial for the chondrogenic phenotype, whilst static loading does not have the same effect (Elder and Athanasiou, 2009).

Work by Angele et al. (2003) exposed MSC pellet cultures in a chondrogenic medium to cyclic hydrostatic pressure that changed from 0.55MPa to 5.05MPa (within the normal physiological range for cartilage) at a frequency of 1Hz. The results of this study demonstrated that pellets increased in size and produced increased levels of proteoglycan and collagen when loaded for 7-14 days (Angele et al., 2003).

The application of hydrostatic pressure to MSCs was also investigated by Miyanishi et al. (2006). In the first of two papers published in 2006, this group loaded human bone marrow MSC pellets with 10MPa hydrostatic pressure at 1 Hz for 4 hours a day for up to 14 days in the presence or absence of TGF- β 3 (Miyanishi et al., 2006b). Intermittent hydrostatic pressure induced the gene expression of the chondrogenic markers Sox 9, collagen type II and aggrecan in the absence of TGF- β 3, and further increased the expression of these markers in pellets cultured with TGF- β 3 (Miyanishi et al., 2006b). In the second paper published in 2006, Miyanishi et al. exposed pellets to 0.1, 1 or 10 MPa cyclic hydrostatic pressure at 1 Hz for 4 hours a day for up to 14 days. The increase in hydrostatic pressure led to a dose dependent increase in the gene expression of aggrecan, whilst Sox 9 expression was induced in all groups and collagen type II only in pellets that received 10 MPa loading (Miyanishi et al., 2006a). In addition to a dose dependent increase in response to load, they also showed a time dependent increase in the production of GAG and collagen over the two week culture period (Miyanishi et al., 2006a).

Investigation into the effect of delaying the application of hydrostatic pressure was performed by Meyer et al. (2011). During this study, 10MPa hydrostatic pressure was applied at 1Hz for 1 hour a day, 5 days a week for up to six weeks to porcine BMSCs seeded into 2% agarose gels in the presence or absence of 10ng/ml TGF- β 3 (after 21 days of preculture) (Meyer et al., 2011). The results of this work showed that, despite

donor variation, hydrostatic loading increased the production of GAG and collagen by stimulated cells in the presence of TGF- β 3, but loading in the absence of TGF- β 3 (after 21 days of preculture) did not induce increased matrix synthesis (Meyer et al., 2011). The relationship between TGF- β and hydrostatic pressure was further studied by Vinardell et al. (2012). Pellet cultures of porcine chondrocytes, synovial membrane derived stem cells and fat pad derived stem cells were exposed to 10MPa hydrostatic pressure at 1Hz for four hours a day over 14 days in the presence of different medium concentrations of TGF- β 3 (Vinardell et al., 2012). In the absence of TGF- β 3, hydrostatic pressure did not induce the expression of markers of chondrogenesis, however hydrostatic pressure did enhance chondrogenesis in the presence of 1ng/ml TGF- β 3. In contrast, load had no extra beneficial effect on cartilage specific markers when applied in combination with 10ng/ml TGF- β 3 (Vinardell et al., 2012). These results demonstrate the close links that exist between mechanical load and TGF- β 3 production; this is discussed in further detail in section 1.9.

The effect of hydrostatic pressure on the stability of the MSC chondrogenic phenotype has also been studied. Carroll et al. (2014) looked at the effect of a 10MPa load administered at 1Hz for 4 hours a day, 5 days a week for up to 5 weeks on porcine MSCs and fat pad derived stem cells embedded in 2% agarose gels. This work showed that load was beneficial for the synthesis of GAG in both types of stem cell and collagen in MSCs only; furthermore, loading also improved the mechanical properties of scaffolds containing both cell types (Carroll et al., 2014). Hydrostatic loading was also shown to reduce calcium deposition in MSCs that were cultured in chondrogenic medium, but not hypertrophic medium. This suggests that hydrostatic pressure has some beneficial effects not only on cartilage-like matrix synthesis in MSCs, but also for preserving the chondrogenic phenotype (Carroll et al., 2014).

Work conducted into the effects of hydrostatic loading show that cyclic application of physiological levels of pressure leads to an increase in the production of matrix molecules by MSCs within scaffolds when applied in combination with TGF- β , but that hydrostatic pressure alone cannot, in all cases, increase the expression of chondrogenic markers above free swelling controls in the absence of TGF- β .

1.8.2 Compression

Compressive load is commonly used to study the effects of mechanical load on chondrogenic cells. Common estimates of physiological deformation caused by joint movement range from 6% (Eckstein et al., 2001) to 20% (Armstrong et al., 1979). The natural frequency of walking in humans is approximately 1Hz so in order to replicate the physiological effects of movement (particularly the effects of movement on the knee) it is common for studies to apply loads of 10-15% compression at a frequency of 1Hz (Huang et al., 2004, Campbell et al., 2006, Mauck et al., 2000, Ng et al., 2006, Thorpe et al., 2010, Schatti et al., 2011), however this is not always the case e.g. Angele et al. (2004) loaded MSC hydrogels at 0.33Hz and 40% compression. The period of loading that the cells were subjected to also varies markedly from study to study e.g. Huang et.al. (2004) applied a load at 1Hz for four hours a day on fourteen consecutive days, whereas Kisiday et.al. (2009) loaded for twelve hours a day for eight days. Compressive loading, using a number of different cyclic regimes has been shown to induce chondrogenesis in MSCs.

Huang et.al. (2004) compared the chondrogenic effects of both TGF- β 1 and compression both separately and in combination. Bone marrow derived rabbit MSCs were seeded in to 2% agarose gels and were then exposed to 10% compression at 1Hz for four hours a day, for up to fourteen days. The data collected showed that load was able to induce chondrogenesis in MSCs in the absence of exogenous TGF- β , and that the relative induction of collagen type II and aggrecan gene expression was similar to that induced by culturing the cells in chondrogenic culture media containing 10 ng/ml TGF- β 1 (Huang et al., 2004).

Longer loading periods of compressive load have also been shown to induce chondrogenesis in MSCs. Kisiday et.al. (2009) applied 7.5% compression on top of 2.5% pre-compression at 0.3Hz for 21 days to equine bone marrow derived MSCs seeded into 2% agarose cells, in the absence of TGF- β (Kisiday et al., 2009). This led to increased levels of proteoglycan synthesis when compared to unloaded controls. However, the cells subjected to load only produced 20-35% of the proteoglycan content of the MSCs cultured in 10ng/ml TGF- β 1 (Kisiday et al., 2009).

Campbell et.al. (2006) seeded human bone marrow derived MSCs into 3% alginate scaffolds and applied a 15% load at 1Hz with and without TGF- β 3 in six hour cycles (1.5 hours loading followed by 4.5 hours rest) for up to eight days. In the absence of TGF- β 3, load was able to induce the expression of aggrecan, Sox 9 and collagen type X (Campbell et al., 2006). The effect of load was also noted not to be synergistic when applied in combination with 10 ng/ml TGF- β 3 (Campbell et al., 2006).

Angele et al. (2004) cultured human bone marrow derived MSCs in a hyaluronan-gelatin scaffold in chondrogenic medium (containing 10 ng/ml TGF- β 1), and scaffolds were loaded at 0.33Hz and 40% compression for four hours for up to three weeks. This loading regime led to an increase in the gene expression of aggrecan, collagen type I and collagen type II, and led to a significant increase in GAG and collagen content in loaded scaffolds compared to unloaded controls at day 21 of culture (Angele et al., 2004).

Further characterisation of the effect of compressive load in the presence of TGF- β was performed by Thorpe et al. (2010). Agarose scaffolds (2%) containing porcine bone marrow derived MSCs were cultured in the presence or absence of TGF- β 3 (10 ng/ml) (after 21 days of preculture) and either kept in free swelling culture or were exposed to compressive load from day 0 to day 42 or from day 21 to 42. The loading regimen consisted of 10 % compression at 1Hz, for one hour a day, five times a week. The results of this study showed that loading from day 0 had a detrimental effect on chondrogenesis and that loading from day 21 (after three weeks of chondrogenic induction) did not positively or negatively affect the outcome of chondrogenic induction (Thorpe et al., 2010). This work also demonstrated that the continuous application of 10 ng/ml TGF- β 3 was the most potent inducer of chondrogenesis in this system.

These studies show that compressive load has a chondrogenic effect on MSCs both in the presence and absence of TGF- β . However, they also highlight the complex relationship between TGF- β and the response of MSCs to mechanical load. Four of the four five studies discussed above noted the lack of synergy between mechanical load and TGF- β application, emphasising the crucial relationship between TGF- β and mechanical load that is discussed further in section 1.9.

1.8.3 Shear

Shear stress, resulting from fluid flow is widely recognised to be important in the response of osteocytes to mechanical loading, but shear stresses have also been shown to have an effect on the activity of MSCs (Lee et al., 2011).

Evidence suggests that MSCs cultured in a 2D monolayer exhibit an osteogenic response when exposed to low levels of shear (fluid flow), leading to the up-regulation of markers such as osteopontin and osteocalcin (Kreke 2005), and at higher magnitudes of shear, markers of endothelial cells such as von Williebrand in MSCs (Wang et al., 2005). The action of shear stress also causes an increase in fluid flow through the matrix (Zahedmanesh et al., 2014). This increase in flow provides improved nutrient movement and availability, as well as more efficient removal of cellular waste, providing a better suited environment for cellular proliferation and metabolic activity.

The application of mechanical shear stimulation alone, in the absence of chondrogenic media, to human bone marrow derived MSCs in fibrin-polyurethane composite scaffolds was investigated as part of a study by Schätti et al. (2011). In this study, shear forces were generated by rotating a ceramic ball on the surface of the scaffolds. This work demonstrated that the application of mechanical shear caused a small up-regulation of chondrogenic markers such as collagen type II, aggrecan and COMP, but the combination of shear and compressive load was required to induce significant chondrogenesis (Schatti et al., 2011).

Other studies have examined the effect of shear forces in the form of fluid flow on MSC chondrogenesis. Alves da Silva et al. (2011) exposed human bone marrow derived MSCs seeded on chitosan poly(butylene terephthalate) (CPBTA) meshes to a flow of chondrogenic media containing 1ng/ml TGF- β 3 at 100 μ l/minute/fibre mesh for 28 days (Alves da Silva et al., 2011). The application of fluid flow had no beneficial effects over the static culture of MSCs on meshes with chondrogenic medium with regards to the gene expression of collagen type II or aggrecan; however it did reduce the gene expression of collagen type I, collagen type X and Runx2, suggesting some chondroprotective effects of fluid flow (Alves da Silva et al., 2011). The effect of fluid flow on MSC pellets was studied by Kock et al. (2014). Human bone marrow derived MSCs were formed into pellets and allowed to differentiate for 18 days in the presence of 10 ng/ml TGF- β 2, followed by exposure of the pellets to a 1.22 ml/min flow of chondrogenic medium for 10 days (Kock et al., 2014). Exposure to fluid flow had a negative effect on chondrogenesis leading to reduced collagen type II gene expression, reduced GAG production and a reduction in histologically detectable GAG and collagen II from the pellets (Kock et al., 2014).

The limited work performed on the effect of shear loading suggests that fluid flow has limited beneficial and potentially negative effects on MSC chondrogenesis. However, the application of shear using a solid surface (such as a ceramic ball) may have some beneficial effects for MSC chondrogenesis, particularly when applied in combination with compressive load.

1.8.4 Shear and compression

Within joints *in vivo*, and particularly within the knee, the forces that most cartilage is exposed to during day to day physiological movements are a combination of compression (caused by gravitational or muscular loading) and shear stress (generated by the movement of the two articular surfaces across each other and the movement of synovial fluid across the surface of the tissue). As a result there is interest in the effect of applying a combination of shear and compression to MSCs to investigate the effect of this joint relevant mechanical load on chondrogenesis.

Different authors have carried out investigations into the combined effects of these two forces using very different bioreactors. Bioreactor designs range from those that provide compression using a linear actuator and the movement of a plate in contact with the samples to provide shear, to the 'ball on stick' design which uses a ceramic hip replacement ball to both rotate on top of the sample providing mechanical shear and press down on the sample to provide compressive loading (Waldman et al., 2007, Wimmer et al., 2004, Frank et al., 2000, Yusoff et al., 2011).

Most of the studies performed have focused on the effect of multiaxial mechanical load on chondrocytes. However, there has been a substantial amount of work performed by our group investigating the effect of combined shear and compression on MSCs, using the 'ball on stick' bioreactor system first described by Wimmer et al. (2004) (figure 1.6). The most relevant work is summarised below, however, this bioreactor has also been used for similar studies that have not been described e.g., studies focusing on the potential use of viral over-expression of genes such as Sox 9 and BMP2 for cartilage tissue engineering in a joint-like environment (Kupcsik et al., 2010, Salzmann et al., 2009, Neumann et al., 2013, Neumann et al., 2015).

Characterisation of this system showed that when human bone marrow derived MSCs were seeded into fibrin-poly(ester-urethane) and exposed to 10% compressive load (on top of 10% pre-compression) and $\pm 25^\circ$ oscillation at a frequency of 1Hz for one hour a day for seven days, the MSCs responded by increasing GAG production and up-regulating collagen type II, aggrecan and lubricin transcription (Li et al., 2010a). This work demonstrated that the response to load was only observable if the constructs were not cultured with TGF- β 1 and that loading in the presence of TGF- β 1 provided no extra chondrogenic effect. Further investigation showed that the application of multiaxial load to MSC seeded constructs in this system led to the induction of endogenous TGF- β 1 production resulting in approximately 1 ng/ml of TGF- β 1 in the culture medium (Li et al., 2010a). This endogenously produced TGF- β 1 induces the chondrogenesis of loaded MSCs, and blocking the TGFBR1 blocks the induction of chondrogenesis in response to multiaxial mechanical load (Li et al., 2010a).

Importantly, this study was therefore able to show that multiaxial mechanical load induces the endogenous production of TGF- β 1 by MSCs which then drives chondrogenesis; loading in the presence of chondrogenic medium containing 10 ng/ml TGF- β 1 masks this beneficial effect of the load on chondrogenesis (Li et al., 2010a).

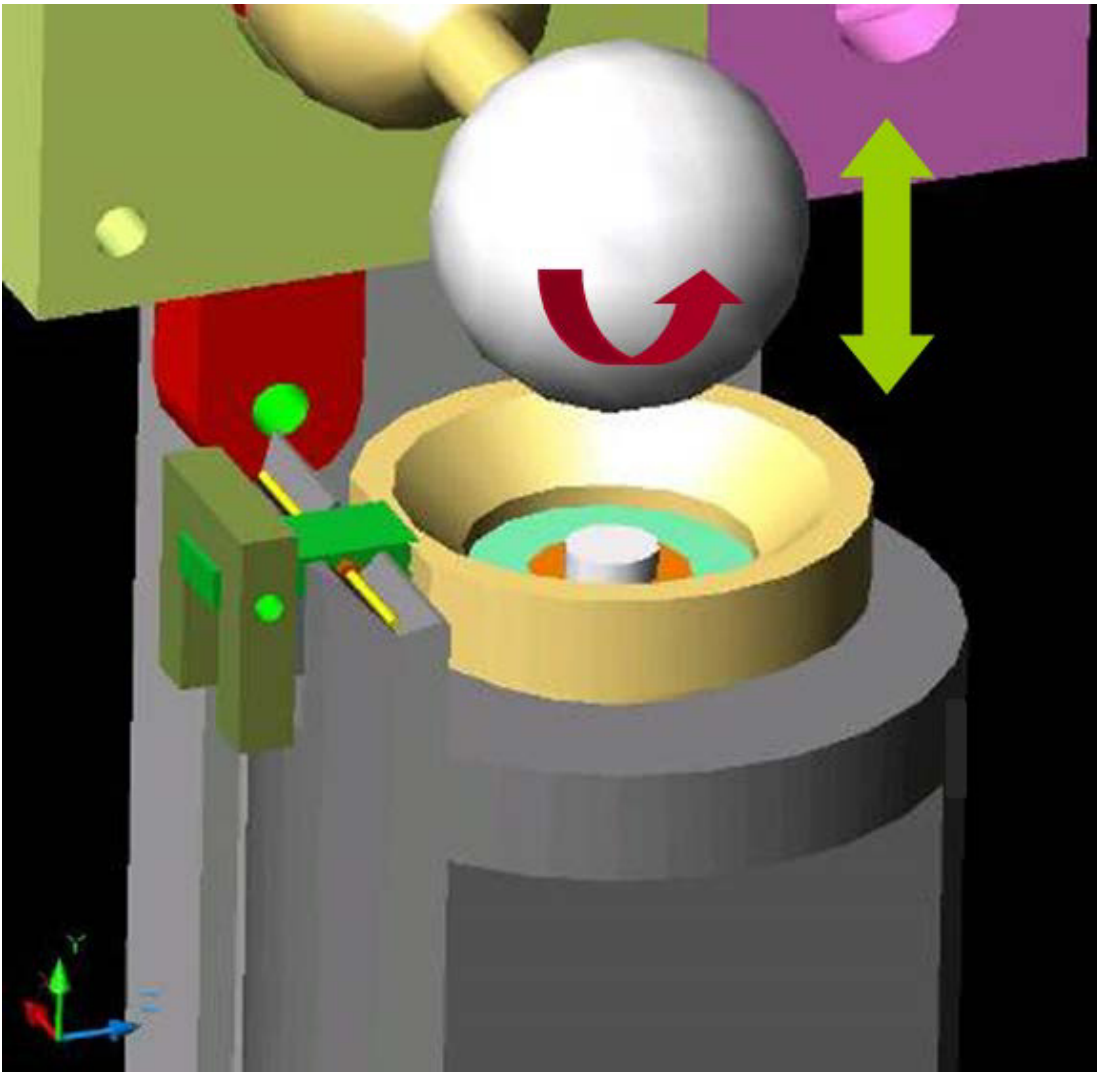


Figure 1.6

A schematic showing the application of compressive and shear load to a fibrin-poly(ester-urethane) scaffold using a ball on stick bioreactor. The white sphere represents a ceramic ball which can be raised and lowered as well as rotated around the axis parallel to the surface of the scaffold. A combination of these two movements results in the generation of both compressive (green arrow) and shear loading (red arrow).

This bioreactor has also been used to investigate the effect of different loading regimes on MSC chondrogenesis (Li et al., 2010b). Li et al. (2010) tested the effects of a range of loading regimes using 5%, 10% and 15% compressive loading at either 0.1Hz or 1Hz for one hour for seven days. Results of this work demonstrated that after seven days pre-culture of MSCs in a chondrogenic medium, an increase in either the frequency of loading, or the amplitude of compressive load led to an increase in GAG production and the expression of collagen type II and aggrecan (Li et al., 2010b). The data collected from this study also showed that the ratios of chondrogenic to fibroblastic, hypertrophic and osteoblast markers were improved by the application of multiaxial load suggesting that the cells are adopting a chondrocyte-like phenotype (Li et al., 2010b).

The application of shear or compressive load alone or in combination to MSCs, in the absence of TGF- β 1 has also been investigated in this system (Schatti et al., 2011). This work used four conditions to establish the effects of the individual loading components; MSC seeded fibrin-poly(ester-urethane) scaffolds were kept as free swelling controls, exposed to compression only at 1Hz 10% compressive load (on top of 10% pre-compression), shear stress only ($\pm 25^\circ$ oscillation at a frequency of 1Hz) and a combined shear and compression group utilising the above loading regimes (Schatti et al., 2011). All three loading regimes led to an increase in GAG production, shear loading alone led to increased gene expression of some chondrogenic markers, but the shear and compression combined group was the only one to show significant increases in the expression of chondrogenic markers such as SOX9 and type II collagen. The results of this work also indicated that there was an increase in the ratios of collagen type II to collagen type I, type X and ALP (Schatti et al., 2011).

The attraction of multiaxial over uniaxial loading is that it better mimics the very complex mechanical loading that occurs within an articulating joint. This combined loading strategy has also been shown to induce the chondrogenesis of MSCs in a way not possible when either shear or compression were applied separately.

1.9 Load and endogenous TGF- β production

The studies summarised in sections 1.8.1, 1.8.2 and 1.8.4 clearly demonstrate that mechanically stimulating MSCs can lead to the induction of a chondrogenic phenotype, even in the absence of exogenous TGF- β . Interestingly, these numerous studies have demonstrated that when one of a number of different types of mechanical load are applied in the presence of exogenous TGF- β there is no synergistic effect between these two chondrogenic stimuli (Huang et al., 2004, Campbell et al., 2006, Kisiday et al., 2009, Li et al., 2010a, Alves da Silva et al., 2011). This is explained by the production of endogenous TGF- β by MSCs in response to mechanical load which has been described by our group and others (Li et al., 2010a, Li et al., 2012, Huang et al., 2004). This body of work was produced using a number of different loading systems with MSCs of different sources and species, suggesting a close link between the application of mechanical load and the production of TGF- β .

The effects of cyclic compression and TGF- β stimulation have been demonstrated on MSCs harvested from the bone marrow of rabbits and implanted into 2% agarose (Huang et al., 2004). Cells in experimental groups were exposed to 10% strain at 1Hz for four hours a day for up to 14 days (Huang et al., 2004). The application of this loading regime, in the presence or absence of TGF- β 1, led to a similar induction of chondrogenic genes as observed when scaffolds were kept in free swelling culture with exogenously applied TGF- β 1. The combined effect of TGF- β and loading was an increase in the production of type II collagen when compared to the cells exposed to TGF- β alone at 14 days of culture, but the overall response between groups was very similar (Huang et al., 2004). The similarity in the effects of loading alone and TGF- β alone suggested that there may be links between the two systems (Huang et al., 2004). This study showed that the loading of cells induced the expression of TGF- β 1 at an mRNA level, suggesting that the chondrogenic induction of MSCs in the loaded group without exogenous TGF- β is driven by endogenously produced TGF- β (Huang et al., 2004).

In a follow-up study, this group investigated the effects of loading on *de novo* TGF- β production (Huang et al., 2005). This study used a slightly altered experimental approach, in which rabbit bone marrow derived MSCs were exposed to 15% compression for four hours at 1Hz for two consecutive days (Huang et al., 2005). This study was designed to determine the temporal production characteristics of various factors associated with the early stages of endogenous TGF- β signalling in loaded rabbit bone marrow MSCs (Huang et al., 2005). The results showed the up-regulation of both parts of the dimeric TGF- β receptor, *T β R-I* and *T β R-II*, as well as up-regulation of the early response genes *c-Jun* and *c-Fos*, which are associated with the ERK and

MAPK pathways, which in turn are associated with TGF- β signalling (Huang et al., 2005). The master chondrogenic transcription factor Sox9 was also found to be up-regulated in loaded cells (Huang et al., 2005).

In applying a combination of shear and compression to MSCs, Li et al. (2010) demonstrated that the beneficial effects of loading on the expression of chondrogenic genes were negated if the cells were exposed to TGF- β within the culture medium. In order to establish the link between loading and production of endogenous TGF- β , MSCs were cultured in a medium containing the T β R-I blocker LY364947 (Li et al., 2010a). The effect of this blocker was to inhibit the chondrogenic response of loaded cells (Li et al., 2010a). This work was therefore able to demonstrate a link between increased TGF- β 1 gene expression and protein production and the chondrogenesis observed in mechanically loaded scaffolds. Similar results have been presented by Li et al. (2012) in a different model of mechanical load, using compression alone. Human bone marrow derived MSCs were seeded into 1.5% alginate beads and allowed to differentiate in chondrogenic medium containing 10ng/ml TGF- β 1 for eight days before being exposed to a pressure of 14-36KPa at 0.25Hz for 1 hour a day for a further seven days in the absence (or presence) of TGF- β 1. The application of load, in the absence of TGF- β 1, led to an increased expression of chondrogenic markers such as collagen type II. This loading regimen also led to an increase in the gene expression, protein production and activation of TGF- β 1 (Li et al., 2012). Blocking the T β R-I with SB431542 led to a decrease in the load-induced up-regulation of chondrogenic markers previously observed (Li et al., 2012). Both of these studies show a direct link between the chondrogenic response to mechanical load and the endogenous production of TGF- β 1 in response to mechanical loading.

The evidence provided by these investigations demonstrates the link between the loading of cells *in vitro*, the up-regulation of *de novo* TGF- β 1 production and the subsequent induction of chondrogenesis in response to this endogenous growth factor production. The effect of load is unlikely to be only limited to increases in TGF- β , although this is currently the best described example. Investigating the effect of load on other growth factors and signalling molecules will provide greater understanding of the response of MSCs to load and may help to advance these cells towards use in the clinic for cartilage repair.

1.10 Thesis Aims

The progression of MSCs towards a hypertrophic, rather than articular cartilage phenotype has hampered their use clinically. It has been hypothesised that the progression of MSCs towards hypertrophy may be due to the loss of the important spatial and temporal signalling networks that exist for chondrogenically differentiating cells *in vivo*. The aim of the work presented in Chapters 3 and 4 was to produce MSC containing fibrin poly(ester-urethane) constructs that were spatially and temporally (in terms of chondrogenic differentiation) heterogeneous. The purpose of this work was to investigate the induction of chondrogenesis and hypertrophy in bone marrow derived mesenchymal stem cells that were stimulated with multiaxial mechanical load in anisotropic scaffolds. It was hypothesised that by producing heterogeneous constructs it would be possible to modulate the induction of chondrogenesis and progression towards hypertrophy through paracrine signalling between cells at different stages of differentiation in the two layers.

TGF- β has been widely used to induce chondrogenesis in mesenchymal stem cells. However, it is also possible to induce MSCs towards chondrogenesis using various forms of mechanical load. The induction of chondrogenesis in response to mechanical load has been linked to the endogenous production of TGF- β by stimulated cells which then drives chondrogenic differentiation. Previous work by our group has shown that the application of mechanical load induces the production of TGF- β 1 by MSCs and subsequently induces chondrogenesis, which could be blocked using a TGF- β receptor I inhibitor. Due to the unexplained differences between groups described in Chapter 4, the work in Chapter 5 aimed to establish new potential markers for the chondrogenic differentiation of MSCs. The work presented in Chapter 5 also aimed to investigate the similarities and differences between these two forms of chondrogenic induction by comparing the secretomes of cells either stimulated with TGF- β or with mechanical load. The purpose of this work was to determine whether the effects of mechanical load are analogous to TGF- β stimulation, or if there are differences between the two forms of induction with regards to the soluble molecules produced by stimulated cells.

The work conducted in this thesis was based upon the principles of regenerative medicine rather than tissue engineering. This appears, superficially, to be a paradox. However, the goal of this thesis was not to produce the highest quality cartilage-like tissue possible *in vitro*, for which the application of 10 ng/ml TGF- β 1 and the use of a better optimised scaffold system would likely induce greater matrix deposition and retention. Instead the goal was to investigate the effect of multiaxial mechanical loading, similar to that which a construct would receive *in vivo*, within a scaffold (fibrin) that is relevant to microfracture and is also approved for clinical use. As most of the

scaffold materials currently used in tissue engineering studies, and TGF- β itself, are not approved for clinical use the experimental design was created to be as translationally, and clinically relevant as possible.

This thesis therefore aimed to address three questions:

Can separate populations of MSCs interact within a scaffold and modulate each other's response to chondrogenic stimuli?

Are the effects of TGF- β 1 stimulation and mechanical load analogous?

Can novel markers of MSC chondrogenesis be found by analysing the secretomes of chondrogenically stimulated cells?

Chapter 2 Materials and Methods

2.1 Materials

All reagents were of analytical grade and purchased from Sigma-Aldrich, Buchs, Switzerland unless otherwise stated. All reagents, plasticware and filtertips used for molecular biology were certified ribonuclease and deoxyribonuclease free.

2.2 Poly(ester-urethane) and Scaffolds Preparation

All preparation of poly(ester-urethane) scaffolds was performed by Dr. David Eglin and Mr. Markus Glarner at the AO Research Institute, Davos, Switzerland.

The poly(ester-urethane) was prepared following a one-step solution poly-condensation and fabricated into a porous structure *via* a salt leaching-phase inverse technique as previously described (Boissard et al., 2009). The poly(ester-urethane) porous sponge was water-jet cut (CUTEC AG, Basel-CH) into cylindrical scaffolds of 8mm in diameter and 4mm in height or 8mm in diameter and 2mm in height, with porosity above 80% and interconnected macro-pores of size ranging from 90 to 300 μ m. Scaffolds of different sizes were used in different experimental set-ups. The design of the multiaxial loading device used in this thesis requires that samples for loading are 4mm deep and 8mm wide. Therefore, either one 4x8 mm scaffold was used (as in Chapter 3 and 4), two 2x8 mm scaffolds were seeded with cells and placed on top of the other (as in Chapter 3) or a cell seeded 2x8 mm scaffold was placed on top of an acellular 2x8 mm scaffold for support during loading (as in Chapter 5),

The scaffolds were sterilised with ethylene oxide in a cold cycle (37°C) and degassed under vacuum for five days.

2.3 MSC Isolation and Proliferation

MSCs were harvested from bone marrow aspirates collected during routine operations with full-ethical approval (KEK-ZH-NR: 2010-0444/0). Detailed information on the donors used in each chapter are provided in the methods section of each individual experimental chapter. All serum was batch tested to ensure MSC multipotency was maintained. The volume of the marrow was determined using a stripette and the sample was then diluted 1:3 with phosphate buffered saline (PBS). The PBS marrow mixture was then repeatedly agitated with a pipette before being passed through a 70µm filter. The tube that contained the PBS marrow mixture and the strainer were then washed with PBS, which was also passed through the filter. Ficoll (Histopaque – 1077) was warmed to room temperature and 2.6ml of Ficoll per 1ml of original marrow sample was transferred to a 50ml polypropylene tube. The marrow sample was carefully layered on top of the Ficoll before the tube was centrifuged at 800 x g for twenty minutes with centrifuge acceleration and deceleration set to the lowest levels. After centrifugation, a plastic dropping pipette was used to collect the interphase (containing mononuclear cells) that had formed between the marrow sample and the Ficoll. The collected interphase was then washed twice with 30ml of alpha Minimum Essential Medium (αMEM) (Gibco, Carlsbad, USA) with 10% foetal bovine serum (FBS) (SeraPlus, Pan Biotech, Aidenbach, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Carlsbad, USA). After washing, the mononuclear cells were counted using a sceptor cell counter (Merck, Darmstadt, Germany). To ensure that only mononuclear cells were counted, cells below 8µm in diameter were gated out of the count. Mononuclear cells were then seeded at a density of 50,000 cells/cm² into culture flasks and left to attach for 96 hours in 10% FBS αMEM supplemented with 5ng/ml basic fibroblast growth factor (bFGF) (Peprotech, Rocky Hill, USA), 100 units/ml penicillin and 100 µg/ml streptomycin. The medium was then changed and attached cells were allowed to grow to 80% confluence before passaging. Cells were again allowed to reach 80%-90% confluence before being frozen and stored in liquid nitrogen.

After thawing, the proliferation of MSCs was carried out before seeding into scaffolds using αMEM, 10% FBS with 5ng/ml bFGF at 37°C with 5% CO₂. Passage 3-4 cells were then trypsinised using 0.05% trypsin-EDTA (Gibco, Carlsbad, USA) and counted using a haemocytometer before being seeded into fibrin-poly(ester-urethane) constructs as described below (section 2.4) (fibrin from Baxter, Vienna, Austria).

2.4 Seeding of Fibrin-Poly(ester-urethane) Scaffolds

The seeding procedure was originally described by Li et al. (2009). In order to carry out the seeding, the required number of culture expanded MSCs (3,600,000 or 4,000,000 cells per 4x8mm scaffold or 1,600,000 or 2,000,000 cells per 2x8mm scaffold depending on the different experimental designs used in each chapter. The cell density was based on previous work on this scaffold system (Li et al., 2009)) per scaffold were resuspended in 33mg/ml fibrinogen (Baxter, Vienna, Austria), 75µl per 4x8mm scaffold or 37.5 µl per 2x8mm scaffold, and transferred to the sterilised cap of an Eppendorf tube. The fibrin cell mixture was then rapidly mixed with an equal volume of 1 unit/ml thrombin (Baxter, Vienna, Austria), using a pipette. A poly(ester-urethane) scaffold, which had been pre-wetted in serum free DMEM (which forms the base of the chondropermissive medium that was applied to the scaffolds after seeding) to decrease the hydrophobicity of the scaffold, was then firmly pressed into the Eppendorf lid using a pair of sterile forceps; removing the pressure from the scaffold allowed the poly(ester-urethane) sponge to regain its original shape and in doing so draw the fibrin cell mixture into the scaffold. The scaffolds were then incubated at 37°C for one hour to allow polymerisation of the fibrin hydrogel. This method has been used previously in a number of studies by this group and results in a homogeneous distribution of cells throughout the scaffold (Zahedmanesh et al., 2014). The final concentrations used for the fibrin gel were 16.5mg/ml fibrinogen and 0.5 units/ml of thrombin.

Once seeded, the scaffolds were transferred to polyether ether ketone (PEEK) holders (Figure 2.1), which were manufactured in house, for further culture. Scaffolds were cultured in a medium consisting of: Dulbecco's modified Eagle medium (4.5g/L glucose (Gibco, Carlsbad, USA), 0.11g/L sodium pyruvate, 50µg/ml L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate, 1×10^{-7} M dexamethasone, ITS+ Premix containing insulin 6.25 µg/ml transferrin 6.25 µg/ml and selenious acid 6.25 ng/ml bovine serum albumin 1.25 mg/ml and linoleic acid 5.35 µg/ml (Corning, Bedford, USA), 1% (v/v) Non-essential amino acids containing 750 mg/L glycine, 890 mg/L L-alanine, 1320 mg/L L-asparagine, 1330 mg/L L-aspartic acid, 1470 mg/L L-glutamic acid, 1150 mg/L L-proline, 1050 mg/L L-serine (Gibco, Carlsbad, USA), 50 µg/ml Primocin (Invitrogen, San Diego, USA) and 5µM 6-aminocaproic acid to reduce fibrin degradation (Kupcsik et al., 2009). Exogenous recombinant human TGF-β1, produced in 293 cells (Fitzgerald, Acton, USA), was added where required as per individual experimental plans.

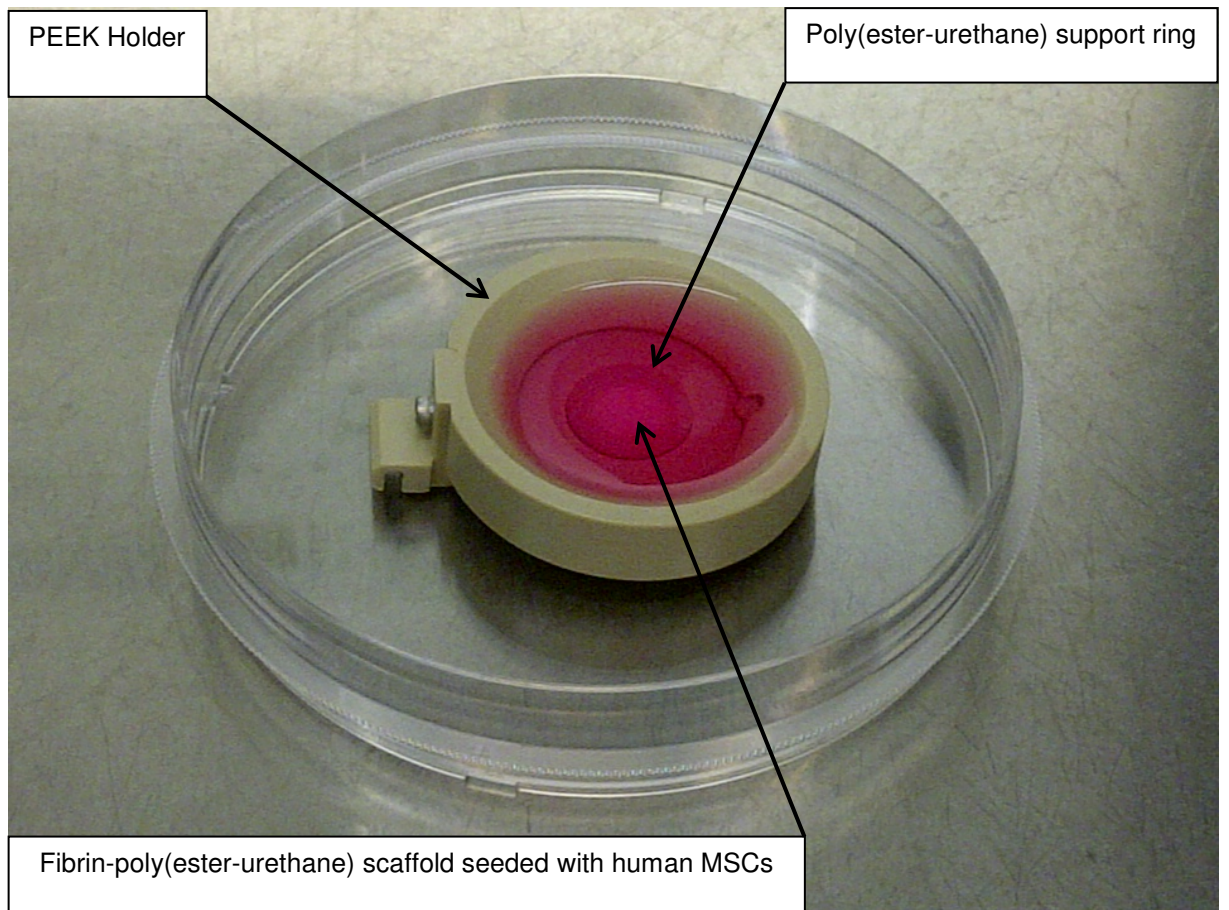


Figure 2.1

A Polyether ether ketone PEEK loading holder containing a fibrin-poly(ester-urethane) scaffold seeded with MSCs.

2.5 Application of Multi-axial Mechanical Load

Scaffolds were loaded using a custom-made multi-axial load bioreactor (Figure 2.2), designed to apply both dynamic shear and compressive loading, based on a tribological system analysis of natural articulating joints (Wimmer et al., 2004). Loaded scaffolds were exposed to 10% compression superimposed on top of a 10% pre-strain and shear loading ($\pm 25^\circ$) at 1Hz for one hour a day five times a week. 10% compression and 25° rotation was chosen based on results from previous work and the limitations of the loading device (Li et al., 2010b). The loading was controlled using LabVIEW software (National Instruments, Ennetbaden, Switzerland) using a customised program designed by ALEA Solutions (Zürich, Switzerland). Load cells, located underneath the PEEK holders, were used to detect contact between the ceramic ball and scaffold in order to apply 10% pre-compression before the initiation of 10% cyclic compression. Loading was carried out in an incubator at 37°C , 5% CO_2 and 80% humidity. Scaffolds were loaded for one, two or four weeks depending on the relevant experimental design. Control scaffolds were kept in free-swelling culture conditions for the culture period (four weeks). The culture media was changed three times per week during the loading period. Multi-axial load, as described here is referred to as joint-like load throughout this thesis.

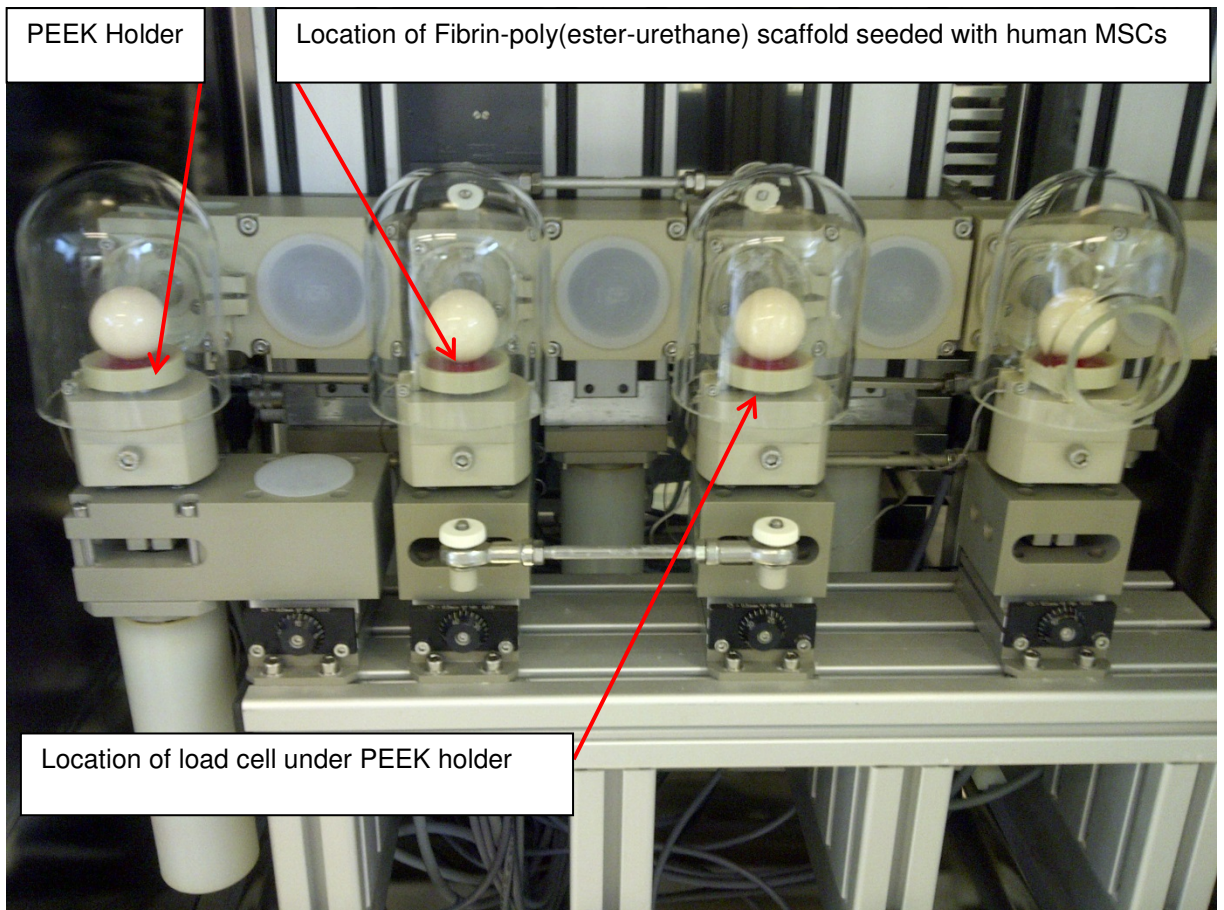


Figure 2.2

The bioreactor used to apply multiaxial load to MSC containing fibrin-poly(ester-urethane) scaffolds.

2.6 Sample Collection and Preparation

Culture media was collected three times a week during the culture period in conjunction with media replenishment, and was then pooled by week, before being stored at -20°C for analysis.

After the required culture period, the scaffolds from each group were harvested for analyses. Twenty-four hours after the final loading scaffolds were cut into halves using a surgical scalpel. The scaffold halves were then prepared for biochemical analysis (GAG/DNA content), gene expression analysis or histological analysis as per the study plan.

TRI reagent® (Molecular Research Centre Inc., Cincinnati, USA) was used for RNA isolation from scaffolds for gene expression analysis. In order to do this one half of the scaffold was placed into a 2ml Eppendorf tube with 1ml of TRI reagent® and a tissue lyser ball bearing. This tube was then placed in a tissue lyser (Retsch & Co., Haan, Germany) for three minutes at 25Hz. After lysis, the tube was centrifuged at 12000 x g for fifteen minutes at 4°C, the supernatant transferred to another tube and 5µl of polyacryl (Molecular Research Centre Inc., Cincinnati, USA) carrier added, to improve the recovery of RNA during isolation, before storage at -80°C.

For biochemical analysis, scaffold pieces were digested in 0.5mg/ml Proteinase K (Roche, Basel, Switzerland) at 56°C for sixteen hours. Proteinase K (PK) was then deactivated with a ten minute incubation at 96°C, and the samples stored at -20°C for analysis.

Portions of scaffold for histological analysis were placed in 100% methanol for fixation (Brenntag, Mülheim, Germany).

2.7 Glycosaminoglycan and DNA Quantification

Glycosaminoglycans (GAGs) are used as a marker for chondrogenesis, the total amount of GAG produced by cells (measured in both collected media and PK digests) was determined using the 1.9-dimethyl methylene blue (DMMB) assay and normalised to the DNA content of each PK digest measured using Hoechst 33258 dye (Polysciences Inc., Warrington, USA). The DMMB method used was based on that of Farndale *et al.* (Farndale *et al.*, 1986)

Quantification of GAG amount released by MSCs into the media was carried out on 50µl of media that had been collected from each sample per week. This method used a standard curve of chondroitin-4-sulfate sodium salt from bovine trachea (Fluka, St. Louis, USA). Standards ranging in concentration from 0.3125 – 10µg/ml were prepared in chondropermissive media (a blank of chondropermissive media was also used). Standards and samples (50µl) were placed into wells of a 96-well plate and 200µl of DMMB added. Absorbance was measured immediately at 535nm using the Victor 3 Micro Plate Reader (Perkin Elmer), and GAG levels calculated following extrapolation from the standard curve.

The quantification of GAG in each PK sample was identical to that used for media samples, except that 20µl of sample and standard were used not 50µl, the blank used was PBS and standards (0.355 – 11.3µg/ml) were made up in PBS not chondropermissive media. Different protocols were used for media and PK quantification due to the different concentrations of GAG in media and PK samples.

Höchst 33258 dye was used to determine DNA content of the PK digests using a method based on that of Labarca *et al.* (Labarca and Paigen, 1980). The standard curve was made using calf thymus DNA (Lubio Science, Luzern, Switzerland) and standards prepared in PBS ranging from 0.3125 - 10 µg/ml. Standards, samples and a PBS blank (40µl) were pipetted into a white 96 well plate and 160µl of 0.01% (v/v) Höchst dye solution added; the plate was then wrapped in aluminium foil to prevent penetration of light, and incubated at room temperature for twenty minutes. Measurements were then made using a wavelength of 360nm (excitation) and 465nm (emission) using the Victor 3 Micro Plate Reader (Perkin Elmer, Waltham, USA), and amounts of DNA calculated following extrapolation from the standard curve.

2.8 TGF- β 1 quantification

Both the amount of total TGF- β 1, and where appropriate the amount of active TGF- β 1, in collected culture media was quantified using the human TGFbeta 1 DuoSet ELISA (R&D systems, Minneapolis, USA). In order to measure the total amount of TGF- β 1 in each sample an acidic activation step was performed as per the manufacturer's instructions. Analyses of the samples without this activation step provided the amount of active TGF- β 1 within the sample. Four parameter logistic analysis was performed using the graph fitting software at www.readerfit.com.

2.9 RNA Extraction

Following on from sample preparation (refer to section 2.6), the RNA TRI reagent supernatants were thawed and 0.1 ml of bromochlorophenol (BCP) added to each 1 ml volume. Tubes were then mixed by inversion for fifteen seconds and placed on a shaker for fifteen minutes. Samples were then centrifuged at 12 000 x g (4°C) for fifteen minutes. The addition of BCP led to a phase separation, the centrifugation step was then used to physically separate the aqueous and organic phases, allowing for the collection of the RNA contained in the aqueous phase.

The RNA within the aqueous phase was precipitated out by adding 0.5 ml of isopropanol. Samples were vortexed, placed on a shaker for ten minutes at room temperature and then centrifuged at 12 000 x g (4°C) for eight minutes.

Following centrifugation, the supernatant was removed and 1 ml of 75% ethanol in DEPC treated water used to wash the RNA pellet. After washing, the sample was centrifuged again at 7500 x g (4°C) for five minutes. The ethanol was removed and the pellets left to air dry for five minutes. Once dry, pellets were resuspended in 20 µl of DEPC water and incubated for fifteen minutes at 56°C to ensure thorough resuspension.

A NanoDrop 1000 spectrophotometer (NanoDrop, Wilmington, USA) was then used to quantify the amount of RNA (measurement at 260nm) and the purity (measured at 230nm and 280nm).

2.10 cDNA Synthesis

Once the concentration of RNA in each sample was determined using the NanoDrop 1000 spectrophotometer, the volume required to provide 1 µg of total RNA per sample for each reaction for work presented in chapter 3 and 500ng for work presented in chapters 4 and 5 was calculated. The generation of cDNA was performed using Perkin-Elmer TaqMan® reverse transcription reagents (Roche, Basel, Switzerland). For results presented in chapter 3 the total reaction volume used was 20 µl, whilst for results presented in chapters 4 and 5 a total reaction volume of 40 µl was used. The decision to change from 20 µl to 40 µl was made in order to allow for the reverse transcription of RNA preparations containing low amounts of RNA. Table 2.1A and B show the reagents used for each 20 µl and 40 µl RT reaction respectively.

A.

Reagents:	µl/tube
10 x TaqMan® RT Buffer	2.0
25 mM Magnesium Chloride	4.4
10 mM dNTP mixture	4.0
50 µM Random Hexamer	1.0
20 U/µl RNase Inhibitor	0.4
50 U/µl Reverse Transcriptase	0.5
RNA Sample + DEPC-water	7.7
Total reaction Volume:	20.0

B.

Reagents:	µl/tube
10 x TaqMan® RT Buffer	4.0
25 mM Magnesium Chloride	8.8
10 mM dNTP mixture	8.0
50 µM Random Hexamer	2.0
20 U/µl RNase Inhibitor	0.8
50 U/µl Reverse Transcriptase	1.0
RNA Sample + DEPC-water	15.4
Total reaction Volume:	40.0

Table 2.1

A. The components required to prepare the required reverse transcription master mix for a single 20µl reaction using Perkin-Elmer TaqMan® reverse transcription reagents (Roche, Basel, Switzerland).

B. The components required to prepare the required reverse transcription master mix for a single 40µl reaction Perkin-Elmer TaqMan® reverse transcription reagents (Roche, Basel, Switzerland).

RT buffer (constituents not made available by supplier), magnesium chloride (final concentration 5.5 mM), dNTP mixture (final concentration 2 mM) and Random Hexamers (final concentration 2.5 μ M) were mixed before the addition of RNase Inhibitor (final concentration 0.4 U/ μ l) and MultiScribe Reverse Transcriptase (final concentration 1.25 U/ μ l) to create a mastermix. Either 12.3 μ l or 24.6 μ l of this was then added to reaction tubes (depending on the final total reaction volume) and the required volumes of DEPC water and cDNA sample added to bring the final reaction volume to 20 μ l or 40 μ l. An Applied Biosystems GeneAmp 5700 thermocycler (Applied Biosystems, Carlsbad, USA) was then used to perform reverse transcription using the following protocol; ten minutes at 25°C for primer annealing, thirty minutes at 48°C for reverse transcription and ten minutes at 95°C for reverse transcriptase inactivation. The samples were then diluted with 60 μ l of DEPC water with Tris-EDTA buffer (0.01 M TRIS, 0.001M EDTA) for samples in chapter 3, or 40 μ l for samples in chapters 4 and 5, the cDNA samples were then stored at -20°C.

2.11 Real-time PCR

Real-time PCR was used to determine the relative expression of target genes of interest in the experimental samples. Oligonucleotide primers and probes were either designed and tested by members of this group using 'Primer Express Oligo Design Software v.1.5' to span exon-exon boundaries and synthesised by Microsynth Ag. (Balgach, Switzerland) (Table 2.2), or purchased as a complete Assay on Demand (angiogenin, angiopoietin 2, BLC, collagen type VI a1 chain, GRO α , leptin, MCP3, MIF, MIP3 α , MMP13, nitric oxide synthase 1, nitric oxide synthase 2, nitric oxide synthase 3, osteoprotegerin, PDGFA, ribosomal 18s RNA and Sox-9 (Applied Biosystems, Carlsbad, USA)). Self-designed primers and probes were reconstituted at a concentration of 45 μ M, then 20 μ l of reverse primer, 20 μ l of forward primer and 20 μ l of probe were mixed with 40 μ l of DEPC water to provide a 10x primer probe mixture.

Gene	Primer Forward	Primer Reverse	Probe (5'FAM/3' TAMRA)	Amplicon Length
human Col1A1	5'-CCC TGG AAA GAA TGG AGA TGA T-3'	5'-ACT GAA ACC TCT GTG TCC CTT-3'	5'-CGG GCA ATC CTC GAG CAC CCT-3'	138bp
human Col2A1	5'-GGC AAT AGC AGG TTC ACG TAC A-3'	5'-GAT AAC AGT CTT GCC CCA CTT ACC- 3'	5'-CCT GAA GGA TGG CTG CAC GAA ACA TAC-3'	77bp
human Col10A1	5'-ACG CTG AAC GAT ACC AAA TG-3'	5'-TGC TAT ACC TTT ACT CTT TAT GGT GTA-3'	5'-ACT ACC CAA CAC CAA GAC ACA GTT CTT CAT TCC- 3'	100bp
human Aggrecan	5'-AGT CCT CAA GCC TCC TGT ACT CA-3'	5'-CGG GAA GTG GCG GTA ACA-3'	5'-CCG GAA TGG AAA CGT GAA TCA GAA TCA ACT-3'	184bp
human Runx2	5'-ACG AAG GTT CAA CGA TCT GAG AT- 3'	5'- TTT GTG AAG ACG GTT ATG GTC AA -3'	5'-TGA AAC TCT TGC CTC GTC CAC TCC G-3'	77bp
human Vascular Endothelial Growth Factor	5'-GCC CAC TGA GGA GTC CAA CA- 3'	5'-TCCTATGTG CTG GCC TTG GT-3'	5'-CAC CAT GCA GAT TAT GCG GAT CAA ACC T-3'	69bp

Table 2.2

Sequences of self-designed primers and probes used for real-time PCR analysis.

The total volume of the reaction mixture used for real-time PCR differed between chapters. In chapter 3 a 20 μ l reaction volume was used (Table 2.3), whilst in chapters 4 and 5 a 10 μ l reaction volume was used (Table 2.4). This change in volume was due to an change in the PCR system used by our laboratory during the course of the project. The reaction mixture was prepared by mixing the required volumes of 2x TaqMan® Master Mix, 20x Assay on Demand or 10x primer probe mix and DEPC treated water (Tables 2.3 and 2.4). The reaction mixture was then pipetted into a Thermo-fast 96 PCR Detection Plate (Thermo Fischer scientific, Waltham, USA) (chapter 3) or a MicroAmp Optical 96 well reaction plate (chapters 4 and 5) (Applied Biosystems, Carlsbad, USA) as per the plate design. cDNA samples (2 μ l) were then added to each well. In order to ensure that there was no contamination of the PCR reaction mix during the preparation of plates for RT-PCR 2 μ l of DEPC water was added to the mastermix as a non-template control. Following pipetting, plates were covered with Absolute QPCR seal, briefly centrifuged (1000 x g, twenty-five seconds) and placed in to an Applied Biosystems 7500 real-time PCR machine (for work presented in chapter 3), an Applied Biosystems StepOnePlus real-time PCR machine (for work presented in chapter 4) or an Applied Biosystems QuantStudio 6 Flex Real-Time PCR System (for work presented in chapter 5) (Applied Biosystems, Carlsbad, USA). The Real-time PCR cycle consisted of ten minutes at 96°C followed by forty cycles of amplification (fifteen seconds at 95°C and one minute at 60°C (chapters 3 and 4) or 2 minutes at 60°C chapter 5). After each cycle of amplification wells are analysed for fluorescence; this measurement was then used to determine the relative abundance of mRNA matching each gene in the sample.

After the measurements had been made the collected data was analysed using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) using ribosomal 18s RNA as the endogenous control. 18s was chosen as the endogenous control due to its stability in response to mechanical load in the bioreactor described in this system as shown previously in chondrocytes and MSCs by previous laboratory members (Lee et al., 2005, Li et al., 2009). The data was normalised to the expression of the cells at the time that they were seeded into the scaffold (day zero after monolayer culture).

Assay on Demand 20 μ l	μ l/reaction
TaqMan Master Mix 2x	10
Assay on Demand 20x	1
cDNA	2
DEPC-water	7
Total	20

Primer/Probe Mix 20 μ l	μ l/reaction
TaqMan Master Mix 2x	10
Primer/Probe Mix 10x	2
cDNA	2
DEPC-water	6
Total	20

Table 2.3

The components required for one reaction for real-time PCR with a total volume of 20 μ l. Human 18s and human Sox9 are bought as 20x primer/probe mixes and therefore different reaction setups.

Assay on Demand 10 μ l	μ l/reaction
TaqMan Master Mix 2x	5
Assay on Demand 20x	0.5
cDNA	2
DEPC-water	2.5
Total	10

Primer/Probe Mix 10 μ l	μ l/reaction
TaqMan Master Mix 2x	5
Primer/Probe Mix 10x	1
cDNA	2
DEPC-water	2
Total	10

Table 2.4

The components required for one reaction of real-time PCR with a total volume of 10 μ l. Human 18s and human Sox9 are bought as 20x primer/probe mixes and therefore different reaction setups.

2.12 Histological Analysis

Methanol fixed samples (section 2.6) were frozen in OCT compound (R. Jung GmbH, Nussloch, Germany) before being sectioned (12 μ m thick) on a cryotome (Carl Zeiss AG, Oberkochen, Germany) and adhered to Superfrost Plus slides (Thermo Fischer scientific, Waltham, USA). Slides were stored at -20°C for future use.

2.12.1 Toluidine Blue staining

Toluidine blue was used to stain for sulphated GAG within histological sections, toluidine blue is a metachromatic dye that stains acidic components blue (e.g. nuclei) and sulphated GAG purple. To stain sections with toluidine blue, slides were first rinsed with distilled water to remove OCT compound. Sections were then placed in 0.1% (w/v) Toluidine blue solution (Fluka, St. Louis, USA) with 0.1% (w/v) sodium tetraborate (Fluka, St. Louis, USA) in deionised water for two minutes before being rinsed with distilled water. Sodium tetraborate was included in the solution as it is a communal stock which is used by other members of the institute who also use the solution for sections embedded in poly(methyl-methacrylate) (PMMA) and it is required to adjust the pH to stain PMMA sections, it does not affect the staining of the fibrin-poly(ester-urethane) scaffolds described in this thesis. Slides were then blotted dry with blotting paper and left to air dry for at least thirty minutes. Slides were then placed in 100% xylene (Brenntag, Mülheim, Germany) for two minutes twice before being mounted using Eukitt mounting medium.

2.12.2 Safranin O/Fast Green staining

Safranin O was used to stain for sulphated GAG within histological sections, Safranin O stains sulphated GAG red/orange. OCT compound was removed from the sections by rinsing in distilled water for ten minutes prior to Safranin O staining. This was followed by a twelve minute incubation in Weigert's Haematoxylin (detailed constituents not provided by manufacturer). The slides were then placed in lukewarm tap water for ten minutes to 'blue' the haematoxylin. Blueing involves the slight elevation of pH which results in a blue/purple staining of the nucleus, this is necessary due to the acidic nature of haematoxylin preparations that results in purple staining before blueing. The result of this process is increased contrast with the red Safranin O stain. After blueing, the slides were briefly washed in distilled water and then placed in 0.02% (v/v) fast green in 0.01% (v/v) acetic acid in deionised water (for five minutes). Fast green staining was followed by thirty seconds in 1% (v/v) acetic acid and then five minutes in 0.1% (w/v) Safranin O solution (Chroma-Gesellschaft Schmid GmbH & Co, Münster, Germany). The slides were then dehydrated by immersing them in 96% ethanol twice (one minute each) and then 100% ethanol twice (two minutes each). Slides were then placed in 100% xylene for two minutes twice before being mounted using Eukitt mounting medium.

2.12.3 Immunohistochemistry

All immunohistology for each target epitope was performed simultaneously on all groups within each biological repeat.

The presence of collagen types I, II, VI, X and TGF- β 1 in sections was determined immunohistochemically using the following primary antibodies COL-1 (Sigma-Aldrich, Buchs, Switzerland), CIIIC1 and 5C6 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, USA), C7974 and MAB240 (R&D systems, Minneapolis, MN, USA).

Slides were brought to room temperature and were then washed in distilled water for ten minutes to remove OCT compound. Slides were transferred to a cuvette containing 0.3% H₂O₂ (Fluka, St. Louis, USA) in methanol (Brenntag, Mülheim, Germany) for thirty minutes in order to inactivate endogenous peroxidases. Slides were then placed on paper towel and allowed to air dry. Once dry, a Dako pen (Dako, Baar, Switzerland) was used to apply a hydrophobic barrier around each section. Slides were then washed twice for five minutes in 0.1% (v/v) PBS-Tween 20.

Slides were then incubated in 0.05-0.5 units/ml bovine testicular hyaluronidase (in 0.1% (v/v) PBS-Tween 20 (PBS-T) at 37°C for thirty minutes before being washed three times for five minutes in 0.1% (v/v) PBS-T.

Sections were subsequently blocked with an appropriate serum for one hour at room temperature; for all antibodies except C7974 this required horse serum (Vector Laboratories, Burlingame, USA) diluted 1:20 in in 0.1% (v/v) PBS-T. C7974 required goat serum diluted 1:20 in in 0.1% (v/v) PBS-T (Vector Laboratories, Burlingame, USA).

Following the block step, the serum was removed without washing and immediately replaced with the primary antibody. The sections were incubated with the primary antibodies for thirty minutes at room temperature. The primary antibodies were diluted with 0.1% Tween 20 as described in Table 2.5.

Slides were then washed three times for five minutes with 0.1% Tween 20 PBS, before being incubated in the relevant biotinylated secondary antibody diluted in 0.1% Tween 20 PBS as described in Table 2.5.

Following this incubation, samples were washed again in 0.1% (v/v) PBS-T and then incubated with ABC solution (Vector Laboratories, Burlingame, USA) for thirty minutes at room temperature, washed again and then incubated with ImmPACT DAB (Vector Laboratories, Burlingame, USA) (Vector Laboratories, Burlingame, USA) for four minutes before being placed into distilled water. Samples were then counterstained with Mayer's haematoxylin for twenty seconds and blued in tap water for five minutes.

Samples were dehydrated in 50%, 70%, 96%, 100%, 100% EtOH before being cleared in xylene and coverslipped with Eukitt mounting medium.

Protein of Interest	Primary Antibody/Dilution (Manufacturer)	Secondary Antibody (dilution)
Collagen type I	COL-1 1:2000 (Sigma)	Biotinylated anti-mouse IgG 1:200 (Vector Laboratories)
Collagen type II	CIICI 1:6 (DSHB)	Biotinylated anti-mouse IgG 1:200 (Vector Laboratories)
Collagen type VI	5C6 1:5 (DSHB)	Biotinylated anti-mouse IgG 1:200 (Vector Laboratories)
Collagen type X	C7974 (R&D systems)	Biotinylated anti-mouse IgM 1:200 (Vector Laboratories)
TGF- β 1	MAB240 (R&D systems)	Biotinylated anti-mouse IgG 1:200 (Vector Laboratories)

Table 2.5

The primary and secondary antibodies used to label proteins of interest during immunohistochemical processing. Each antibody was diluted in 0.1% Tween 20 PBS before use, the dilution factor used for each antibody is provided in this table along with the manufacturer of the antibody.

2.13 Microscopy

Images of histologically prepared sections were taken using an Axioplan 2 microscope, an AxioCamHR camera and Carl Zeiss AxioCamHR V.5.07.03 software (Carl Zeiss AG, Oberkochen, Germany). Images presented in Chapter 3 were taken at 4x magnification and images presented in Chapter 4 were taken at 2.5x, 20x or 40x magnification.

2.14 Statistical analysis

Data are presented, where appropriate, as mean \pm standard deviation. Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, USA), and statistical significance was defined as being $P \leq 0.05$.

The D'Agostino & Pearson omnibus normality test was used to determine if data sets exhibited a normal distribution before statistical analysis. Samples were tested for equal variance as part of parametric analysis. If the data sets showed a normal distribution a T-test was used where there were only two groups, or a one-way ANOVA with a Tukey's multiple comparison *post-hoc* test where there were more than two groups. For data sets that did not show a normal distribution the Mann-Whitney test was used where two groups were compared or a Kruskal-Wallis test with a Dunn's multiple comparisons test where there were more than two groups being compared.

Due to the variation in the type of statistics used between chapters the statistics used have been detailed individually within each results chapter.

Chapter specific methods are provided in the experimental plan section of each chapter.

Chapter 3 Investigating the potential for crosstalk between mesenchymal stem cells at different stages of chondrogenic differentiation within multi-layer fibrin poly(ester-urethane) constructs

Aim

The aim of this chapter was to investigate the development of paracrine signalling between MSCs at different stages of chondrogenic differentiation and determine the effect on chondrogenesis, and hypertrophy, in the presence and absence of mechanical load.

3.1 Introduction

One of the major challenges with using mesenchymal stem cells for cartilage tissue engineering is the progression of the cells towards hypertrophy and terminal differentiation rather than to a stable, hyaline, articular cartilage phenotype (Mueller and Tuan, 2008, Mueller et al., 2010). A potential cause for this undesirable fate selection is the use of differentiation conditions that do not properly support or coordinate the progression of MSC chondrogenesis, leading to the development of an unstable hypertrophic phenotype (Pelttari et al., 2006). This hypertrophic MSC phenotype is characterised *in vitro* by the upregulation of gene expression and production of proteins such as type X collagen and MMP13 as well as markers normally associated with bone, such as Runx2 (Mueller and Tuan, 2008).

In order to improve the quality of tissue engineered cartilage that can be generated from MSCs this work aimed to provide a more structured and coordinated environment for MSCs to undergo chondrogenesis. The idea behind this system came from the growth plate.

The growth plate is a band of hyaline cartilage that sits in the metaphysis of long bones; it has a precisely defined structure that allows concurrent chondrogenesis of the epiphyseal aspect of the plate, and the calcification of the diaphyseal aspect of the plate (Kronenberg, 2003). This controlled process of dual chondrogenesis and calcification allows for elongation of the bone as new cartilaginous tissue is laid down by the upper layers of the plate which then go on to ossify in the lower regions of the plate. The growth plate consists of several layers of chondrocytes (Figure 3.1); the periarticular cells and resting chondrocytes, the proliferating zone, the prehypertrophic zone, and the hypertrophic zone (Mackie et al., 2011). Indian hedgehog (IHH), a key vertebrate morphogen, is produced by pre-hypertrophic and hypertrophic chondrocytes in the prehypertrophic and hypertrophic zones of the growth plate (Deckelbaum et al., 2002, Kronenberg, 2003). The effect of IHH signalling is two-fold: firstly it acts to increase the number of chondrocytes in the proliferating zone entering into terminal differentiation; this acts to drive the progression of chondrocytes from a resting, or proliferative state, towards hypertrophy (Kronenberg, 2003, Lai and Mitchell, 2005). In so doing, IHH induces the ossification of the cartilaginous growth plate. Secondly, the production of IHH by pre- and hypertrophic cells, which are in the later stages of chondrogenesis, also induces the expression of parathyroid hormone related protein (PTHrP) from the periarticular cells, which are at a much earlier stage of chondrogenic development (Kronenberg, 2003). The PTHrP in turn prevents the proliferating chondrocytes from progressing towards terminal differentiation by up regulating the expression of chondrogenic genes (Kronenberg, 2003, Mau et al., 2007). PTHrP blocks

the action of IHH signalling by binding to, and activating, the PTH receptor 1, leading to the induction of the protein kinase A (PKA), protein kinase C (PKC) and inositol trisphosphate (IP3) signalling cascades (Kim et al., 2008, Mau et al., 2007). In particular, the activation of the PKA signalling pathway leads to the up regulation of the expression of the transcription factor Gli3, which has a negative effect on the production of IHH target genes (Kim et al., 2008). The result of the interaction between these two factors is the formation of a negative feedback loop which tightly regulates the lengthening of the growth plate by providing a proliferative and chondrogenic environment in the upper layers, whilst the process of terminal differentiation and calcification occurs in a self-limiting manner in the lower regions (Vortkamp et al., 1996, Kobayashi et al., 2005). The salient part of this negative feedback loop for the work presented in this chapter is not IHH or PTHrP or their specific interactions in the growth plate. It is instead the interaction of cells at different stages of chondrogenic differentiation, late stage hypertrophic cells and early stage periarticular cells, and the dramatic effects that they can have on the behaviour of the other cell type. The standard method for inducing chondrogenesis in MSCs *in vitro*, is to culture the cells in a 3D system (e.g. pellet culture) in the presence of an isoform of transforming growth factor beta (TGF- β) but in the absence of mechanical load (Johnstone et al., 1998). The chondrogenic induction of MSCs in this system leads to a synchronous progression of the cells towards chondrogenesis and hypertrophy. As a result there is no potential for signalling to occur between cells at different stages of differentiation (i.e. early and late) as all of the cells receive the same stimuli and respond in a similar manner.

The objective of this work was to culture MSCs in a system that would introduce the potential for cells that had undergone one or two weeks chondrogenic differentiation to interact with naïve undifferentiated MSCs. This was not an attempt to directly copy or replicate the growth plate, but to use cells at different stages of the chondrogenic differentiation pathway to modulate each other's phenotype and behaviour through crosstalk between the two cell populations.

Our group has previously reported the use of a custom made bioreactor (Figure 1.6) which can be used to apply multiaxial shear and compression load to tissue engineered fibrin-poly(ester-urethane) constructs. This multiaxial load environment allows for tissue engineered constructs to be studied in an environment that mimics the one they might encounter *in vivo*, allowing for a greater understanding of how these cells might act within a scaffold *in vivo*.

Previous work with our multiaxial load bioreactor has demonstrated that primary human MSCs can be directed towards chondrogenic differentiation and matrix deposition, in

the absence of any growth factors, through the exposure to a combination of shear and compressive load that broadly mimics the loading that implants would receive within a joint (Li et al., 2010a, Li et al., 2010b, Kupcsik et al., 2010, Neumann et al., 2013). Li *et al.* showed that the application of multi-axial load leads to the endogenous production of TGF- β 1 by MSCs within the loaded scaffolds, which then drives the deposition of cartilage like matrix (Li et al., 2010a).

The work presented in this chapter was designed to investigate the potential for paracrine signalling between MSCs at different stages of chondrogenic differentiation in a mechanical environment that mimics that of the joint *in vivo*. I hypothesised that creating constructs containing layers of MSCs at different stages of differentiation may modify the induction of chondrogenesis and hypertrophy by introducing the potential for paracrine signalling relationships that are not present in constructs that only contain cells at the same stage of differentiation.

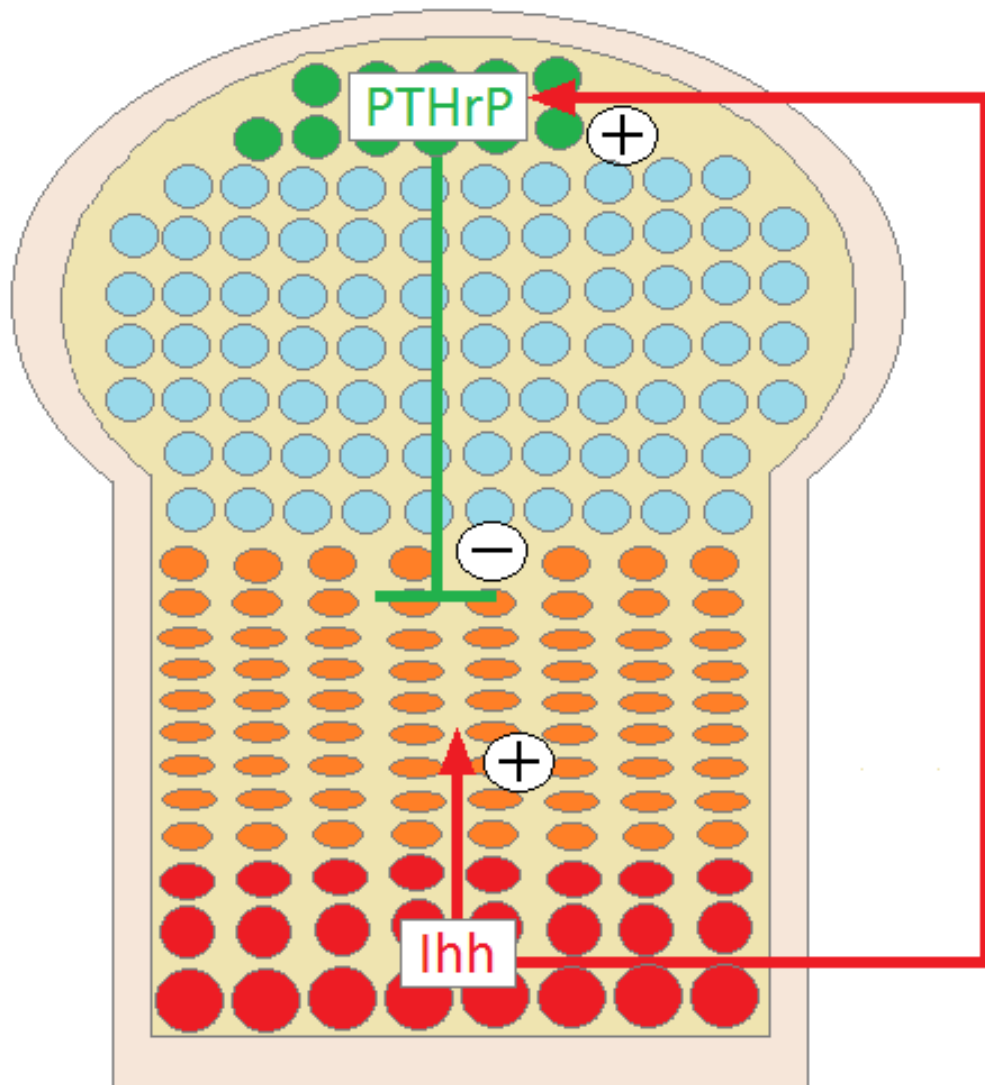


Figure 3.1

The epiphyseal growth plate, showing the PTHrP producing periarticular chondrocytes in green, resting zone chondrocytes in blue, proliferating zone cells in orange and Ihh producing hypertrophic cells in red. Arrows indicate the PTHrP-Ihh axis which controls the rate of growth plate development by regulating the progression of chondrocytes towards terminal differentiation; from resting zone chondrocytes to hypertrophic cells.

3.2 Materials and Methods

3.2.1 Donor Information

This work was carried out using cells from three bone marrow donors. Two aspirates were taken from iliac crests (one male twenty-four years old, one female forty years old) and one from the 4th thoracic vertebral body (female eighteen years old).

3.2.2 Experimental Design

In order to investigate the effect of cross talk between cells at different stages of differentiation an experiment was designed with four groups (Figure 3.2). On day 0 of the experiment two and a half million MSCs were seeded into each of six 2x8mm poly(ester-urethane) scaffolds (three free swelling controls and three for loading), these were the "bottom" scaffolds of group 4 constructs. The scaffolds were then placed into PEEK holders and cultured in chondropermissive media with 10ng/ml TGF- β 1 for two weeks, the media was changed three times a week. On day 7 of the experiment another set of six scaffolds were seeded with two and a half million MSCs each, these were cultured for one week in chondropermissive media with 10ng/ml TGF- β 1, these were the "bottom" scaffolds group 3 constructs. On day 14 of the experiment twelve fibrin-poly(ester-urethane) were seeded with MSCs and placed on top of the scaffolds seeded on day 0 and day 7 within the PEEK holders, these are referred to in the text as "top" scaffolds. As a result group 3 constructs contained a bottom layer of cells chondrogenically pre-differentiated for one week with naïve MSCs on top and group 4 constructs contained a bottom layer of cells chondrogenically pre-differentiated for two weeks with naïve MSCs on top. On day 14, control constructs were also produced consisting of two and a half million naïve MSCs seeded in to both "top" and "bottom" scaffolds (both 2x8mm poly(ester-urethane) sponges) (group 1) and 5 million MSCs seeded into a 4x8mm poly(ester-urethane) sponge as a comparison with previous work (group 2). Within each experimental repeat all of the cells seeded into scaffolds on day 0, 7 and 14 were from the same donor and were seeded at the same passage (passage 3 or 4). On day 14, all of the media was replaced with chondropermissive media without TGF- β 1. Half of the scaffolds from each group were kept in freeswelling culture and the other half were exposed to multiaxial mechanical load.

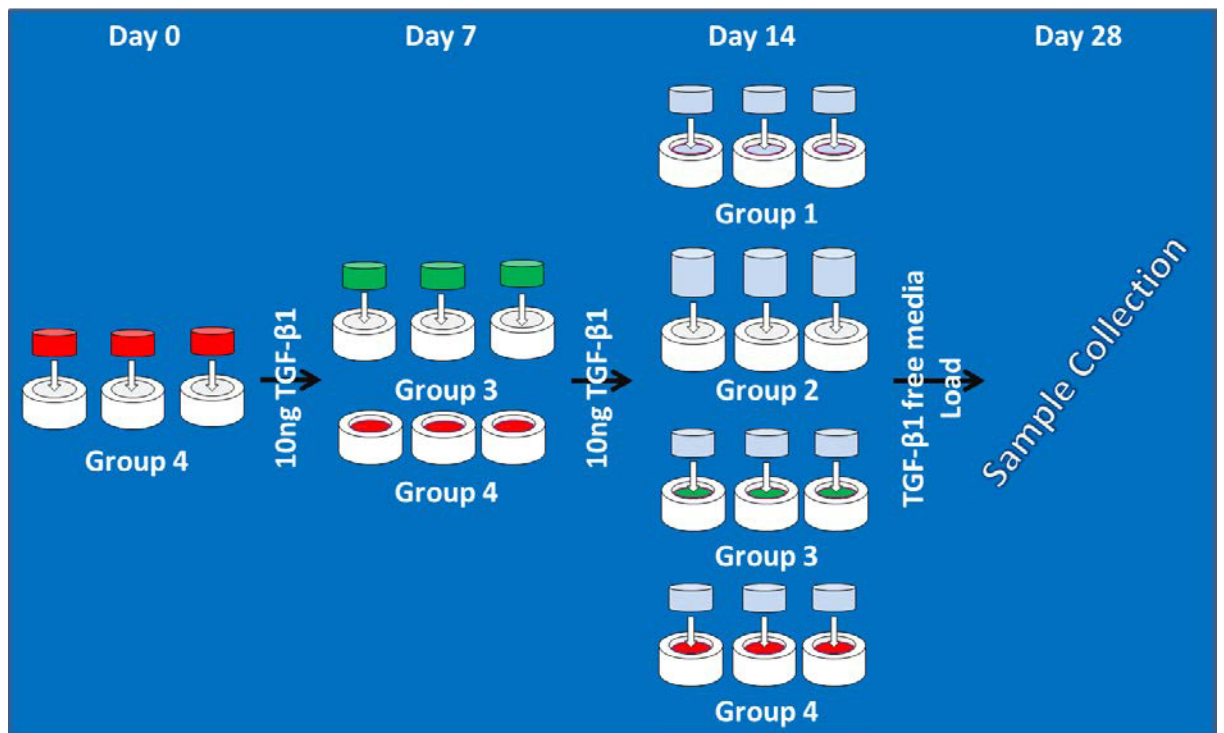


Figure 3.2

A schematic showing the experimental design for the work carried out in this chapter. Bilayer fibrin-poly(ester-urethane) constructs were produced with MSCs at different stages of chondrogenic differentiation in upper and lower scaffolds in order to investigate crosstalk between cells within constructs, and the effect of co-culturing cells at different stages of differentiation.

Group 4 bottom scaffolds (red), 2x8mm, containing two and a half million MSCs, were pre-differentiated for two weeks before another set of 2x8mm scaffolds containing two and a half million naïve MSCs (light blue) were seeded on top of them on day 14.

Group 3 bottom scaffolds (green), 2x8mm, containing two and a half million MSCs, were pre-differentiated for one week before another set of 2x8mm scaffolds containing two and a half million naïve MSCs (light blue) were seeded on top of them on day 14.

Group 1 and 2 scaffolds were seeded with naïve MSCs on day 14. Group 1 constructs consisted of two 2x8 mm scaffolds, one on top of the other, each containing two and a half million MSCs. Group 2 constructs consisted of one 4x8mm scaffold, containing five million MSCs.

3.2.3 Mechanical Loading Regimen

Scaffolds were loaded using a custom-made multiaxial load bioreactor based on tribological principles. Loaded scaffolds were exposed to 10% compression superimposed on top of a 10% pre-strain and shear loading ($\pm 25^\circ$) at 1Hz for one hour a day five times a week up to a total of 10 cycles. In contrast, control scaffolds were kept in free-swelling culture for the loading period (two weeks).

3.2.4 Sample Collection and Storage

Media was collected three times a week at media changes and was pooled by week before storage at -20°C for GAG and TGF- $\beta 1$ quantification. At the end of the culture period of two of the experimental repeats, three scaffold halves per group were harvested for biochemical analysis (GAG and DNA quantification) and three scaffold halves per group for RNA isolation/real-time PCR. In one of the three repeats, three scaffold halves per group were taken for real-time PCR, three scaffold quarters were taken for biochemical analysis and three scaffold quarters were fixed in methanol for histological analysis. Analysis was performed as described in Chapter 2.

3.2.5 Statistical Analysis

Results for all real-time PCR (freeswelling and load, and free swelling alone) as well as all GAG, DNA and TGF- $\beta 1$ quantification results represent data from three independent experimental repeats each carried out in triplicate using cells from a different MSC donor.

An unpaired T-test was used to test the difference of media collected during week 1 of culture, whilst the Mann-Whitney test was used to compare the TGF- $\beta 1$ content of media collected during weeks 2-4.

A one-way ANOVA with a Tukey's multiple comparison test was then used to determine statistical differences between groups for media GAG by week, DNA and GAG/DNA data. The Kruskal-Wallis test with a Dunn's multiple comparisons test was used to test the difference of groups for all real-time PCR results and both media TGF- $\beta 1$ and GAG content from week 2-4.

Two-way ANOVA and Sidak's multiple comparison test were used to test the significance of differences between loaded and control samples at each time point for cumulative media GAG and media GAG presented by group over time.

3.3 Results

Due to the use of primary human cells in this work there was a high degree of variability between biological repeats. Due to this high level of variation and the relatively low numbers of donors tested many of the analyses do not reach significance at the 5% level. As a result both significant changes and non-significant trends within the data collected are described and discussed, where relevant, in this chapter.

3.3.1 Differential gene expression in response to co-culture and mechanical load

No significant differences were detected in the expression of aggrecan mRNA despite a trend towards an increase with load in group 1 scaffolds (Figure 3.3A). As observed with other genes, there was a high degree of variation within this data set due to inter-donor variation in the expression of aggrecan mRNA. There was also a low n-number for some groups due to low expression of aggrecan mRNA in one donor, particularly in the absence of load (group 1 control top n=5, group 1 load top n=9, group 1 control bottom n=5, group 1 load bottom n=7, group 2 control n=5, group 2 load n=8, group 3 control top n=7, group 3 load top n=9, group 3 control bottom n=6, group 3 load bottom n=6, group 4 control top n=5, group 4 load top n=9, group 4 control bottom n=8, group 4 load bottom n=7). The lack of response to load in aggrecan production was also observed for the collagen type II in this donor.

Loading led to significant increases in the expression of Sox9 in group 1 load top ($P=0.004$) and group 1 load bottom ($P=0.025$) scaffolds over group 1 control top scaffolds (Figure 3.3B). Group 1 ($P=0.034$) and 4 ($P=0.017$) load top scaffolds also showed significantly higher levels of Sox9 expression than group 3 control top scaffolds. The expression of Sox9 was not significantly different between loaded bottom scaffolds between groups (all comparisons $P>0.999$). Load did not lead to a significant change in expression between top and bottom scaffolds in group 1, 3 or 4 (all comparisons $P>0.999$). No significant differences were seen within group 3 or group 4 despite a trend towards an increase in expression in response to load.

Statistical analysis of collagen type II gene expression showed no significant differences between groups, despite a non-significant trend towards an increase in expression in group 1 scaffolds (top and bottom) in response to load (Figure 3.3C). The collagen type II data presented here was not normalised to day 0 cells, as the other genes were, but instead to group 2 loaded scaffolds. The other groups were normalised to group 2 loaded scaffolds for two reasons; firstly as group 2 consisted of 4x8mm scaffolds seeded with five million MSCs the scaffolds were similar to those

used to previously characterise this system, this allows for a comparison of the new scaffold layout presented in this chapter with the work that has been performed previously using fibrin poly(ester-urethane) constructs in this bioreactor. Secondly, the loaded group was chosen as collagen II is only consistently detected in loaded, but not free swelling constructs in this system. Due to the variability in the mRNA response to load collagen type II, was not detected in all groups, the "n-number" of the control groups (and group 1 loaded scaffolds) was, therefore, lower than for loaded groups (group 1 control top n=4, group 1 load top n=7, group 1 control bottom n=3, group 1 load bottom n=6, group 3 control top n=4, group 3 load top n=8, group 3 control bottom n=5, group 3 load bottom n=9, group 4 control top n=4, group 4 load top n=8, group 4 control bottom n=6, group 4 load bottom n=9). The difficulties encountered in the detection of collagen type II in non-loaded scaffolds is due to its highly selective nature as a marker of chondrogenesis in this system; a similar pattern of expression is reported in Chapter 4. Group 2 control scaffolds are not shown as no collagen type II mRNA was detected in these scaffolds. As with collagen type I mRNA detection, there was a high degree of variation within the expression levels of collagen type II in the cells; this appears to result from high inter-donor variation in the production of collagen type II, rather than the high intra-donor variation observed in collagen type I, and this variation may have contributed to the lack of significant differences for this gene.

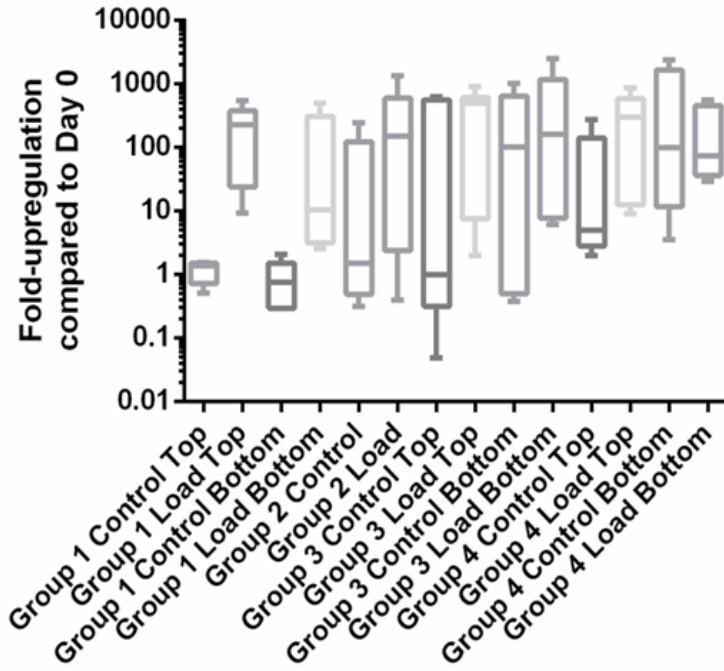
Loading led to a significant increase in collagen type X expression between group 1 top control and loaded scaffolds ($P=0.002$). The expression of collagen type X was also significantly higher in group 1 top loaded scaffolds than group 1 bottom control scaffolds ($P=0.027$). Free swelling group 3 top control scaffolds displayed a significantly lower level of collagen type X gene expression than group 3 ($P=0.028$) top loaded scaffolds. Significantly higher levels of collagen type X mRNA were also detected in group 3 and 4 top loaded and group 4 bottom loaded scaffolds than group 1 and 2 control scaffolds (group 1 control top compared to group 3 load top $P=0.001$, group 1 control top compared to group 4 load top $P<0.001$, group 1 control top compared to group 4 load bottom $P=0.004$, group 1 load top vs group 2 control $P=0.007$, group 1 control bottom compared to group 3 load top $P=0.014$, group 1 control bottom compared to group 4 load top $P=0.004$, group 2 control vs group 3 load top $P=0.003$, group 2 control compared to group 4 load top $P=0.001$, group 2 control compared to group 4 load bottom $P=0.016$, Figure 3.3D). This shows that chondrogenic stimulation, both in the form of load and predifferentiation, acts to increase the expression of collagen type X in this system.

Real-time PCR analysis of collagen type I gene expression indicated that there was no significant effect in response to load, or differences in the stages of differentiation of cells within the scaffolds (Figure 3.3E). Compared to day 0, values for these results

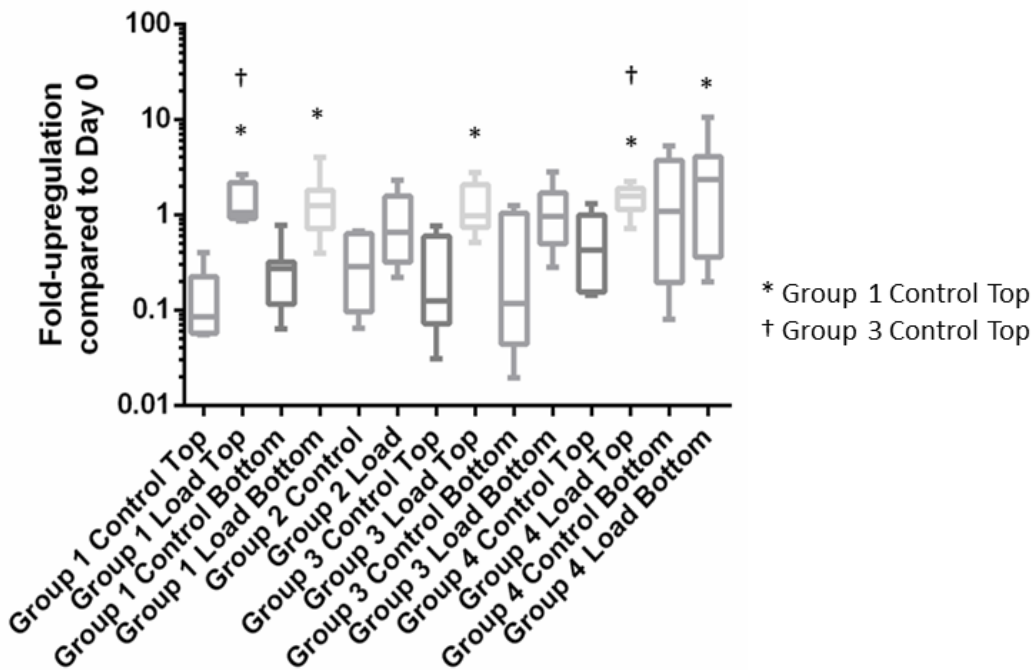
ranged from a mean 2.016-fold upregulation to a mean 5.704-fold upregulation; however, there was a high degree of variation within some groups e.g. mean 5.704 \pm 7.782-fold upregulation in group 2 control, group 2 load mean 4.813 \pm 5.352-fold upregulation in group 1 control bottom and mean 4.879 \pm 4.539-fold upregulation compared to day 0. This variation appears to result from intra-donor variation rather than inter-donor variation.

In order to clarify the results obtained from the real-time PCR analysis by removing the effects of mechanical load, the results for the freeswelling control scaffolds have also been presented separately from the results for the loaded scaffolds (Figure 3.4). Statistical analysis was also performed on these free swelling groups alone, in isolation from loaded groups. The results demonstrate that the expression of aggrecan mRNA in group 4 control bottom scaffolds was significantly higher than in group 1 control bottom scaffolds ($P=0.033$) (Figure 3.4A). The expression of collagen type X was also higher in group 4 top control scaffolds than group 1 control top scaffolds ($P=0.010$ and 0.035 respectively) (Figure 3.4D). Type X collagen expression was also higher in group 4 control top than group 2 control scaffolds ($P=0.040$) (Figure 3.4D). These statistical differences were not apparent when the data set was analysed as a whole, and may have arisen due to a reduction in the number of groups being analysed.

A. Aggrecan



B. Sox 9

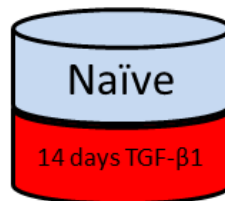
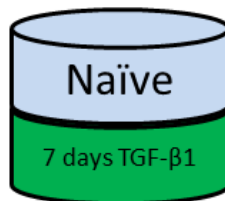
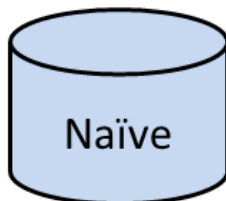
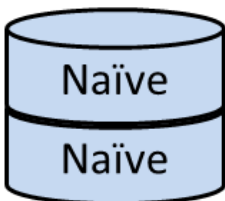


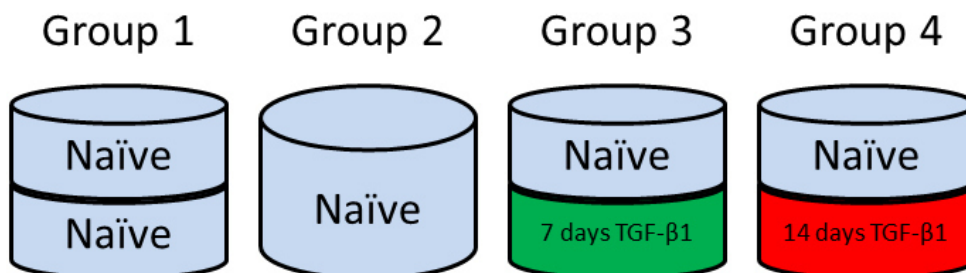
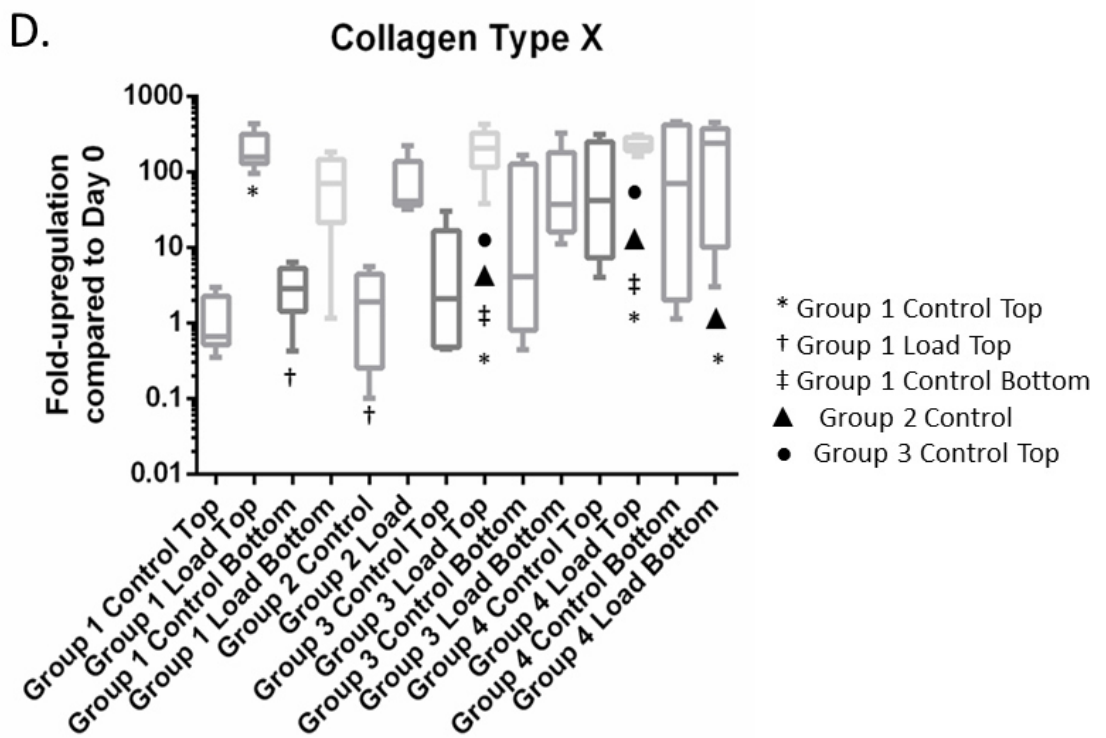
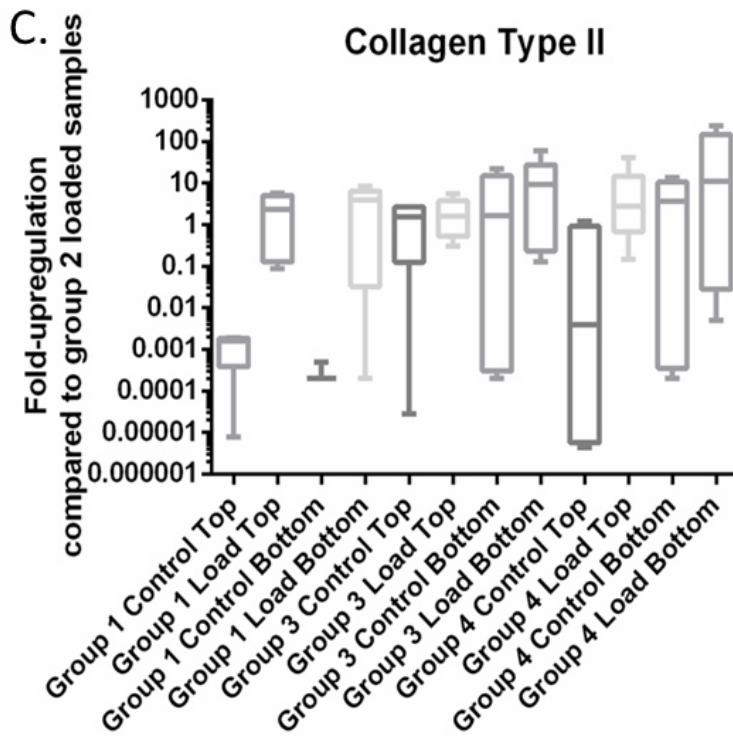
Group 1

Group 2

Group 3

Group 4





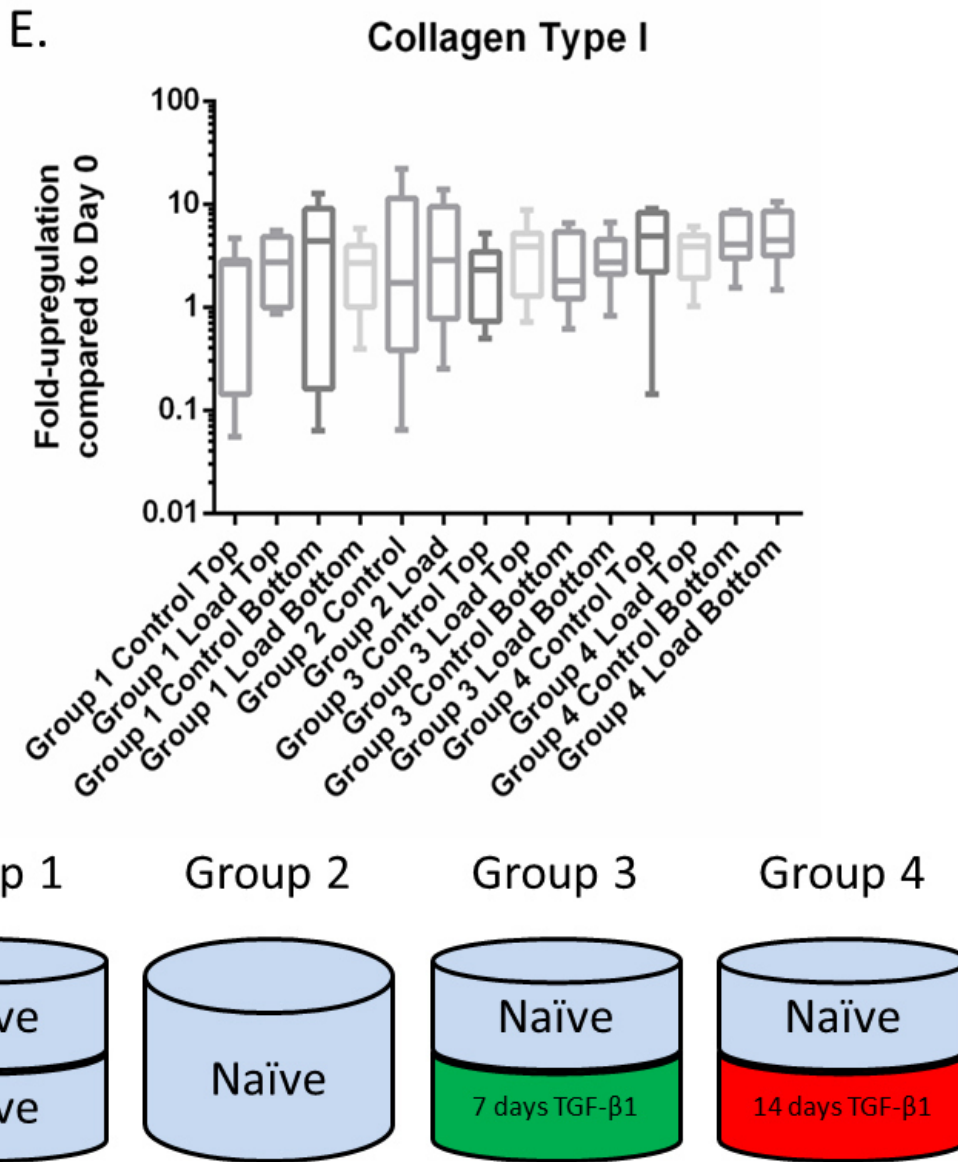
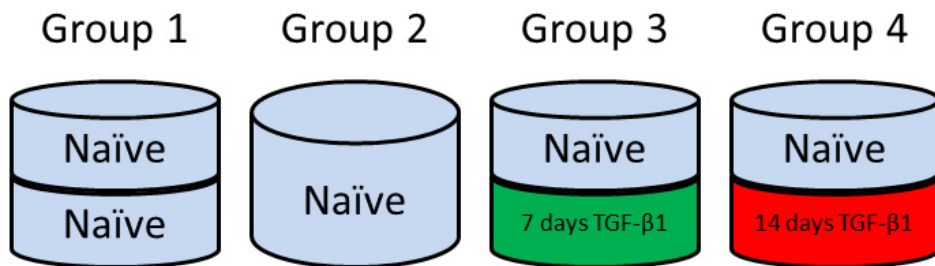
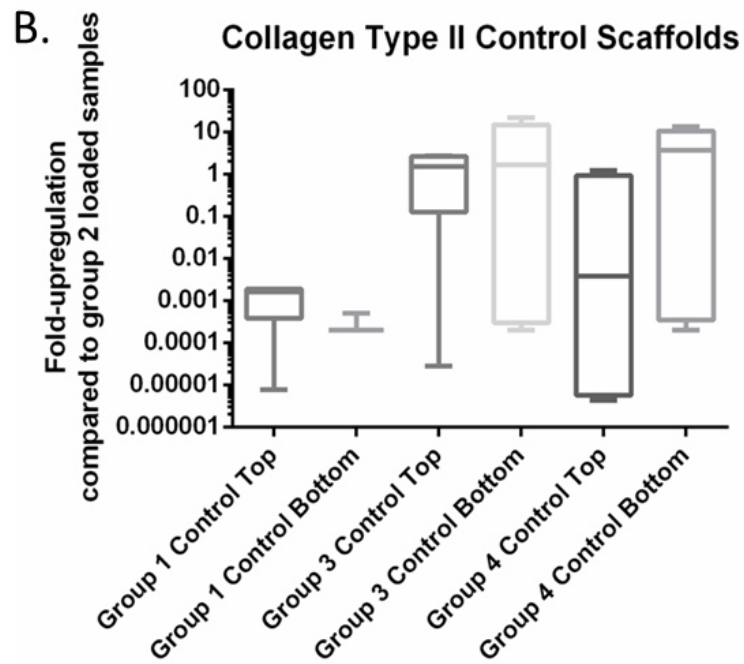
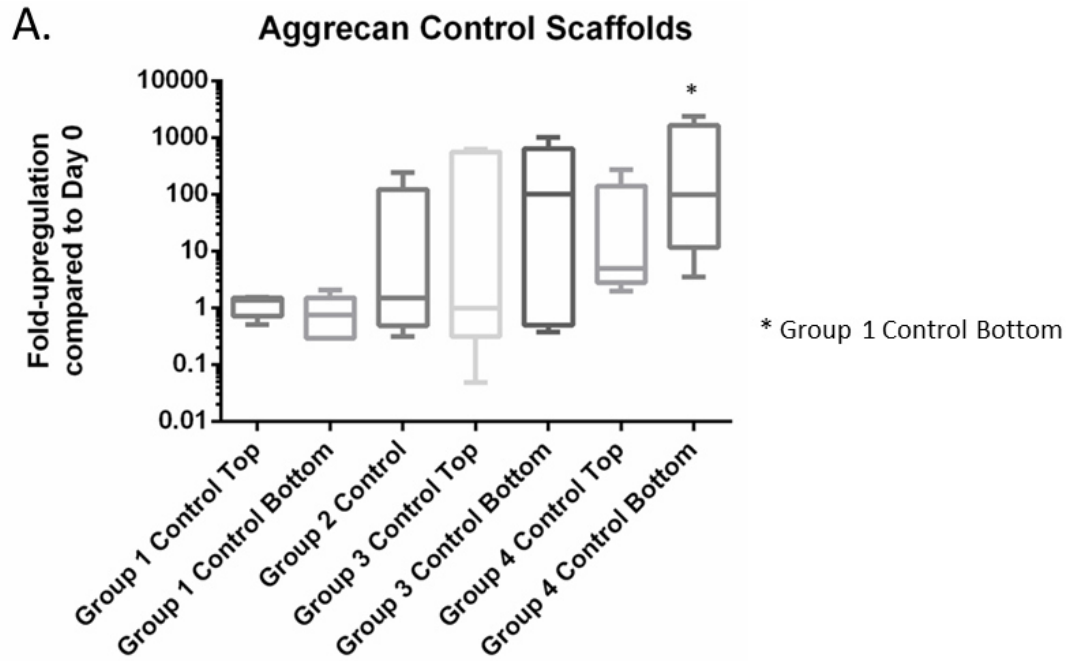


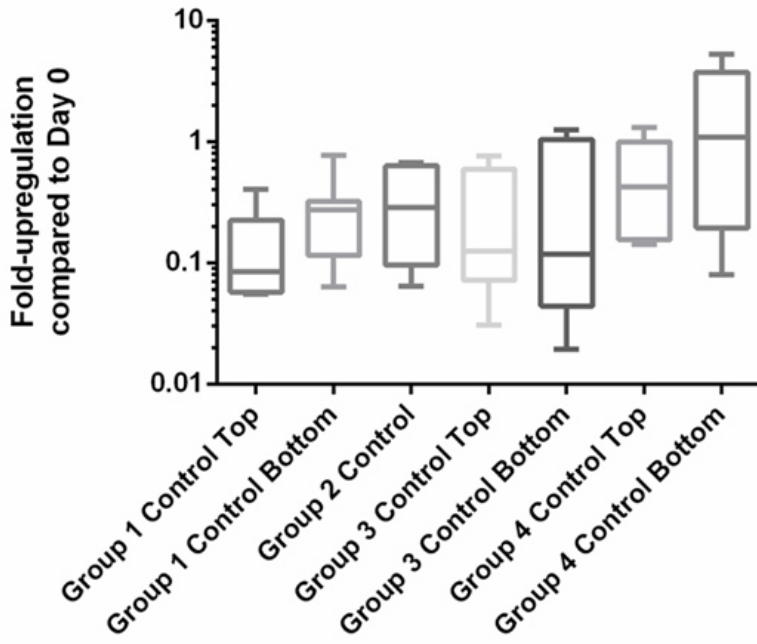
Figure 3.3

Gene expression measured at day 28 of culture, using real-time PCR, demonstrates the effect of co-culturing MSCs at different stages of differentiation on chondrogenesis in response to mechanical load. (A) aggrecan (B) Sox9 (C) collagen type II (D) collagen type X (E) collagen type I. Data represents results from three biological repeats carried out in triplicate (unless otherwise stated in results section). Genes were normalised to monolayer culture expanded MSCs (day 0) using the $\Delta\Delta C_t$ method, apart from collagen type II which was normalised to group 2 loaded scaffolds. Statistical significance was defined as $P \leq 0.05$ and determined using the Kruskal-Wallis and Dunn's multiple comparison tests. Significant differences are displayed on the plots using markers; the legend at the side of the plot indicates which group is represented by each marker. The presence of a marker over a group indicates a significant difference between that group and the group represented by the marker.



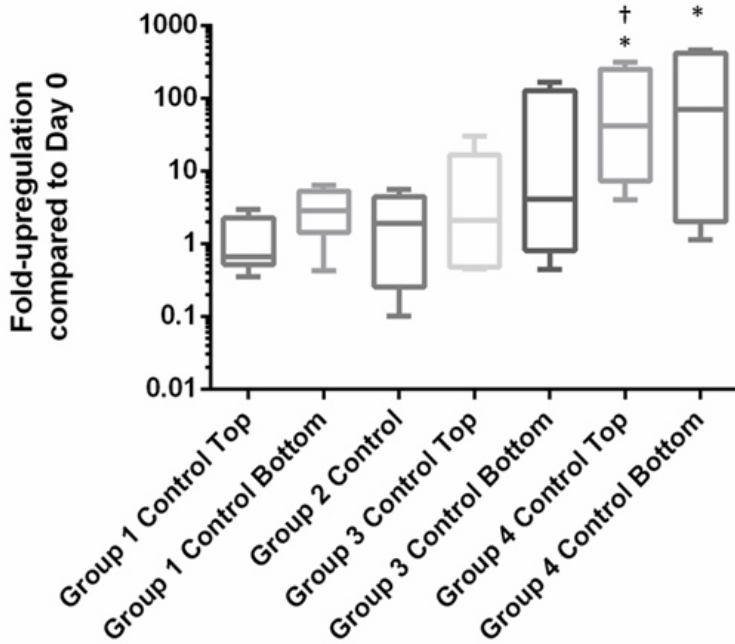
C.

Sox 9 Control Scaffolds



D.

Collagen Type X Control Scaffolds



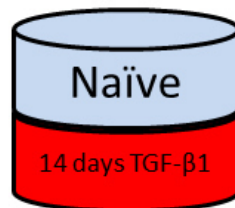
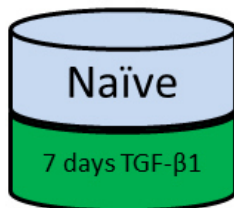
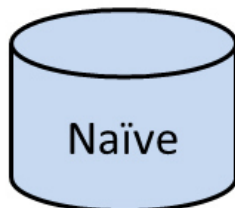
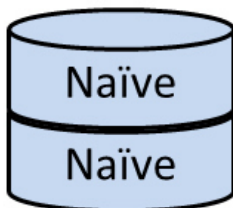
* Group 1 Control Top
 † Group 2 Control

Group 1

Group 2

Group 3

Group 4



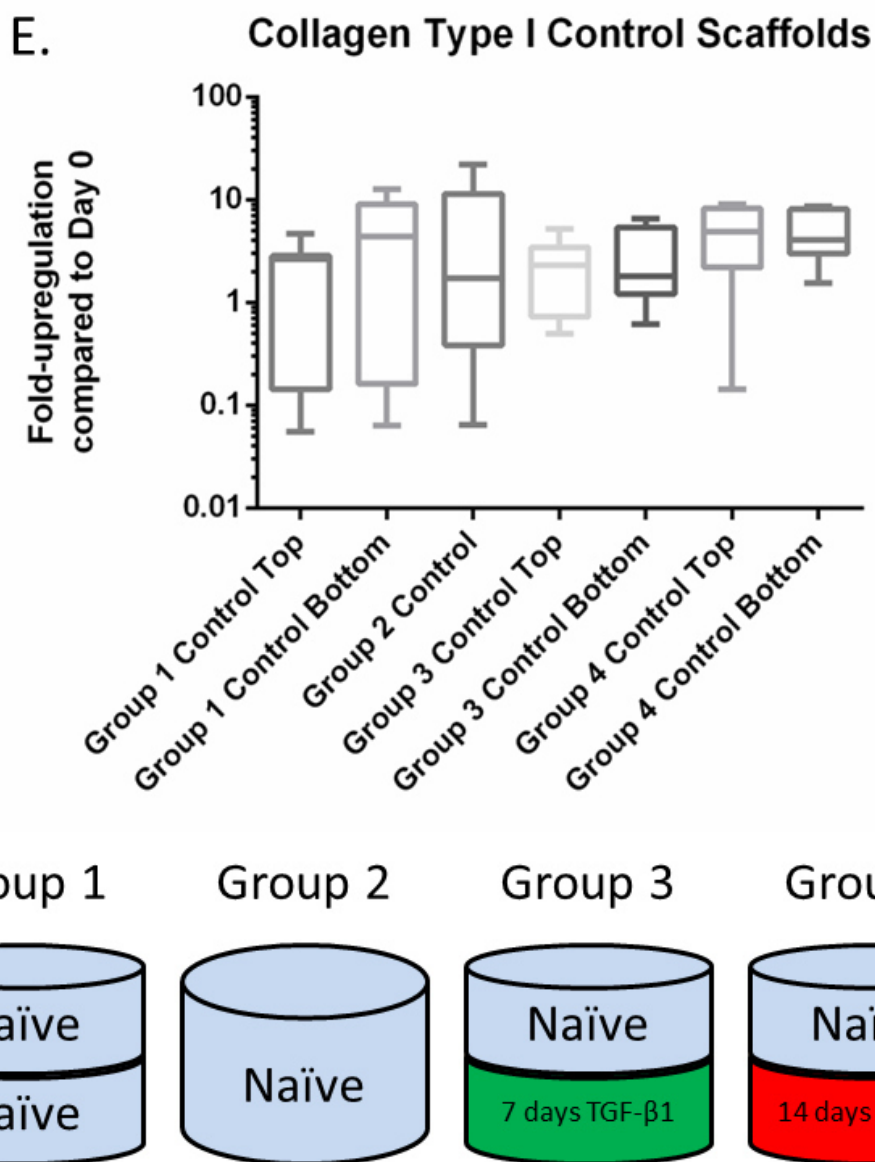


Figure 3.4

Gene expression measured at day 28 of culture using real-time PCR demonstrates the effect of co-culturing MSCs at different stages of differentiation on chondrogenesis in free swelling culture. (A) aggrecan (B) collagen type II (C) Sox9 (D) collagen type X (E) collagen type I. Data represents results from three biological repeats carried out in triplicate (unless otherwise stated in results section). Genes were normalised to monolayer culture expanded MSCs (day 0) using the $\Delta\Delta C_t$ method apart from collagen type II which was normalised to group 2 loaded scaffolds. Statistical significance was defined as $P \leq 0.05$ and determined using the Kruskal-Wallis and Dunn's multiple comparison tests. Significant differences are displayed on the plots using markers; the legend at the side of the plot indicates which group is represented by each marker. The presence of a marker over a group indicates a significant difference between that group and the group represented by the marker.

3.3.2 Quantification of TGF- β 1 release from MSCs in response to co-culture and mechanical load

Quantification of TGF- β 1 detected in the media, using an ELISA, confirmed that over the first two weeks of culture cells received equal amounts of exogenous TGF- β 1 (Figure 3.5A and B).

Quantification of week three medium (representing the first week of mechanical loading) showed that overall, load was beginning to have a positive effect on endogenous production of TGF- β 1 but had not caused significant changes within groups (Figure 3.5C).

Week four media, representing the second week of mechanical loading, demonstrates a much clearer effect of load on the concentration of TGF- β 1 released into the media compared to week 3 (Figure 3.5D). Load led to a significant increase in TGF- β released into the medium in group 1 and 2 compared to controls and media from all four loaded groups contained significantly more TGF- β 1 than group 1 and 2 control scaffolds (group 1 load compared to group 1 control $P=0.002$, group 2 load compared to group 2 control $P=0.005$). There was no significant difference between the control scaffolds in the four groups or between the loaded scaffolds across the four groups. However, the level of TGF- β 1 detected in the media of group 3 and 4 control scaffolds was higher than in group 1 and 2 controls, although not significantly, which may explain the lack of significance between loaded scaffolds in groups 3 and 4 and group 3 and 4 controls. This rise in TGF- β 1 detected in the media from cells seeded in group 3 and 4 controls may result from the effects of predifferentiation on the cells in the bottom scaffolds or from the release of TGF- β 1 trapped within the scaffold during preculture. This shows that by the second of week of load the cells are consistently producing endogenous TGF- β 1 in response to mechanical load.

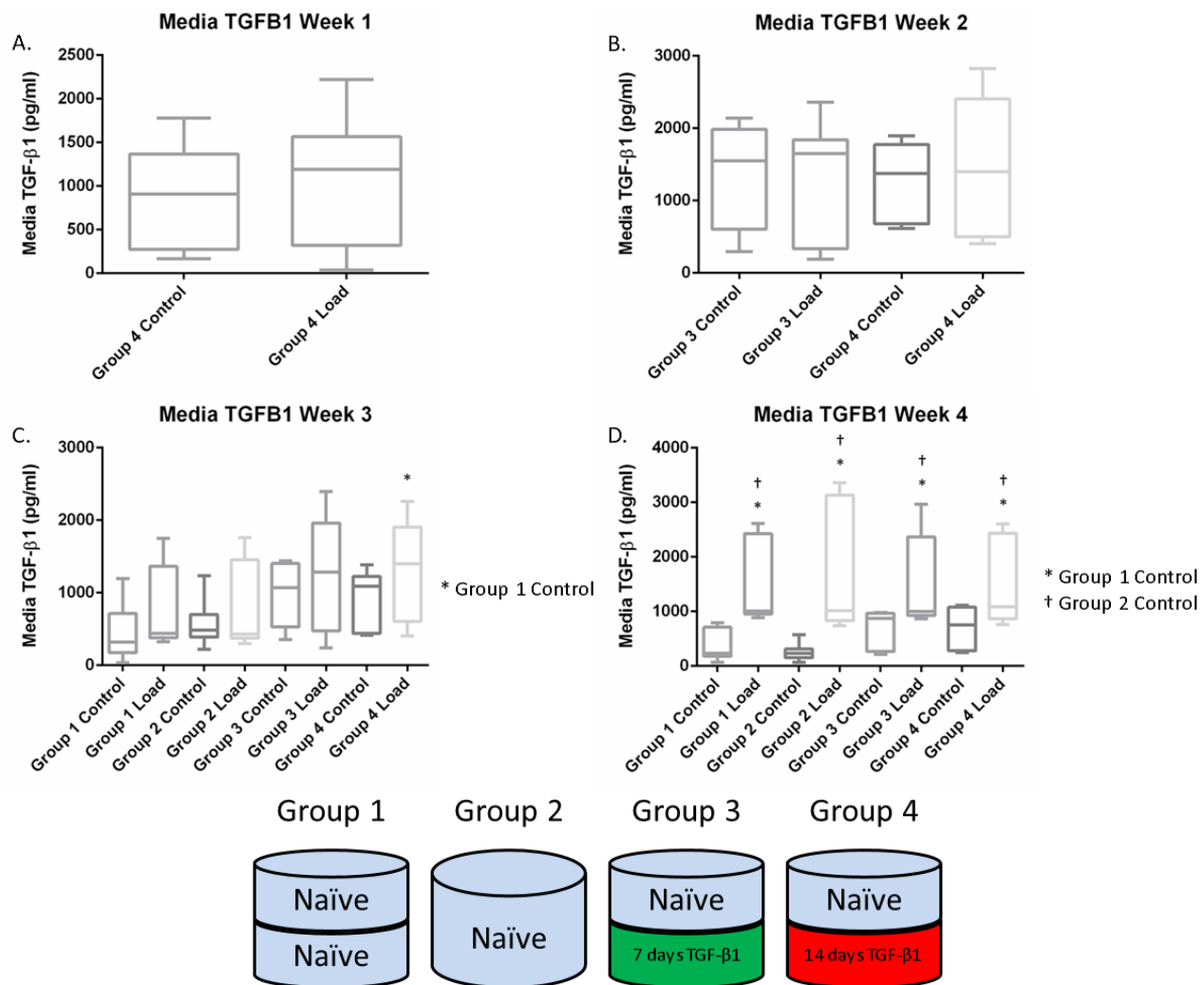


Figure 3.5

The total amount of TGF-β1 present in the media (pg/ml), as determined in each week of culture using an ELISA, demonstrating the effect of co-culturing MSCs at different stages of differentiation on chondrogenesis in response to mechanical load. Results represent data from three experimental repeats carried out in triplicate. Statistical significance was defined as $P \leq 0.05$ and determined using an unpaired T-test for data collected during week 1 and the Kruskal-Wallis and Dunn's multiple comparison tests for media collected in week 3-4. Significant differences are displayed on the plots using markers; the legend at the side of the plot indicates which group is represented by each marker. The presence of a marker over a group indicates a significant difference between that group and the group represented by the marker.

3.3.3 Quantification of DNA and GAG content of scaffolds containing MSCs and GAG released into the culture media

Hoechst 33258 quantification of scaffold DNA content showed that at the end of the culture period group 2 scaffolds contained approximately twice the amount of DNA of scaffolds in other groups (Figure 3.6A). This reflects the size of group two scaffolds which were 4x8 mm compared to the other groups which utilised 2x8 mm scaffolds. There was no significant difference in DNA content between group 1, 3 and 4 constructs, or between group 2 control and loaded scaffolds.

The amount of GAG present within each scaffold was determined from proteinase K digests using the DMMB assay. The results indicated that there were no significant differences in GAG content from different scaffold groups or between loaded and free-swelling scaffolds (Figure 3.6B).

The amount of GAG was also quantified in samples of culture media collected at media changes and pooled by week. The results of this analysis have been presented in two ways; by week, to compare the amount of GAG released between groups at each time point (Figure 3.7), and over time to show the release from each group over the four weeks in culture (Figure 3.8).

Analysis of GAG release into the media after 1 week of culture demonstrated no difference between group 4 control and loaded scaffolds, as the application of mechanical load had not begun and both groups were being supplemented with chondrogenic media containing 10ng/ml TGF- β 1 (Figure 3.7A). After the second week of culture, GAG levels detected in the media collected from group 4 scaffolds contained significantly more GAG than group 3 scaffolds (group 3 control compared to group 4 control $P=0.029$, group 3 load compared to group 4 load $P=0.014$) (Figure 3.7B). This difference is due to the extra week of culture group 4 scaffolds had had compared to group 3 scaffolds and represents differences in the stage of differentiation of the cells within the scaffolds.

Week three represents the first week of the cells being exposed to mechanical loading. Quantification of the release of GAG into the media, by week, shows that there is a trend towards an increase in GAG release in group 1, 3 and 4 in response to load (Figure 3.7C), however only media from group 3 loaded scaffolds contained significantly more GAG than their respective freeswelling controls ($P=0.031$). This may be due to the increased levels of TGF- β 1 that were released from group 4 control scaffolds, compared to other control scaffolds, which might subsequently have resulted in a significantly higher amount of GAG being released compared to group 1 control scaffolds. This would then decrease the perceivable difference in production between group 4 loaded and control scaffolds ($P=0.042$). The amount of GAG detected in the

culture media of group 3 and 4 loaded scaffolds was significantly higher than group 2 loaded scaffolds (group 4 load compared to group 2 load $P=0.012$, group 4 load compared to group 3 control $P=0.015$). The GAG released into the media from group 4 loaded scaffolds was also significantly higher (1.6 fold mean increase) than group 1 loaded scaffolds ($P=0.028$).

In contrast to the results obtained from the quantification of GAG released into the media in week 3, the results from week 4 show no significant difference in GAG content between any of the groups or between loaded samples and free swelling controls (Figure 3.7D).

The results obtained for the release of GAG into the media over time indicated that in groups 1 and 2 the amount of GAG released increased between weeks 3 and 4 (Figure 3.8A and B). The GAG release was higher in loaded scaffolds than in controls in week 4 for group 2 but not group 1 scaffolds ($P=0.048$) (Figure 3.8A and B). There was no difference in GAG release in the pre-culture period (week 1 and 2) between control and loaded scaffolds for group 3 and 4 constructs (Figure 3.8C and D). In week 3, the release of GAG into the media was significantly higher in loaded scaffolds compared to their respective controls in both group 3 and 4 ($P < 0.001$ and 0.001 respectively) (Figure 3.8C and D); however, in week 4 the release of GAG by loaded constructs was only higher in group 3 scaffolds ($P=0.008$).

Quantification of the cumulative GAG released into the culture media has been presented both by week (Figure 3.9) and by group over time (Figure 3.10) as for media GAG.

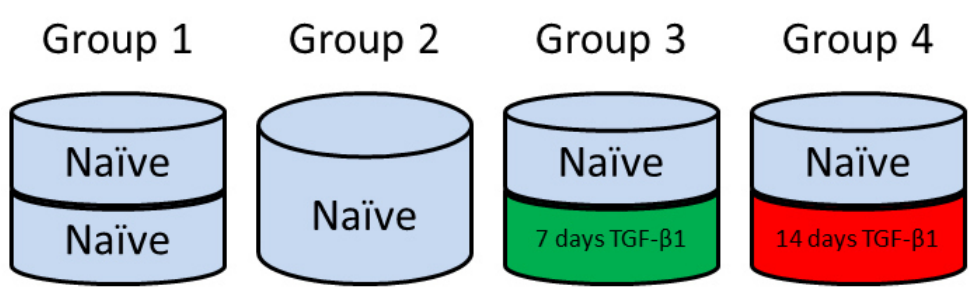
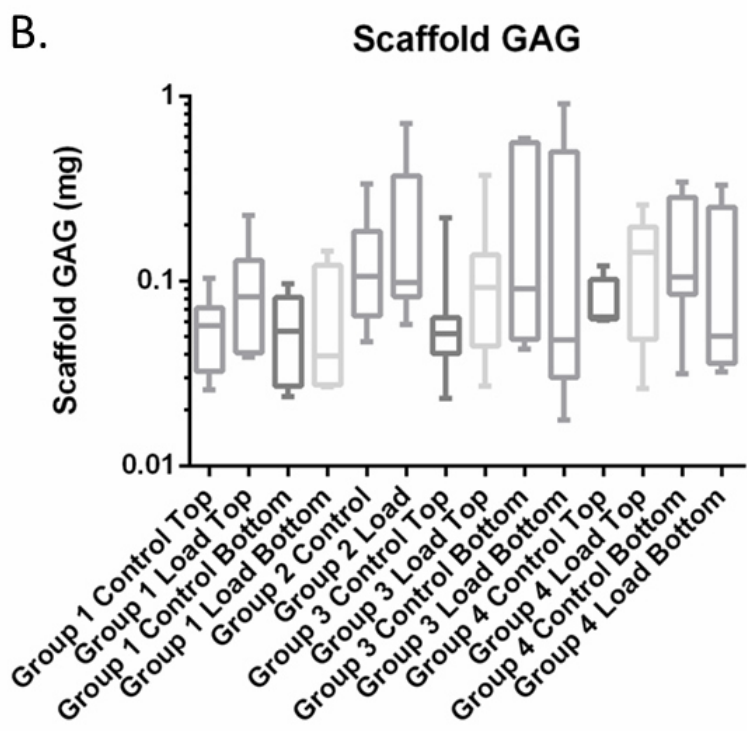
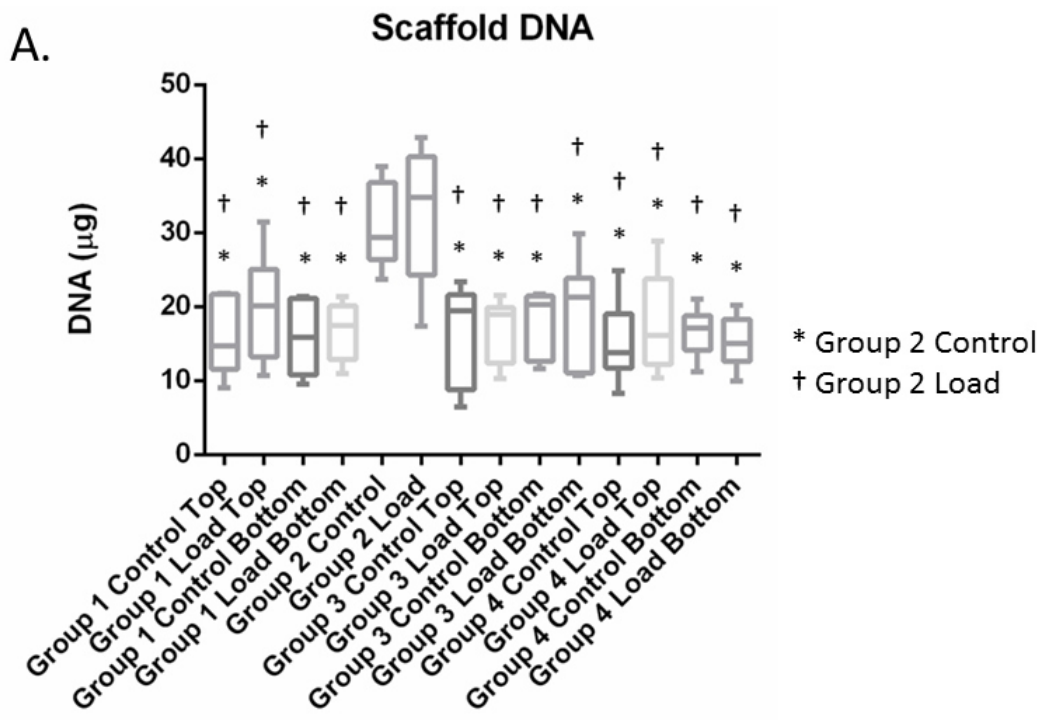
Results of cumulative GAG released into the media by week demonstrated, as would be expected, that in week 1 and 2 there was no significant difference within groups 3 or 4 (between control and loaded scaffolds) as loading had not yet been applied to the scaffolds and all groups were receiving 10 ng/ml TGF- β 1. By week 3 group 4 control scaffolds had released significantly more GAG to the medium than group 1 and 2 control scaffolds (Figure 3.9C) (group 1 control compared to group 4 control $P < 0.001$ a 4.2 fold mean increase, group 2 control compared to group 4 control $P < 0.001$ a 3.92 fold mean increase). Group 4 loaded scaffolds had also released significantly more GAG to the medium than group 1 and 2 loaded scaffolds by week 3 (group 1 load compared to group 4 load $P=0.004$ a 3.43 fold mean increase, group 2 load compared to group 4 load $P=0.003$ a 3.55 fold mean increase) (Figure 3.9C). No significant differences were detected within groups. The pattern of cumulative release was the same at week 4 as in week 3 (group 1 control compared to group 4 control $P < 0.001$ a 1.91 fold mean increase, group 1 load compared to group 4 load $P=0.002$ 1.87 fold mean increase, group 2 control compared to group 4 control $P < 0.001$ a 2.00 fold mean

increase, group 2 load compared to group 4 load $P=0.003$ a 1.83 fold mean increase) (Figure 3.9D).

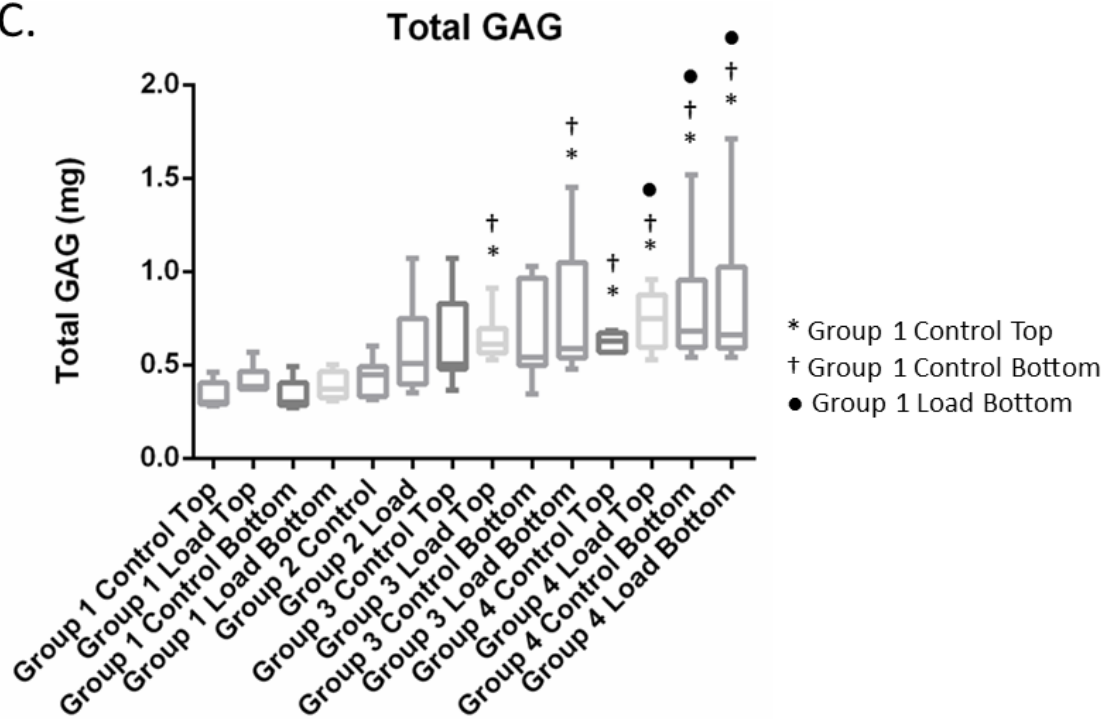
Calculation of the cumulative GAG released into the media, by cells embedded in the scaffolds in each group over time, also showed that significant differences were observed between loaded and control groups for group 2 in week 4 ($P=0.005$ a 1.21 fold mean increase), group 3 during week 3 and 4 ($P=0.006$ and <0.001 respectively, a 1.20 and 1.24 fold mean increase respectively) and group 4 in week 4 ($P=0.038$ a 1.11 fold mean increase) (Figure 3.10B, C and D). Figure 3.10E demonstrates the cumulative GAG data for all four groups on one graph to give an impression of the relative levels of release from the different groups.

In order to normalise the amount of GAG produced by each scaffold to the number of cells within the scaffold, the total amount of GAG produced by each sample (as measured in both the scaffold and in the media) was divided by the DNA content of each scaffold to produce a GAG/DNA ratio. In this study, two separate scaffolds contributed to the "media GAG" value, and given the experimental design it was not possible to determine the exact source of the GAG in the media (from top or bottom scaffolds).

Therefore, to produce a ratio the total amount of GAG within each scaffold was added to the total amount of GAG in the media collected from that construct, so both top and bottom scaffolds were attributed the same amount of media GAG (Figure 3.6B). This "total GAG" value was then divided by the scaffold DNA content to give a GAG/DNA ratio (Figure 3.6D). Results of "total GAG" quantification demonstrated no significant changes within groups but significantly higher levels of expression in group 3 and 4 scaffolds compared to group 1 control top and bottom scaffolds in both control and loaded constructs. Results of GAG/DNA quantification indicated increased levels of GAG production in group 3 and 4 scaffolds over group 1 and 2 scaffolds but no significant changes within groups or between groups 3 and 4; however, interpretation of this data requires caution as described later in the discussion of this chapter.



C.



D.

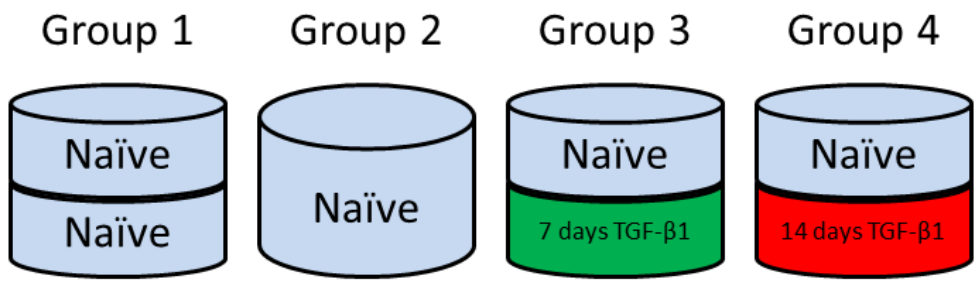
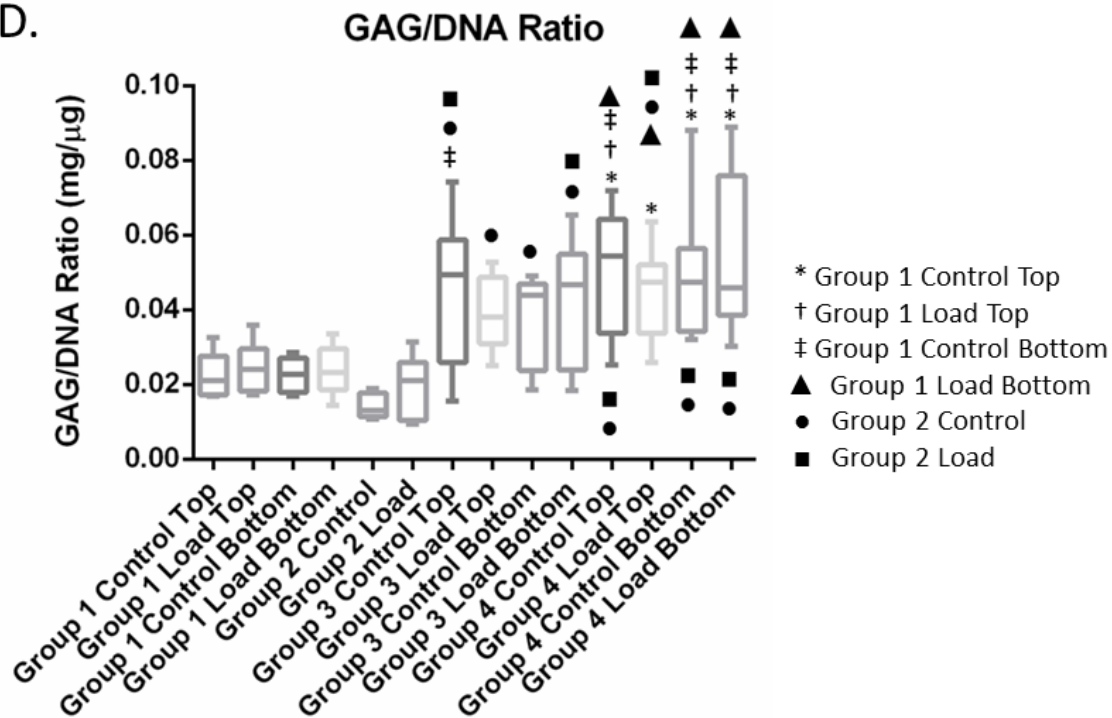


Figure 3.6

The amount of GAG and DNA present within scaffolds as determined using the DMMB assay and Hoechst 33258 dye respectively, showing the effect of co-culturing MSCs at different stages of differentiation on chondrogenesis in response to mechanical load.

(A) The total DNA content of proteinase K digests of scaffolds. (B) The total GAG content of proteinase K digests of scaffolds. (C) The total combined amount of GAG measured in scaffolds and in the culture media. (D) The GAG/DNA ratio for each set of scaffolds. These data represent three biological repeats carried out in triplicate.

Statistical significance was defined as $P \leq 0.05$ and determined using the Kruskal-Wallis and Dunn's multiple comparison tests. Significant differences are displayed on the plots using markers; the legend at the side of the plot indicates which group is represented by each marker. The presence of a marker over a group indicates a significant difference between that group and the group represented by the marker.

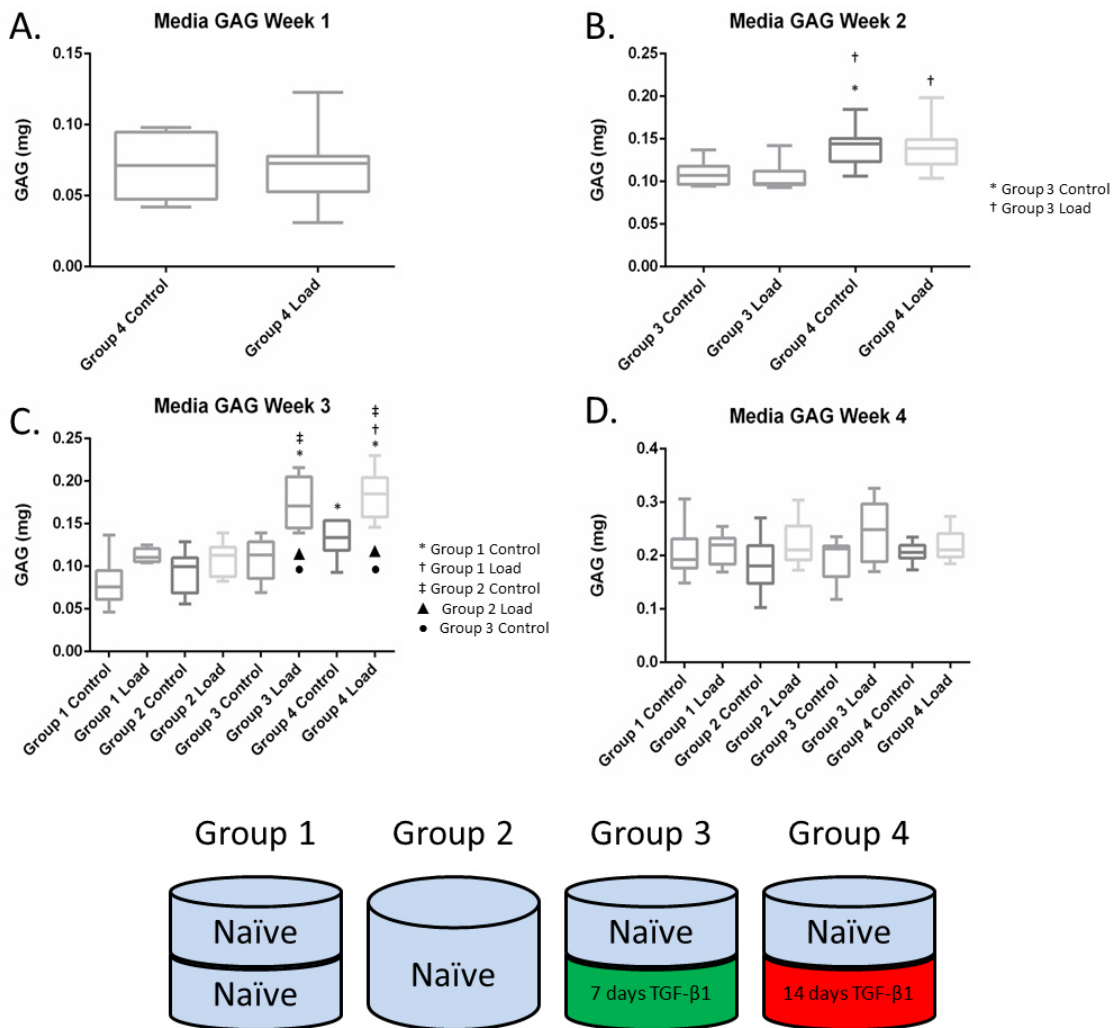


Figure 3.7

The total amount of GAG present in the culture media as determined in each week of culture using the DMMB assay, showing the effect of co-culturing MSCs at different stages of differentiation on chondrogenesis in response to mechanical load. These data represent three biological repeats carried out in triplicate. Statistical significance was defined as $P \leq 0.05$ and determined using an unpaired T-test for data collected during week 1 and the Kruskal-Wallis and Dunn's multiple comparison tests for media collected in week 3-4. Significant differences are displayed on the plots using markers; the legend at the side of the plot indicates which group is represented by each marker. The presence of a marker over a group indicates a significant difference between that group and the group represented by the marker.

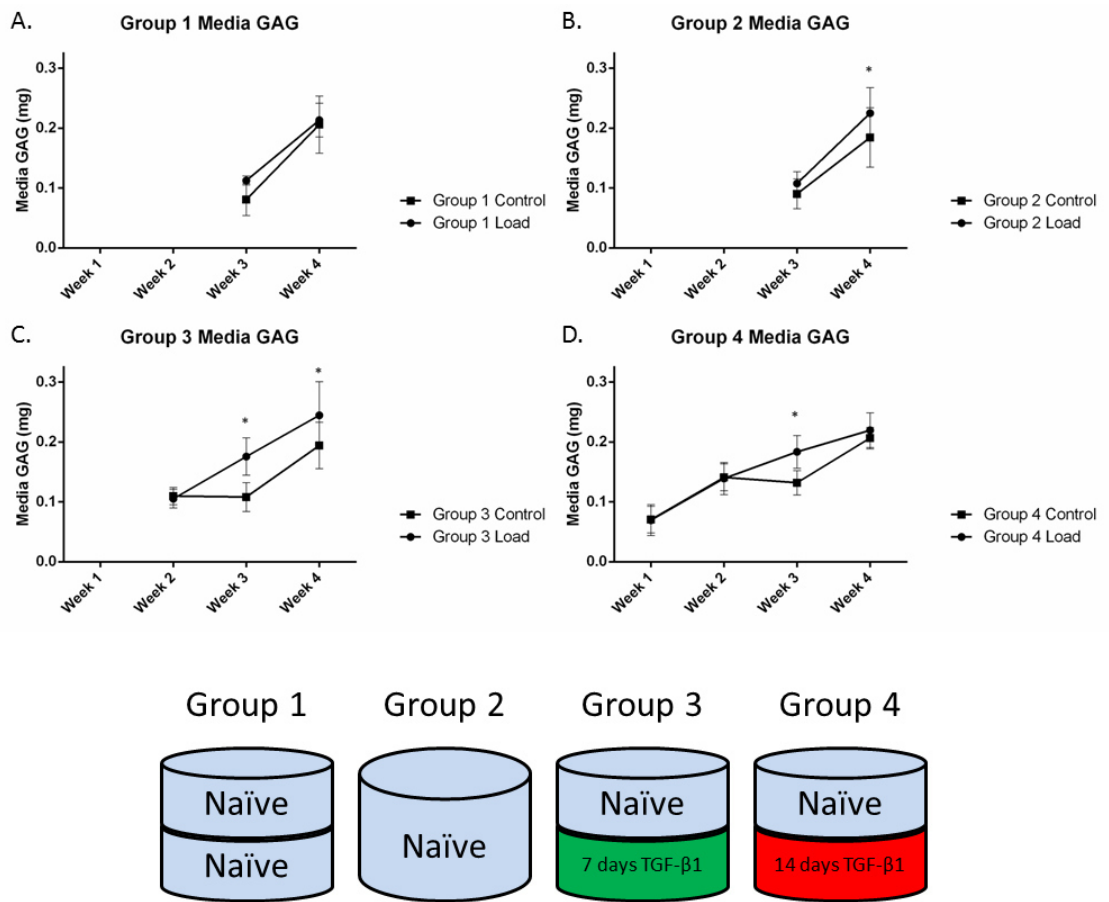


Figure 3.8

The amount of GAG released in to the culture media by week, shown over time by group. These data represent three biological repeats carried out in triplicate. Statistical significance was defined as $P \leq 0.05$ and determined using two-way ANOVA and Sidak's multiple comparison test. Significant differences between loaded and control samples at each time point are displayed using a star.

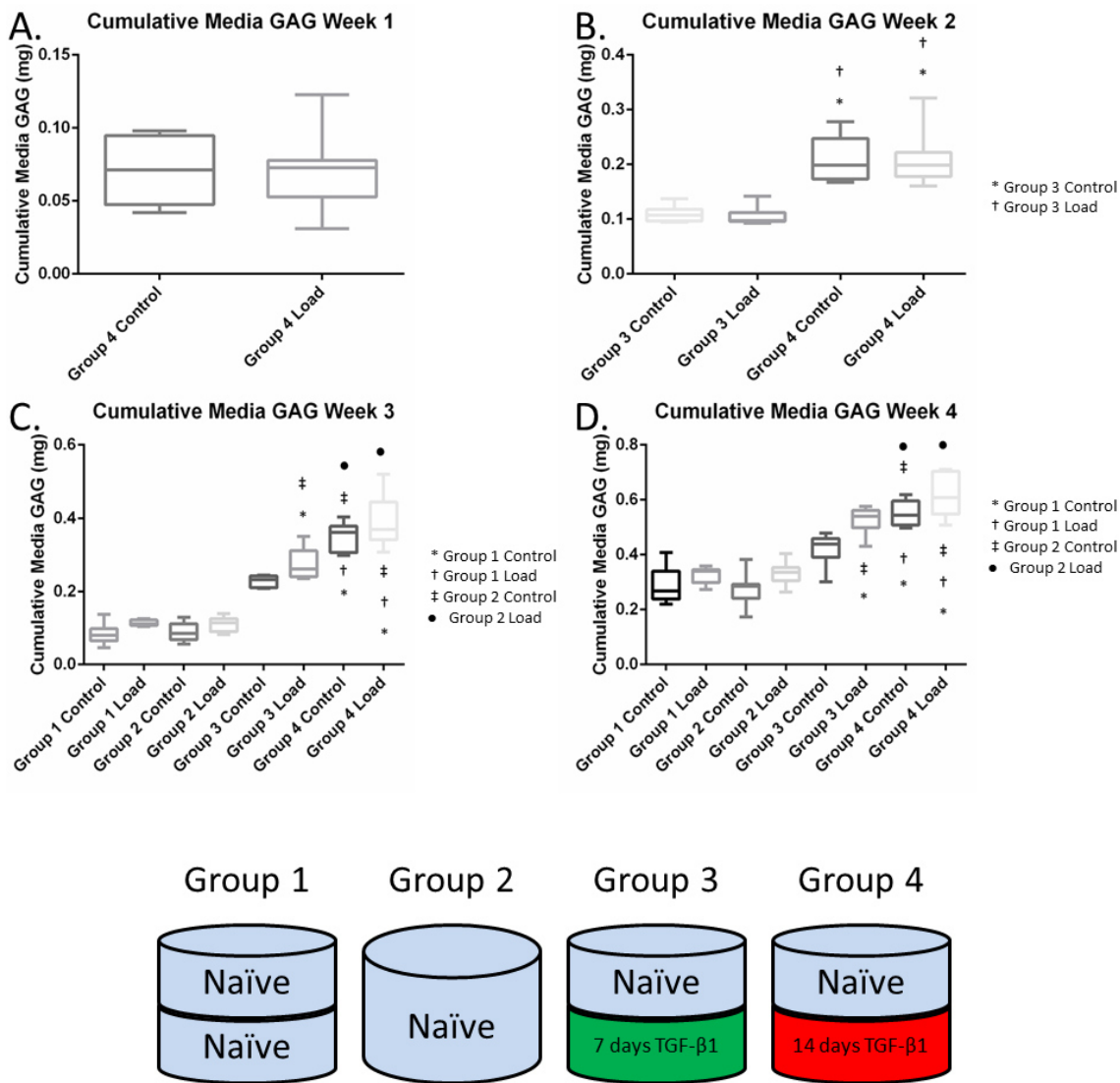


Figure 3.9

The cumulative amount of GAG present in the culture media as determined in each week of culture using the DMMB assay, showing the effect of co-culturing MSCs at different stages of differentiation on chondrogenesis in response to mechanical load. These data represent three biological repeats carried out in triplicate. Statistical significance was defined as $P \leq 0.05$ and determined using an unpaired T-test for data collected during week 1 and the Kruskal-Wallis and Dunn's multiple comparison tests for media collected in week 3-4. Significant differences are displayed on the plots using markers; the legend at the side of the plot indicates which group is represented by each marker. The presence of a marker over a group indicates a significant difference between that group and the group represented by the marker.

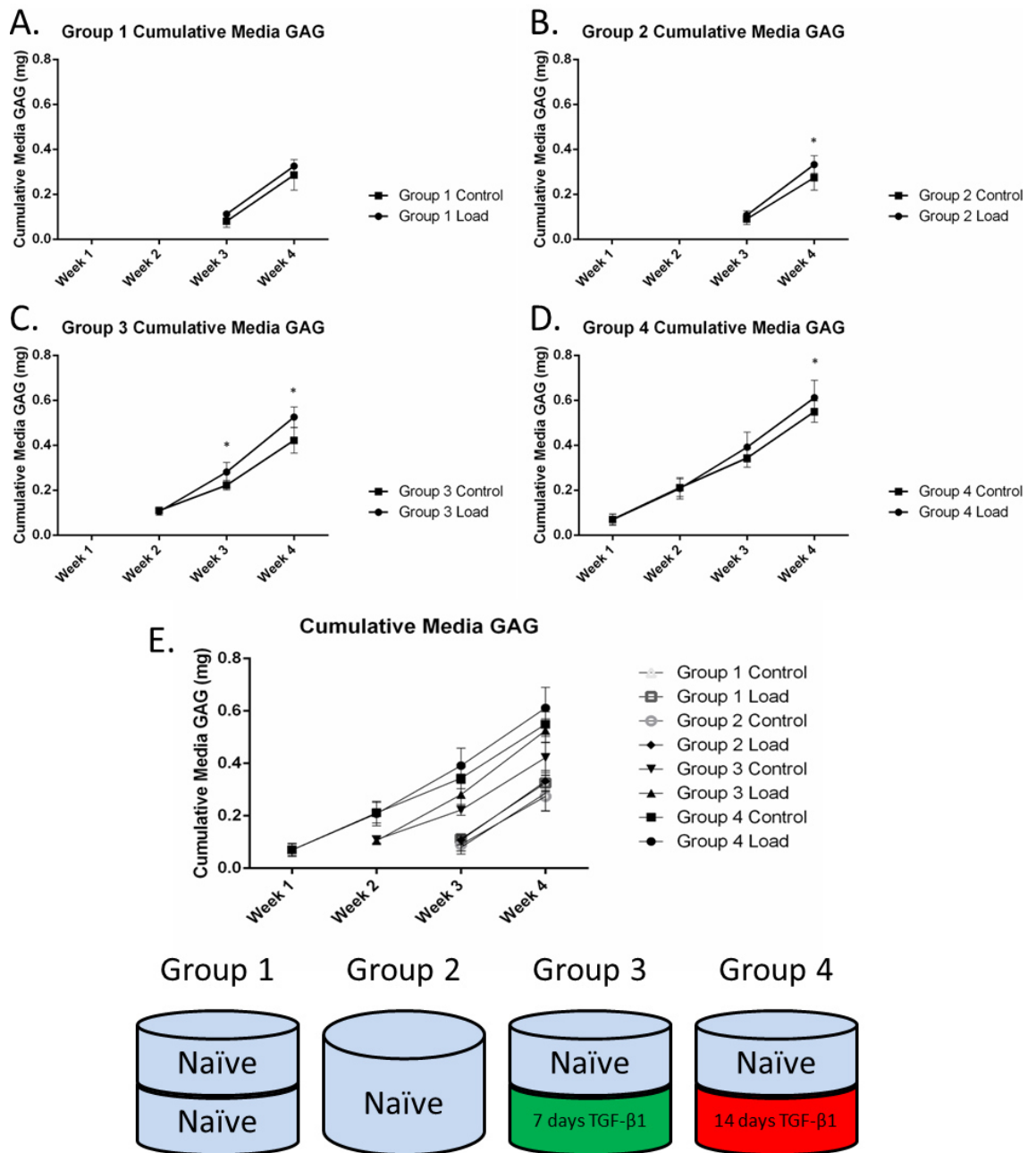


Figure 3.10

The cumulative amount of GAG released from cells embedded in scaffolds over time, as illustrated by group. These data represent three biological repeats carried out in triplicate. Statistical significance was defined as $P \leq 0.05$ and determined using two-way ANOVA and Sidak's multiple comparison test. Significant differences between loaded and control samples at each time point are displayed using a star.

3.3.4 Histology

Samples fixed in methanol for histology were frozen in OCT compound and sectioned on a cryotome. The sections were then stained with Safranin O in order to investigate the distribution of sulphated GAGs within the scaffolds.

Two sets of scaffolds (technical repeats) were collected from each group from one biological repeat and were processed for histology. The orientation of the scaffolds in the images provided in Figures 3.11-14 does not reflect their orientation with regards to each other during culture as this spatial relationship was unfortunately disturbed during histological processing.

In both technical repeats of group 1 scaffolds (Figures 3.11 and 3.13), positive safranin O staining was only present in group 1 loaded top scaffolds and not in either top or bottom control scaffolds.

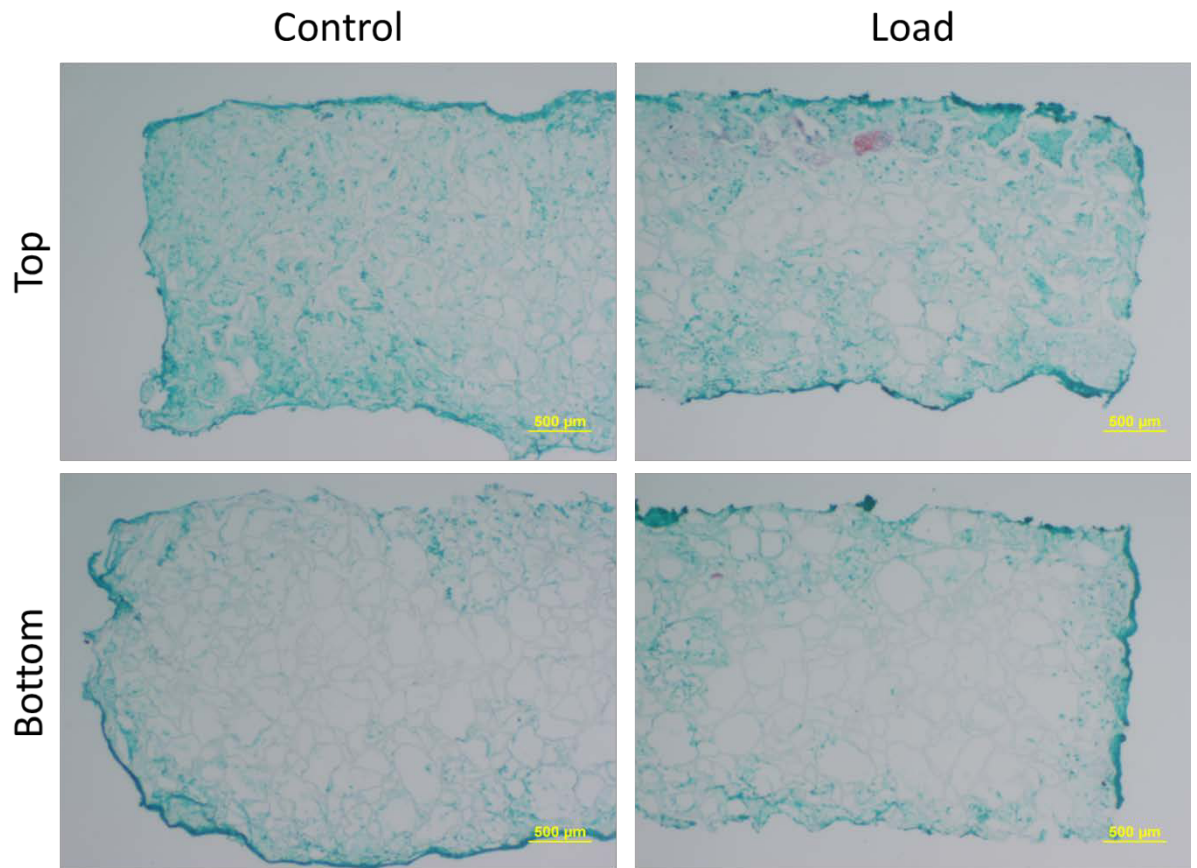
In group 2 scaffolds, safranin O staining was again only observed in loaded scaffolds but not in control scaffolds (Figures 3.11 and 3.13). Group 2 scaffolds are made up of one 4x8mm poly(ester-urethane) scaffolds rather than two 2x8 mm scaffolds as in group 1, 3 and 4. The reason for including these larger scaffolds was to provide a comparison to previous work carried out in this system for the multilayer scaffolds in group 1, 3 and 4 (Li et al., 2009, Li et al., 2010a, Schatti et al., 2011). The staining observed in these scaffolds matches that detected in previous studies.

Due to sectioning artefacts in the sections prepared from sample 1, it is difficult to compare the results from the two sets of scaffolds in group 3 (Figures 3.12 and 3.14). However, in both control groups there was more safranin O staining present in the top scaffold compared with the bottom scaffold. In sample 2, more staining was present in the bottom loaded scaffold compared to the top and in sample 1 the converse was observed with little staining in the bottom loaded scaffold and more in the top loaded scaffold (Figure 3.12).

The staining pattern of group 4 scaffolds, as with group 3, differs between technical repeats. In both sets of loaded scaffolds there was staining in both the top and bottom, with a greater amount of GAG staining positively in the bottom scaffold compared with the top scaffold (Figures 3.12 and 3.14). In control scaffolds from sample 2, there was staining in both top and bottom scaffolds, with more staining in the top scaffold.

Conversely, in sample 1 scaffolds there was no staining in the top scaffold but staining in the bottom scaffold (Figure 3.12).

Sample 1 Group 1



Sample 1 Group 2

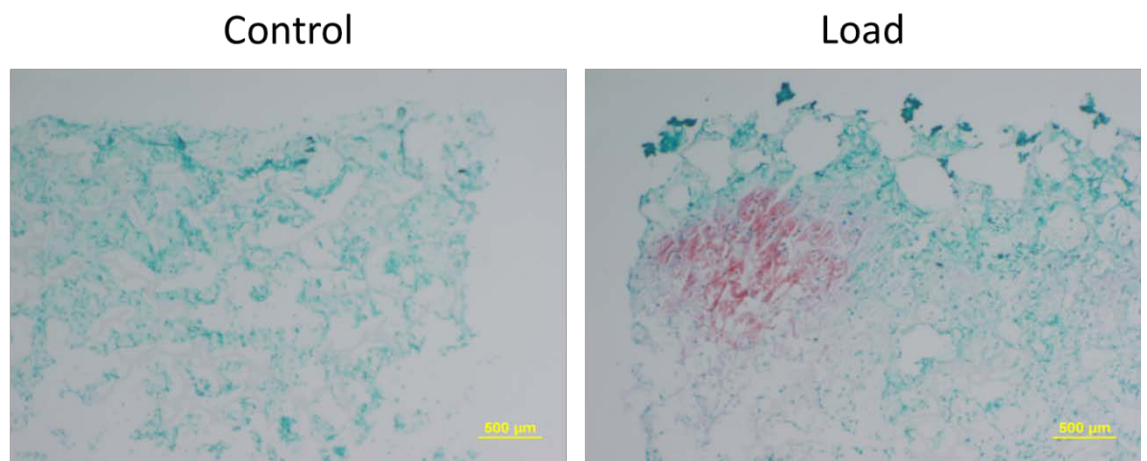


Figure 3.11

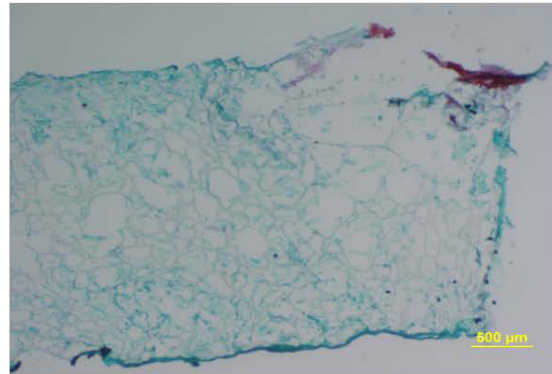
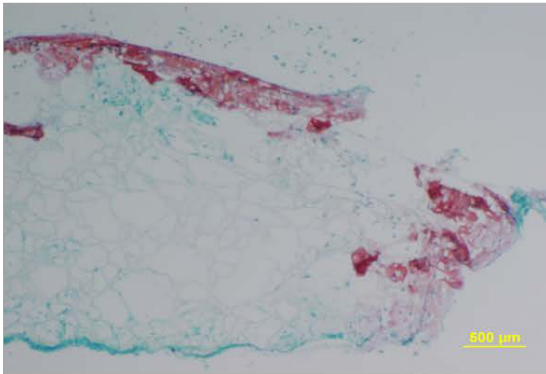
Histological images showing safranin O fast green staining of sample 1 (technical repeat number 1) scaffolds from group 1 and 2 subjected to mechanical load and harvested on day 28 of culture; non-loaded scaffolds served as controls. Scaffolds were fixed in 70% methanol and cryosectioned (12 μ M thickness) prior to histological analysis.

Sample 1 Group 3

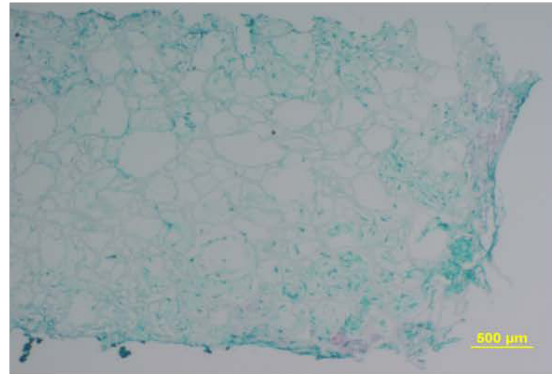
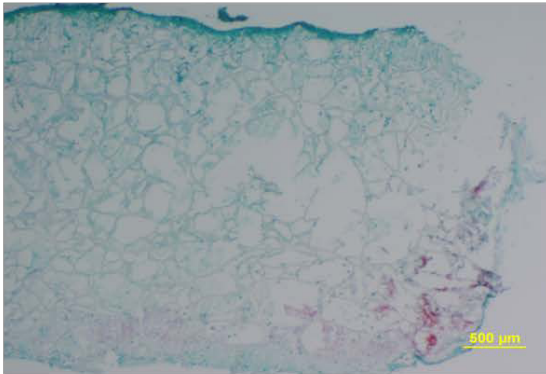
Control

Load

Top



Bottom

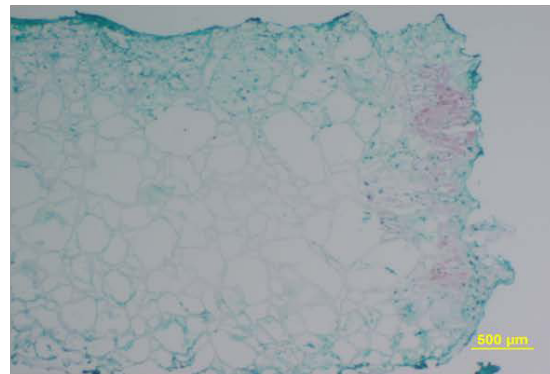
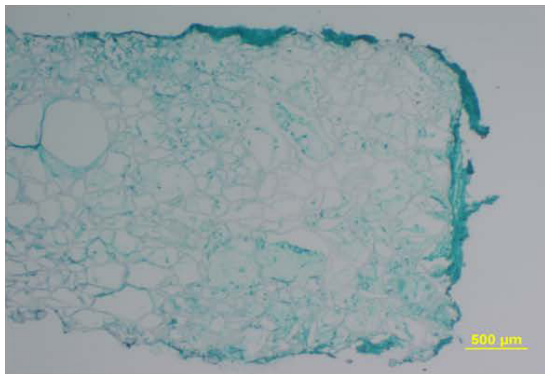


Sample 1 Group 4

Control

Load

Top



Bottom

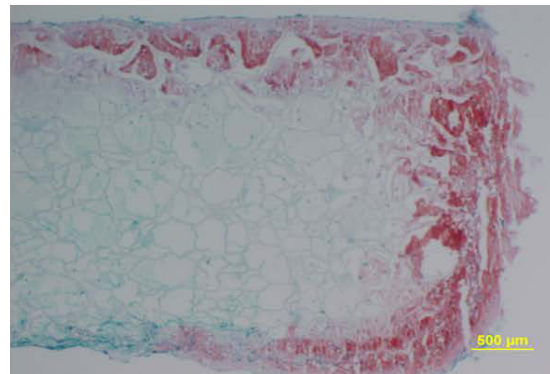
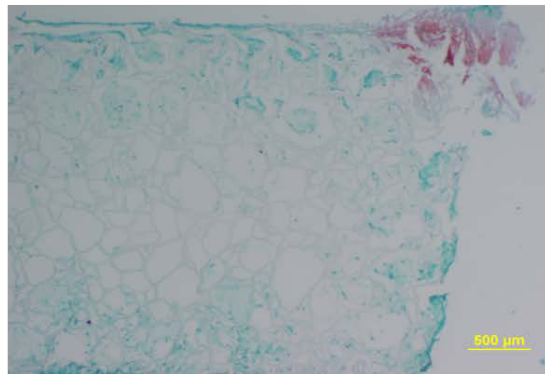
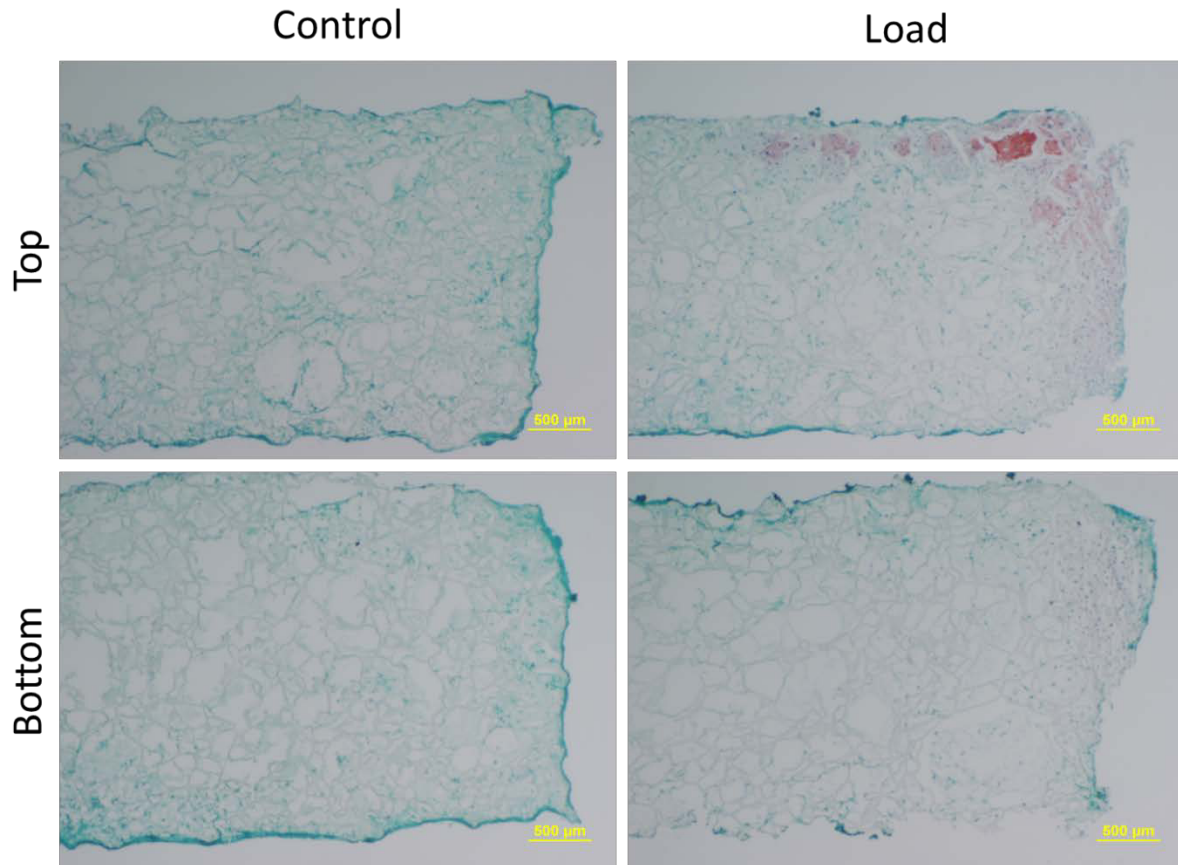


Figure 3.12

Histological Images showing safranin O fast green staining of sample 1 (technical repeat number 1) scaffolds from group 3 and 4 subjected to mechanical load and harvested on day 28 of culture, non-loaded scaffolds serve as controls. Scaffolds were fixed in 70% methanol and cryosectioned (12 μ M thickness) prior to histological analysis.

Sample 2 Group 1



Sample 2 Group 2

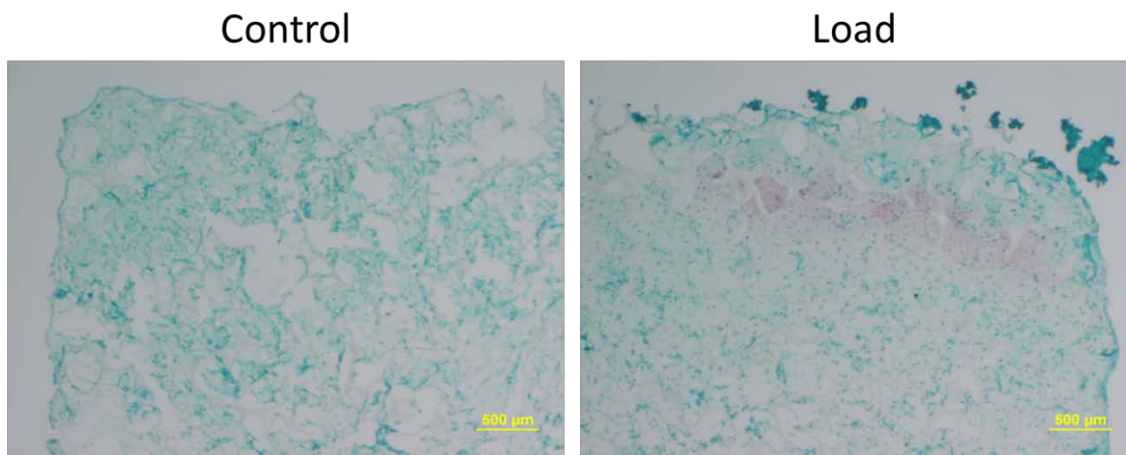
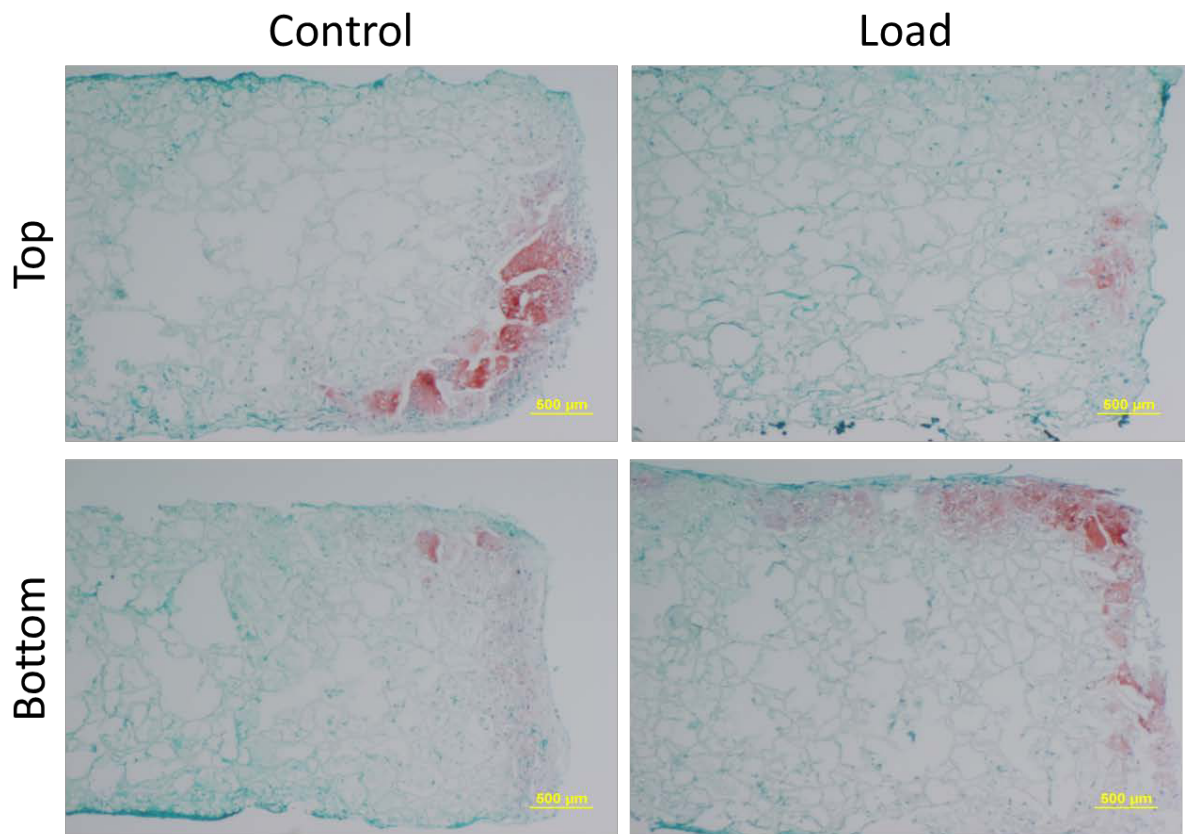


Figure 3.13

Histological images showing safranin O fast green staining of sample 2 (technical repeat number 2) scaffolds from group 1 and 2 subjected to mechanical load and harvested on day 28 of culture, non-loaded scaffolds serve as controls. Scaffolds were fixed in 70% methanol and cryosectioned (12µM thickness) prior to histological analysis.

Sample 2 Group 3



Sample 2 Group 4

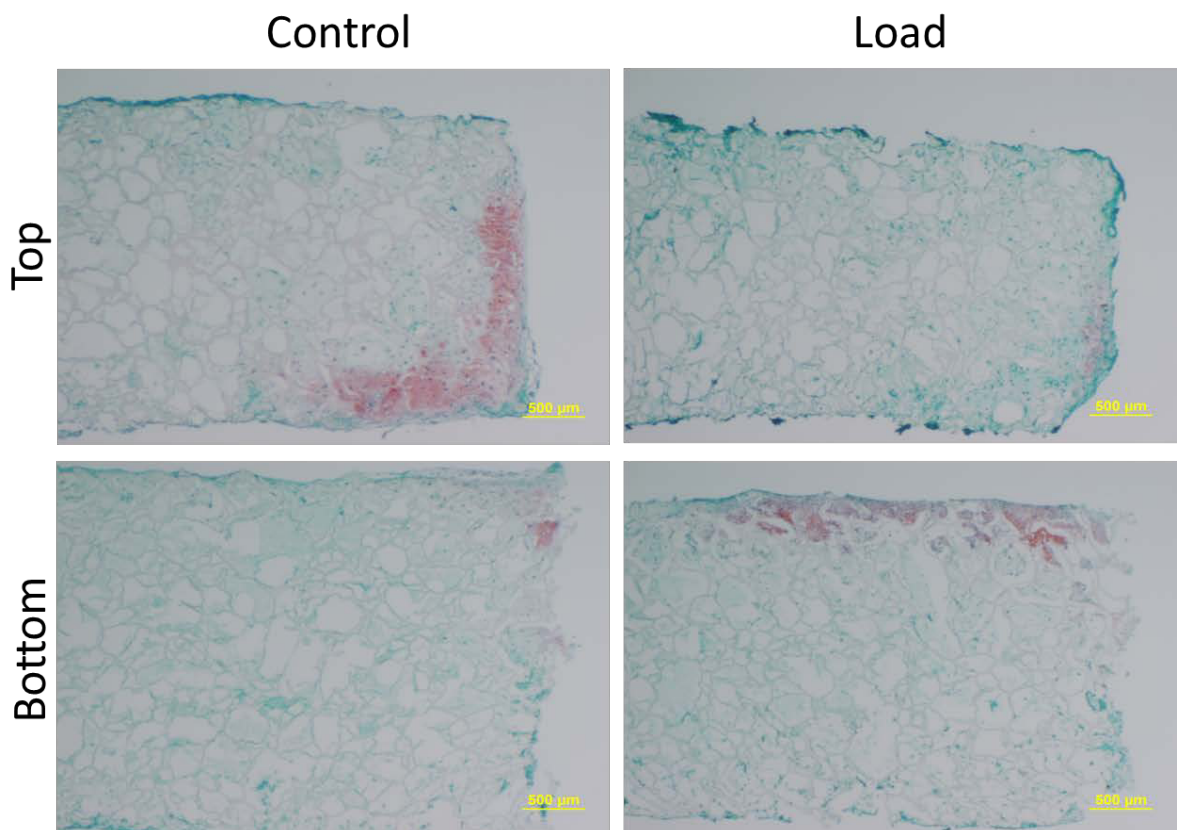


Figure 3.14

Histological images showing safranin O fast green staining of sample 2 (technical repeat number 2) scaffolds from group 3 and 4 subjected to mechanical load and harvested on day 28 of culture, non-loaded scaffolds serve as controls. Scaffolds were fixed in 70% methanol and cryosectioned (12 μ M thickness) prior to histological analysis.

3.4 Discussion

The work presented in this chapter aimed to investigate the effect of crosstalk between MSCs at different stages of chondrogenic differentiation on the process of chondrogenesis and hypertrophy in the presence of mechanical load. In order to do this, bilayer constructs were produced by stacking 2x8 mm fibrin-poly(ester-urethane) scaffolds containing MSCs on top of each other. In order to generate MSCs at different stages of differentiation, the "bottom" scaffolds in group 3 and 4 were predifferentiated in chondrogenic medium containing 10 ng/ml TGF- β 1, for one or two weeks respectively, before placing "top" scaffolds containing naïve MSCs on top of them.

The RNA used for gene expression analysis was taken from individual scaffolds at the end of the culture period; as a result, unlike the biochemical analysis these data represent the gene expression of the cells within each individual scaffold. There were few clear statistical differences with the gene expression data, due in part to large intra- and inter-donor variations in the degree and pattern of gene expression. For genes such as aggrecan and collagen type II this may also be related to the low n-number of some groups, resulting in a low number of degrees of freedom and therefore a higher threshold for a significant result, and the high number of groups used which may reduce the likelihood of achieving significance due to the increased number of comparisons.

Despite the high levels of variability and the lack of significance within some of the genes, there is a defined pattern of expression related to the cells' response to mechanical load within the data. In group 1, there was an increase in both top and bottom scaffolds in the mRNA expression of aggrecan, collagen type II, collagen type X and Sox9 in response to load, although only significant for collagen type X and Sox9 transcripts. In groups 3 and 4, there was also an increase in the expression of these genes for top scaffolds in response to load, but the increase was reduced in group 3 bottom scaffolds, and further reduced in group 4 bottom scaffolds compared to group 1. The smaller differences between control and loaded scaffolds in group 4 bottom scaffolds are likely to result from the predifferentiation phase of culture. The predifferentiation of the MSCs in the bottom scaffolds will have directed the cells towards chondrogenesis resulting in an increase in the expression of genes associated with MSC chondrogenesis (e.g. Sox9 and collagen type X). This is demonstrated by the, non-significant, increases in gene expression of collagen type II and X as well as Sox9 mRNA in group 4 control bottom scaffolds over the control bottom scaffolds of group 1 and 3. As a result the application of load is less likely to induce an increase in chondrogenic gene expression in these MSCs, which were already undergoing chondrogenesis, than the naïve MSCs seeded in group 1 which had received no

previous stimulation. Should this be the case, it would also be expected that load would have a more demonstrable effect on group 3 scaffolds that were only predifferentiated for one week rather than the two weeks predifferentiation that group 4 scaffolds were exposed to; the broad patterns in the real-time PCR data do appear to corroborate this. However, due to the high degree of variation, low 'n' number for some genes and low number of statistically significant differences within these data caution should be exercised about any interpretation as it is not possible to draw firm conclusions.

Freeswelling control scaffolds were also analysed in isolation from loaded scaffolds to remove the effect of load from the system. The results of this showed that there were significant differences between the free swelling groups that were not visible when the data set was analysed as a whole. Of note is the significant increase in collagen type X mRNA expression in group 4 control top scaffolds over group 1 control top scaffolds and group 2 control scaffolds, and the trends (not significant) towards increased expression of aggrecan and Sox9 mRNA in group 4 control top scaffolds compared to group 1 scaffolds, and of collagen type II gene expression in group 3 top control scaffolds compared to group 1 scaffolds. These results show that there appears to be a possible chondrogenic effect in group 3 and 4 top control scaffolds, despite these scaffolds never being exposed to culture media containing TGF- β 1, or exposed to mechanical load, this therefore appears to be linked to the predifferentiation of the bottom scaffold and the interaction of the cells in the two scaffolds during the co-culture period. This effect could be caused by TGF- β 1 retained in the bottom scaffolds during pre-culture, that when subsequently released from the bottom scaffolds induces chondrogenesis in the top scaffolds, or through the paracrine signalling effects of cells in the bottom scaffolds that are further down the chondrogenic lineage than the MSCs in the top scaffold. However, it was not possible with the data collected during this work to confirm this chondrogenic effect in freeswelling top scaffolds in group 3 and 4.

Measurement of scaffold DNA contents showed that at the end of the culture period group 1, 3 and 4 scaffolds all contained the same amounts of DNA whilst group two scaffolds, which were twice the size of the other scaffolds, contained twice as much DNA. Characterisation of this model by our group has previously shown that there is little proliferation of MSCs in this scaffold system resulting in the relative stability of scaffold DNA contents over time in culture (Li et al., 2009).

Quantification of the amount of GAG released into the culture media from week 1 and 2, demonstrated no differences within groups in GAG production, but an increase in release from group 4 scaffolds compared to group 3 scaffolds; this was to be expected given that all groups are receiving chondrogenic media and that group 4 scaffolds had

a week longer in culture than group 3 scaffolds. The application of mechanical load for one hour a day began in week 3. It is in the media collected during this week that the clearest changes occurred in the media GAG content (presented by week). Load increased the amount of GAG released into the media in group 3 and 4 over their respective free swelling controls, although only significantly in group 3. This clear effect of load on GAG release was, however, not replicated in week 4 media, where there was no clear effect of load or any significant differences between the groups. This suggests that the GAG being released into the media from group 3 and 4 is not being synthesised in response to the application of load in week 3, rather it is GAG being washed out of the scaffolds that had been laid down during the preculture period. This trend was also observed in the release of GAG from scaffolds plotted over time for each group, significant differences between control and loaded groups were detected in both group 3 and 4. The level of GAG detected in the media was significantly higher in media collected from group 4 control scaffolds than group 1 loaded scaffolds. This demonstrates that the build-up of GAG during the preculture period in group 4 leads to the substantial release of GAG to the medium in week three and four even in the absence of mechanical load; however, the application of mechanical load increased the release of GAG even further. A small increase in the media GAG level was seen between group 1 loaded scaffolds compared to controls. This effect must be due to the chondrogenic effect of the load applied during week 3, this effect would be similar to the effect felt in group 3 and 4 loaded scaffolds, however the response to load in group 3 and 4 dwarfs that of group 1, suggesting that either the synthesis of GAG is greatly enhanced in response to load by the predifferentiated bottom scaffold in group 3 and 4 or that GAG is being washed out of the bottom scaffolds in response to load.

The hypothesis that the increased levels of GAG released into the media in week 3 are likely to be an artefact caused by loading predifferentiated, GAG-containing scaffolds, was further supported by the results of TGF- β 1 quantification from the culture medium. TGF- β 1 has previously been shown to be produced by MSC laden fibrin-poly(ester-urethane) scaffolds in response to mechanical load in this system (Li et al., 2010a). The release profile of TGF- β 1 in week three media shows that there is a clear and consistent increase in all groups in response to load, but with no significant differences within groups (between loaded and freeswelling scaffolds). The effect of load then appears increased in the media collected during week 4, which shows clear differences between loaded and freeswelling scaffolds, with significant differences between all four loaded groups and group 1 and 2 control scaffolds. This pattern of TGF- β 1 release clearly shows that the synthesis and release of TGF- β 1 builds up gradually from the initiation of loading and is therefore higher in week 4 than in week 3. The lack of a significant difference between group 3 and 4 loaded scaffolds and control scaffolds

may result from the higher levels of TGF- β 1 in group 3 and 4 control groups; this is likely to be due to the effect of the period of preculture on these scaffolds and may be caused by release of TGF- β 1 retained in the scaffolds during preculture or the autoinduction of TGF- β 1 expression in the differentiating MSCs (Van Obberghen-Schilling et al., 1988). The comparison of the release of GAG from scaffolds during week 3 and 4 with the more predictable pattern of TGF- β 1 release suggests that the GAG measured in week 3 media results from a "burst" release of stored GAG as opposed to a biosynthetic response to the application of mechanical load.

The reduced release of GAG into the culture medium in week 4 compared to week 3 could also be caused by increased matrix retention in week 4 due to the build-up of matrix within the scaffolds. This could be investigated by collecting samples for histology at the end of each week of culture. Another factor could be an inhibitory effect of one of the cell populations on matrix production e.g. through a chondro-inhibitory soluble protein such as IL-1. However, the effects that the two populations have on each other have been shown by this work to be subtle and therefore unlikely to cause such a large and rapid decrease in GAG production/release.

Quantification of the cumulative release of GAG from scaffolds into the media over the course of culture suggests an increased response to load in group 3 constructs compared to group 4 constructs. This is evidenced by the significant difference in cumulative GAG release (presented over time) at both week 3 and 4 of culture for group 3 scaffolds, load and control, whereas a significant difference was only detected at week 4 for group 4 scaffolds. The results of cumulative GAG release from scaffolds shown by week indicated that there was also no significant difference in the total amount of GAG released from group 3 and group 4 scaffolds over the total time in culture, despite a trend towards higher levels in the control and loaded groups relative to each other. This may be due either to a plateauing or even slowing of the release of GAG from group 4 scaffolds in the later stages of culture, or to increased GAG production in group 3 constructs. Given the trend towards higher GAG release from group 3 scaffolds compared to group 4 scaffolds in week 4, and the relatively higher levels of cumulative GAG in group 4 control and loaded scaffolds compared to their corresponding scaffolds in group 3, this is more likely to result from a slowing of GAG release from group 4 scaffolds in the fourth week of culture. This could be caused by decreased GAG synthesis within the scaffolds, or a build-up of matrix molecules in the scaffold (resulting from four weeks of chondrogenic stimulation) increasing the amount of GAG retained within the scaffolds, causing a corresponding decrease in GAG released to the medium.

Although useful for providing an idea of what is happening within this system, the analysis of the GAG and TGF- β 1 content needs to be interpreted with caution. The results for the media analysis presented here provide one result per "construct" as a whole but do not differentiate between the top and bottom scaffolds and their contribution to that overall result. The changes in GAG released into the media in week 3 demonstrates the ability of the bottom scaffolds, which had undergone predifferentiation, to contribute GAG to the culture media in response to mechanical loading, despite the GAG having to diffuse through the scaffolds themselves as well as through the poly(ester-urethane) rings which were used to surround the scaffold to keep the top and bottom layers in position relative to one another. The results from this media analysis therefore provide a broad idea of what is going on within the construct but no resolution between the two scaffolds within the construct. It would be possible to indirectly determine the contribution of each set of scaffolds to the overall GAG content of the media by repeating the experiment with extra groups where either the top or bottom scaffold is replaced with an acellular fibrin-poly(ester-urethane) scaffold. Replacing either the top or bottom scaffold with an acellular scaffold would allow for the histological investigation into the diffusion of GAG from the bottom scaffolds in to the top scaffold e.g. in group 3 and 4 control scaffolds where it appears that more GAG is present in the top scaffold compared to the bottom scaffold. Repeating this with an acellular top scaffold, it would be possible to determine if the GAG detected simply diffuses in from the bottom scaffold or is laid down by the cells in the top scaffold driven by crosstalk with cells in the bottom scaffold. However, using empty scaffolds would also remove any effect of crosstalk between scaffolds, and may therefore affect the results. Another option would be to supplement the preculture culture media with the radioactive sulphur isotope ^{35}S in a "pulse-chase" fashion. The presence of this radioactive isotope could then be determined in top scaffolds and the culture media. Both of these approaches could be used to show where GAG produced in the two scaffolds could later be detected but, neither of these systems allow for a determination of the ratios of GAG in the media with regards to scaffold origin.

The GAG/DNA ratio of a construct provides an extremely useful metric for normalising the amount of GAG produced by a construct to the number of cells within the construct. Previous work in this system has calculated the "total GAG" for each scaffold by adding the amount of GAG determined using DMMB in the proteinase K digest of the scaffold at the end of culture and the media samples collected at every media change to give a total amount of GAG produced by the cells in each scaffold over the whole length of culture (Li et al., 2009, Li et al., 2010a). That total GAG value was then divided by the scaffolds DNA content to produce a GAG/DNA ratio. In this system the bulk of GAG that is produced is lost to the culture media rather than being retained within the

scaffold. As a result the value for the media GAG is generally higher than that for the scaffold GAG, especially under loading (Li 2009, Li 2010). The percentage of GAG found in the scaffold in this study ranges from $16.07\% \pm 6.39$ to $28.98\% \pm 15.73$ in control scaffolds (group 1 control bottom and group 2 control respectively) and between $15.19\% \pm 9.00$ and $31.37\% \pm 19.03$ in loaded scaffolds (group 1 load bottom and group 2 load respectively). This means that with the majority of GAG being lost to the media it is not possible to determine which scaffold it came from, top or bottom. Looking at the scaffold GAG content alone is also not a solution to this problem as a large amount of the GAG is lost from the scaffolds, and load tends to reduce the scaffold GAG content more than freeswelling culture as the physical application of load washes GAG out of the scaffold, as demonstrated in this chapter with the media GAG results from week 3. This problem could be overcome by using a culture system such as agarose or alginate which would better retain any GAG produced, however, the scaffold also needs to be resilient enough to withstand the repeated application of multiaxial mechanical load. The purpose of this work was to observe the effects of mechanical load on MSCs within a fibrin scaffold in response to load, rather than to optimise this scaffold to retain matrix molecules, that is however an important issue for future work.

After consideration it was decided to present the "total GAG" as the summation of the scaffold GAG for each scaffold with the total amount of GAG in the media (i.e. the GAG released into the media from both scaffolds) from all weeks of culture. This means that the media GAG component of group 1 and 2 scaffolds is made up of results from week 3 and 4, whilst group 3 results represent week 2, 3 and 4 and group 4 scaffolds from week 1-4. This inclusion of GAG produced during the pre-culture period suggests that there was an inherently higher amount of total GAG and a higher GAG/DNA ratio, regardless of the actual rates of synthesis in response to load in weeks 3 and 4. The reason it was decided to include all of the measurements made within the total GAG value is that in week 3 a large amount of GAG, synthesised in the pre-culture period, was released into the culture media in response to mechanical load. This means that even if just the week 3 and 4 results are taken for the GAG/DNA ratio the pre-culture period would still artificially inflate the results from group 3 and 4 scaffolds. As a result it was decided to include the data from the entire culture period and to highlight the inherent bias within these data. In order to determine the relative contributions of the pre-differentiation and loading periods of culture, a separate set of group 3 and 4 scaffolds could be seeded and harvested for analysis at day 14. This would allow for the determination of GAG production during the pre-differentiation phase and allow for a better comparison to be made between the effect of load over the third and fourth weeks of culture on group 3 and 4 scaffolds compared to group 1 and 2.

The results of Safranin O staining on two technical repeats highlighted both similarities and differences in the deposition of GAG within scaffolds. In both sets of group 1 scaffolds positive GAG staining was present only in group 1 top loaded scaffolds. This suggests that, at a histological level, load is only inducing changes in the top scaffolds and not the bottom scaffolds. This is in contrast to the Sox9 real-time PCR results which suggested the induction of some chondrogenic gene expression in bottom scaffolds in response to load. This suggests that within this experimental model system load had a stronger effect in the top scaffolds, which were directly exposed to the shear component of the load, than the underlying bottom scaffolds, where load was able to induce the expression of Sox9 but not induce detectable matrix deposition. This may be linked to the importance of shear load in the induction of chondrogenesis as discussed in Chapter 4. In line with previous work, loaded 4x8mm scaffolds in group 2 stained positively with Safranin O whilst group 2 control scaffolds did not. Results from group 3 and 4 scaffolds showed interesting trends, but were inconsistent. In group 4 control scaffolds there was a greater amount of matrix deposited in top scaffolds than in bottom scaffolds in both repeats; the same was observed for one of the repeats in group 4. This suggests that the predifferentiated bottom scaffold may, in the absence of any other stimuli for the top scaffolds, either be inducing chondrogenesis in the top scaffold or releasing GAG which then diffused in to the other where it was then detected. As the scaffolds were separated during histological processing it is not possible to analyse the scaffolds in the orientation that they were cultured in. Should the staining occur in abutting regions of the two scaffolds then it would be extremely hard to tell if the matrix in the top scaffold was produced there in response to chondrogenic stimuli from the lower scaffold, or if the GAG was produced in the lower scaffold and had diffused in to the top scaffold. However, if the staining in the top scaffold occurred away from the areas stained positively in the bottom scaffold then it would be likely that the GAG deposition in the top scaffold was driven by a local chondrogenic response by the cells rather than diffusion of GAG throughout the system. Improved histological processing would allow for the scaffolds to be kept in the same relative positions that they were cultured in, providing important additional spatial data about the deposition of matrix relative to the source of load and relative to the other scaffold in the construct. This would be possible e.g. by placing the scaffolds in a mould with OCT compound and then snap freezing; the scaffolds could then be cryosectioned together from the same block and then fixed postsectioning, before staining. This would also allow for a choice of fixative depending on the requirements of more sensitive techniques such as immunohistochemistry, or in-situ hybridisation which would be another method for investigating the source of GAG within the constructs. A chondrogenic effect of the bottom scaffold on the top scaffold could either be mediated

by endogenously produced factors, such as TGF- β , or by exogenously administered TGF- β 1 taken up by the scaffold during the preculture period which is then slowly released over the course of culture, however given the short half-life of activated TGF- β *in vivo* this is the less likely option (Coffey et al., 1987, Wakefield et al., 1990). The deposition of GAG in group 3 and 4 freeswelling control scaffolds correlated with the gene expression data that suggested an increase in the expression of genes associated with MSC chondrogenesis in group 3 and 4 control scaffolds.

GAG deposition as denoted by Safranin O staining is concentrated around the periphery of the scaffolds. This is similar to the pattern of staining seen in Chapter 4, and may relate to the potentially reduced levels of nutrition in the center of the fibrin-poly(ester-urethane) used in this system or the loss of cells from the center of scaffolds as described in Chapter 4.

Histological analysis demonstrated some evidence of potential crosstalk between scaffolds containing cells at different stages of differentiation and has provided more evidence for chondrogenic responses in group 3 and 4 top control scaffolds that were suggested in PCR and biochemical analysis. However, the disruption of scaffold orientation during processing and variation between scaffolds means that it is not conclusive.

This discussion has highlighted a number of problems associated with the model used in this chapter and some of the analytical techniques used to generate the results. One of the problems highlighted in this discussion was intra- and inter-donor variation. The best way to reduce variation would be to carry out more repeats to generate more data and provide a fuller understanding of the behaviour of cells within this system which would allow for more concrete conclusions to be drawn.

3.5 Conclusions

The work presented in this chapter has shown that despite some potential histological and gene expression evidence for crosstalk between cells at different stages of chondrogenic differentiation no clear evidence was obtained that the co-culture model system had an effect on the induction of chondrogenesis or hypertrophy in MSCs in the presence or absence of load. The model system used in this chapter also provided difficulties for data analysis and interpretation. As a result it was decided that a simpler model involving spatial but not temporal differences (in terms of stages of cellular differentiation) should be developed and used for further work, the development and characterisation of this model is described in detail in Chapter 4.

Chapter 4 Asymmetrical seeding of MSCs into fibrin-poly(ester-urethane) scaffolds and its effect on mechanically induced chondrogenesis

Aim

The aims of the work presented in this chapter were to investigate the effect of seeding a layer of MSCs on the loaded surface of MSC containing fibrin-poly(ester-urethane) constructs on mechanically induced chondrogenesis, and to investigate the effect of joint like mechanical load on the activation of endogenously produced TGF- β 1 in response to mechanical load.

Sections of this Chapter have resubmitted following initial review and revision as two separate manuscripts entitled "Asymmetrical seeding of MSCs into fibrin-poly(ester-urethane) scaffolds and its effect on mechanically induced chondrogenesis" and "Joint Mimicking Mechanical Load Activates TGF β 1 in Fibrin-Poly(ester-urethane) Scaffolds Seeded with Mesenchymal Stem Cells" to Tissue Engineering and Regenerative Medicine.

4.1 Introduction

The results of investigations presented in Chapter 3 suggested that when two separate populations of MSCs, at different stages of chondrogenic differentiation, were cultured together in a fibrin-poly(ester-urethane) constructs; predifferentiated cells (that were further down the pathway of chondrogenic differentiation) could, potentially, induce chondrogenic characteristics in naïve MSCs. This demonstrates that the interaction between different populations of MSCs within the same scaffold could potentially be used to modify the cells responses to chondrogenic stimuli. However, the restrictions of the culture system used made this interaction difficult to verify. The work in this chapter followed on directly from the work presented in Chapter 3 by investigating the effect of seeding two different populations of MSCs within the same construct. However, to remove some of the confounding factors associated with the work performed in Chapter 3 the temporal factor (using MSCs at different stages of differentiation) was removed and a single 4x8 mm scaffold was used for culture instead of the two 2x8 mm scaffolds used to produce the constructs described in Chapter 3. The two separate populations were instead created by seeding cells within the fibrin-poly(ester-urethane) scaffolds (as previously described) and also seeding a layer of cells on the loaded surface of the scaffold.

The creation of a second population, within a construct, and the potential for that population to influence the process of chondrogenesis within the construct as a whole was driven by the results seen in Chapter 3. However, the location of the second population was chosen based on a previous study carried out in the same mechanical loading device described in thesis by another member of our group who studied the importance of the two individual components of the load, namely compression and shear. Compression and shear were applied both individually, and in combination to MSC loaded fibrin-poly(ester-urethane) scaffolds. The results of this work showed that the shear component of the load was critical in the induction of chondrogenesis (Schatti et al., 2011). When considering that shear loading is important for the chondrogenic response, it would suggest that under joint like load, cell location, specifically the exposure of the cells to the shear component of the load, may play a role in the results that can be obtained. It may be possible to exploit the potentially chondrogenic effect of shear load in order to improve the chondrogenic induction and matrix deposition of human bone marrow mesenchymal stem cells in the absence of any exogenous growth factors by asymmetrically seeding scaffolds with cells to maximise the exposure of a small population to the shear component of the load.

TGF- β is secreted by cells in an inactive, latent form in which the active TGF- β peptide is bound to the latency associated peptide (LAP) and a latent TGF- β binding peptide

(LTBP). For TGF- β to bind to, and activate, a target receptor (Section 1.7, Figure 1.5) the mature TGF- β peptide must first be released from the LAP and if present the LTBP (Robertson and Rifkin, 2013). TGF- β activation can occur in a variety of ways including protease degradation (e.g. the serine protease plasmin), mechanical stimulation, deglycosylation or the application of a number of physiochemical stimuli such as heat, extremes of pH and UV light (Robertson and Rifkin, 2013, Lyons et al., 1990). Mechanical forces, in a number of different forms, have been shown to activate TGF- β . Work by Annes et al. and Wipff et al. has shown that integrin binding and subsequent cell generated traction forces are involved in the activation of TGF- β that is bound to the extracellular matrix via LTBP, whilst the mechanical activation of TGF- β has also been demonstrated in fluid environments in response to the application of fluid shear stress or stirring forces (Ahamed et al., 2008, Albro et al., 2012, Annes et al., 2004, Wipff et al., 2007). Work by our group using a custom built bioreactor has shown that the application of a combination of shear and compressive load, which mimics the load of a diarthrodial joint, induces the chondrogenesis of human MSCs via the induction of TGF- β 1 expression and secretion in stimulated cells, but the activation state of TGF- β 1 has not been investigated (Li et al., 2010a).

In this chapter the response of MSCs to multi-axial mechanical load that mimics the mechanical environment of an articulating joint was investigated. The hypothesis was that induction of chondrogenesis, and the deposition of cartilage-like matrix by MSCs in response to multi-axial load, could be improved, while maintaining total cell number, by seeding the scaffolds with two populations of cells, one population inside the scaffold as previously described and a second population on the surface of the scaffold, directly exposed to shear load. In order to do this, the scaffolds were seeded asymmetrically with a small proportion (10%) of the total number of cells seeded on the scaffold's loaded surface, whilst the majority of the cells (90%) were seeded within the scaffold itself. As a control, a third group consisting of only 10% of the total number of cells were seeded directly on the surface of otherwise acellular fibrin filled scaffolds. This was to establish whether the cells applied to the surface of the scaffold were completely responsible for the observed response, or whether any changes resulted from interactions between cells seeded on the surface of the scaffold and those seeded within the scaffold. This work also aimed to further investigate the effect of joint like load on MSCs and the TGF- β 1 pathway by quantifying not only the effect of multiaxial load on the overall production of TGF- β 1 but also its activation. The term joint like load is used in this chapter to refer to the application of multiaxial shear (generated by a 25° rotation of a ceramic sphere on the surface of the scaffold at 1Hz) and compression (cyclic 10% compression on top of 10% prestrain at 1Hz).

4.2 Materials and Methods

4.2.1 Donor Information

MSCs were used from four different marrow aspirates from vertebral bodies; two females aged eighteen and forty-nine years and two males aged twenty-two and seventy-six years old, as well as one aspirate from the tibial plateau of a forty-eight year old male.

4.2.2 Experimental design

Seeding of fibrin-poly(ester-urethane) scaffolds with different cell distribution patterns

The number of cells seeded into each scaffold and the location of the cells within the scaffolds varied by group. Group 1 scaffolds contained four million cells seeded evenly throughout each 4x8mm fibrin-poly(ester-urethane) scaffold. Group 2 scaffolds contained three million six hundred thousand cells seeded throughout the scaffolds with four hundred thousand cells allowed to adhere to the upper surface of the scaffold. The final set of scaffolds (group 3) were filled with fibrin alone and four hundred thousand cells were then allowed to adhere to the upper face of the scaffolds (Figure 4.1).

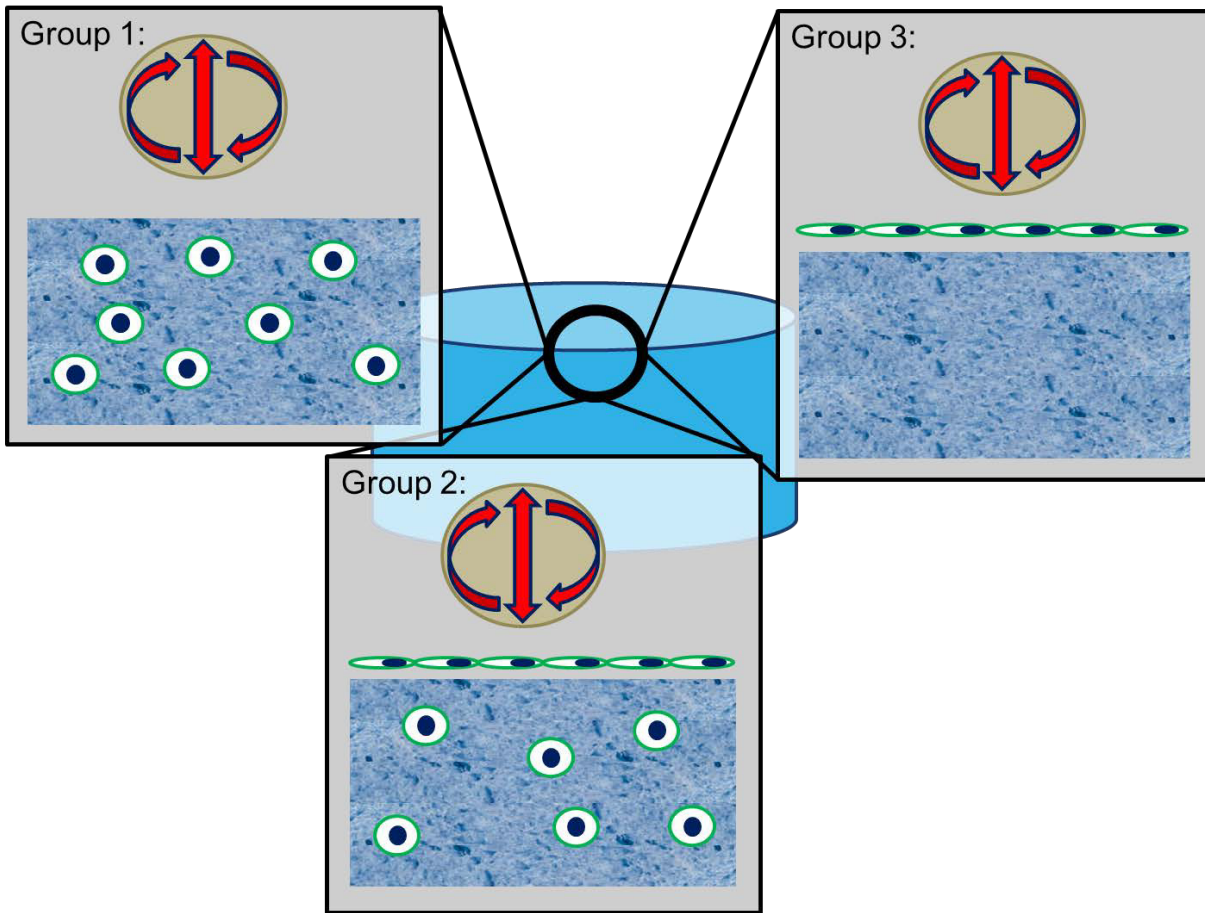


Figure 4.1

Schematic showing the different seeding patterns used in this study. In group 1, four million cells were evenly seeded throughout scaffolds. In group 2, three million six hundred thousand cells were seeded evenly throughout the scaffold and four hundred thousand seeded on the loaded surface of the scaffold. Group 3 scaffolds were not seeded with cells within the scaffold but only with four hundred thousand cells on the loaded surface.

4.2.3 Seeding of cells on to fibrin-poly(ester-urethane) scaffolds

In order to seed cells on top of the fibrin-poly(ester-urethane) scaffolds, the scaffolds were first seeded with fibrin and cells or fibrin alone as described (Section 2.4 and 4.2.3), and left to polymerise for one hour. During this incubation period four hundred thousand MSCs were resuspended in 100µl of serum free DMEM per scaffold, and after one hour this cell suspension was dripped on to the surface of the polymerised fibrin-poly(ester-urethane) scaffolds and left in place for twenty minutes at 37°C. After this second incubation step the scaffolds were removed from the Eppendorf tube lids and placed into PEEK holders for further culture.

4.2.4 Membrane labelling of MSCs with the fluorescent dye PKH26 and seeding of labelled MSCs into fibrin-poly(ester-urethane) scaffolds

An additional six scaffolds were seeded with a mixture of unlabelled MSCs and cells that had been labelled with the red fluorescent dye PKH 26 (Sigma-Aldrich, Buchs, Switzerland); the role of this fluorescent dye was to establish the location and distribution of MSCs in both free-swelling and loaded scaffolds after four weeks in culture. In order to apply the dye to MSCs, the cells were first trypsinised and counted before being washed in serum free DMEM and resuspended in 500µl of 'diluent C'; this was supplied, pre-made, by the company with the dye. A further 500µl of diluent C containing 1µl of PKH26 dye per million cells was added to the cell suspension. The tube containing the cells was then wrapped in aluminium foil and placed on an orbital shaker for five minutes at room temperature. Following incubation, 1ml of serum was added to terminate the reaction between the cells and the dye. The stained cells were washed three times with 10% FBS DMEM before being used for seeding.

Two group 1 scaffolds (one non-loaded control and one loaded sample) were seeded with four million stained cells that were distributed evenly throughout the scaffold. Two scaffolds were seeded as in group 2 with three million six hundred thousand unlabelled hMSCs seeded within the scaffold itself and four hundred thousand labelled cells seeded on the surface of the scaffold. Two final scaffolds were seeded as group 3 scaffolds with four hundred thousand labelled cells on top of otherwise acellular scaffolds.

One of each of these pairs of scaffolds were exposed to mechanical load using the same protocols and duration of loading as used for scaffolds in group 1,2 and 3 whilst the other scaffold was kept in free-swelling culture.

4.2.5 Mechanical Loading

Scaffolds were loaded using a custom-made multiaxial load bioreactor based on tribological principles (Wimmer et al., 2004). Loaded scaffolds were exposed to 10% compression superimposed on top of a 10% pre-strain and shear loading ($\pm 25^\circ$) at 1Hz for one hour a day five times a week up to a total of 20 cycles. In contrast, control scaffolds were kept in free-swelling culture conditions for the entire culture period of four weeks.

4.2.6 Sample collection and storage

Media was collected three times a week at media changes and was pooled by week before storage at -20°C for analysis. After 28 days of culture, scaffolds were harvested for biochemical analysis (three scaffold halves per group), RNA isolation/real-time PCR (four scaffold halves per group) and histology (one scaffold half per group), and analysed as described previously in the Chapter 2 (Section 2.6).

4.2.7 Statistical Analysis

The data presented represents combined data from 3 (real time PCR) or 4 (GAG/DNA and TGF- β 1 analysis) individual experiments with each performed using different donors in quadruplicate (real time PCR and TGF- β 1 analysis) or triplicate (GAG/DNA). Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA), and significance was defined as $P \leq 0.05$. The D'Agostino & Pearson omnibus normality test was used to determine if the distribution of data within each data set was normal. Following this, the Kruskal-Wallis and Dunn's multiple comparison tests were used to analyse biochemical, real-time PCR and protein analysis with the exception of collagen type II real time PCR where the Mann-Whitney test was used as only two groups were being compared.

4.3 Results

Due to the use of primary human cells in this work there was a high degree of variability between biological repeats. Due to this high level of variation and the relatively low numbers of donors tested many of the analyses do not reach significance at the 5% level. As a result both significant changes and non-significant trends within the data collected are described and discussed, where relevant, in this chapter.

4.3.1 Fluorescence membrane labelling of MSCs seeded in to fibrin-poly(ester-urethane) scaffolds

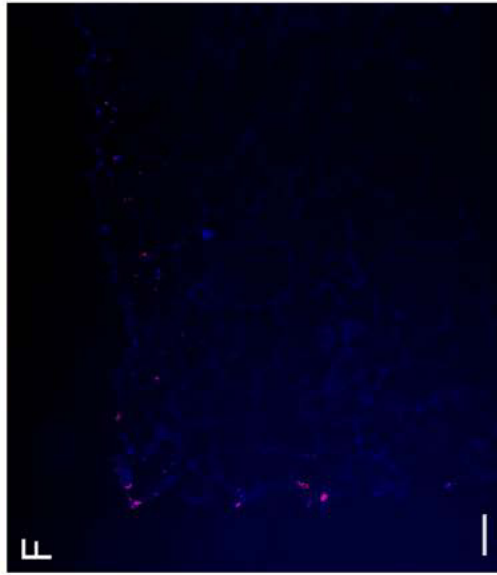
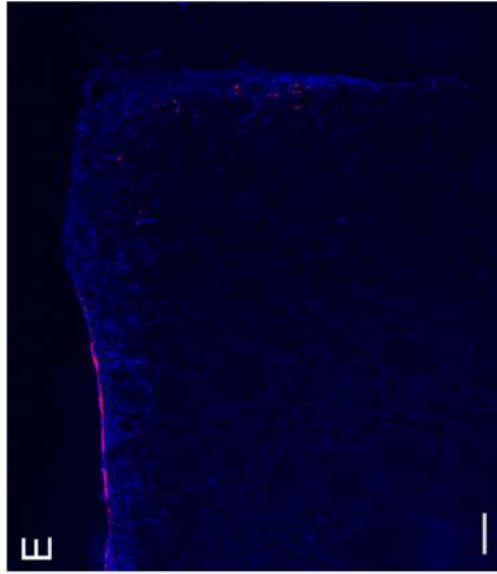
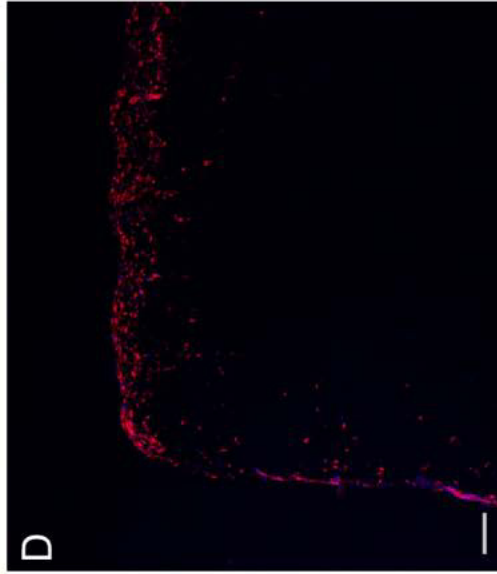
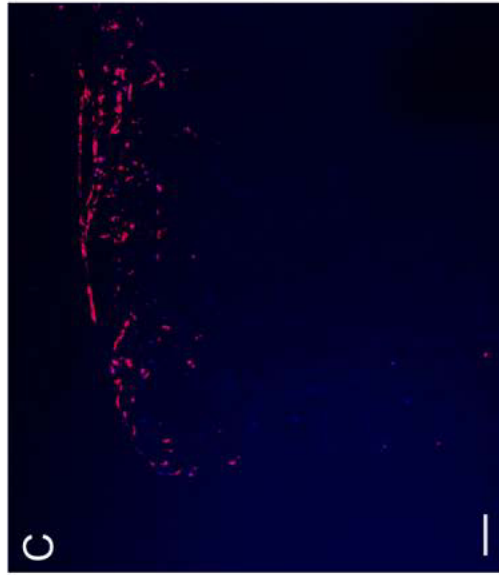
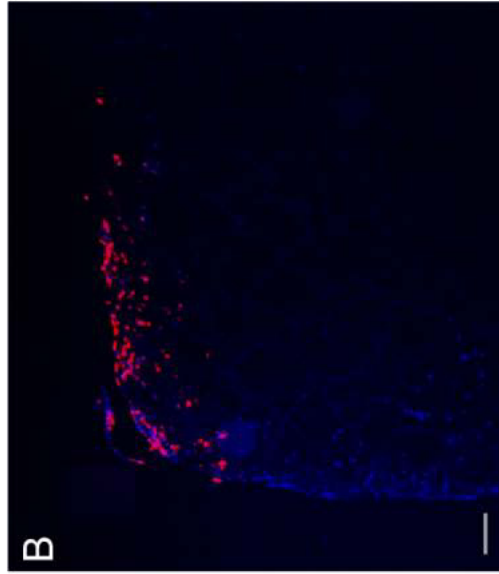
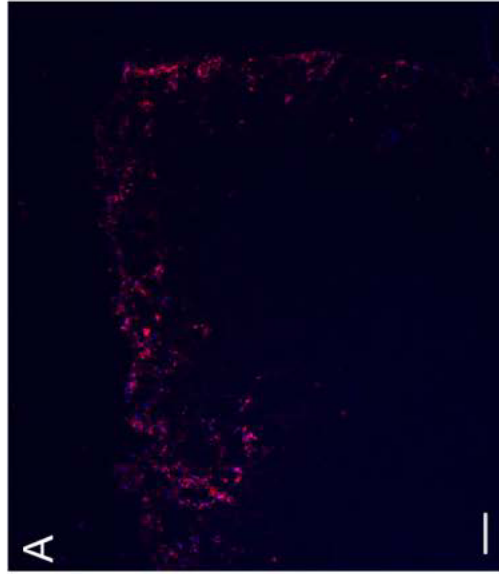
Fluorescent cell labelling was used to determine the location and distribution of seeded cells after four weeks in culture (Figure 4.2). In group 1 scaffolds, where all the cells seeded within the scaffold were labelled, the labelled MSCs are present around the four edges of the scaffold but not within the center of the scaffold (Figure 4.2A and D). The lack of cells in the center of the scaffold after four weeks in culture is likely to be due to the restricted nutrition in these areas, leading to cell death or migration of cells towards the edge of the scaffolds. The labelling may also be lost as a result of proliferation; however, MSCs only undergo limited proliferation in this scaffold system.

In group 2 and 3 scaffolds, the cells seeded on to the scaffold surface were labelled, whereas cells seeded within the scaffold itself were not (Figure 4.2B, C, E and F). In these two groups, the labelled cells clearly remain on the upper surface of the scaffold after twenty-eight days of culture. In group 2 and 3 control scaffolds (Figure 4.2B and C), the labelled cells are distributed throughout the surface layers of the scaffolds. However, in group 2 loaded scaffolds the distribution of labelled cells is restricted to the upper most layer of the scaffold surface with few detectable cells visible below this region (Figure 4.2E), but this was not replicated in group 3 loaded conditions (Figure 4.2F). The change in cell distribution between group 2 control and loaded scaffolds suggests that the application of multiaxial load modifies the distribution of cells seeded on the scaffold surface in this model.

Group 1

Group 2

Group 3



Control

Load

Figure 4.2

Representative fluorescent images showing the location of cells labelled with the membrane marker PKH26 cells after four weeks of multi-axial load. Cell membranes labelled with PKH26 appear red and cell nuclei, counterstained with DAPI, are blue. Images A, B and C show control scaffolds from groups 1, 2 and 3 respectively, whilst images D, E and F show the equivalent loaded scaffolds. Scale bar represents 200µm.

4.3.2 Safranin O and toluidine-blue staining of fibrin-poly(ester-urethane) scaffolds seeded with MSCs

In order to show the deposition of sulphated GAGs, scaffolds were stained with safranin O and toluidine blue (Figure 4.3 and 4.4). As expected, no staining was observed in groups 1, 2 or 3 control scaffolds (Figure 4.3 and 4.4A,C and F), however, moderate positive safranin O and metachromatic toluidine blue staining was present along the upper surface of group 1 loaded scaffolds (Figure 4.3 and 4.4B). Stronger positive staining was present in the same region of group 2 loaded scaffolds (Figure 4.3 and 4.4D), whilst no staining was observed in group 3 scaffolds (Figure 4.3 and 4.4F).

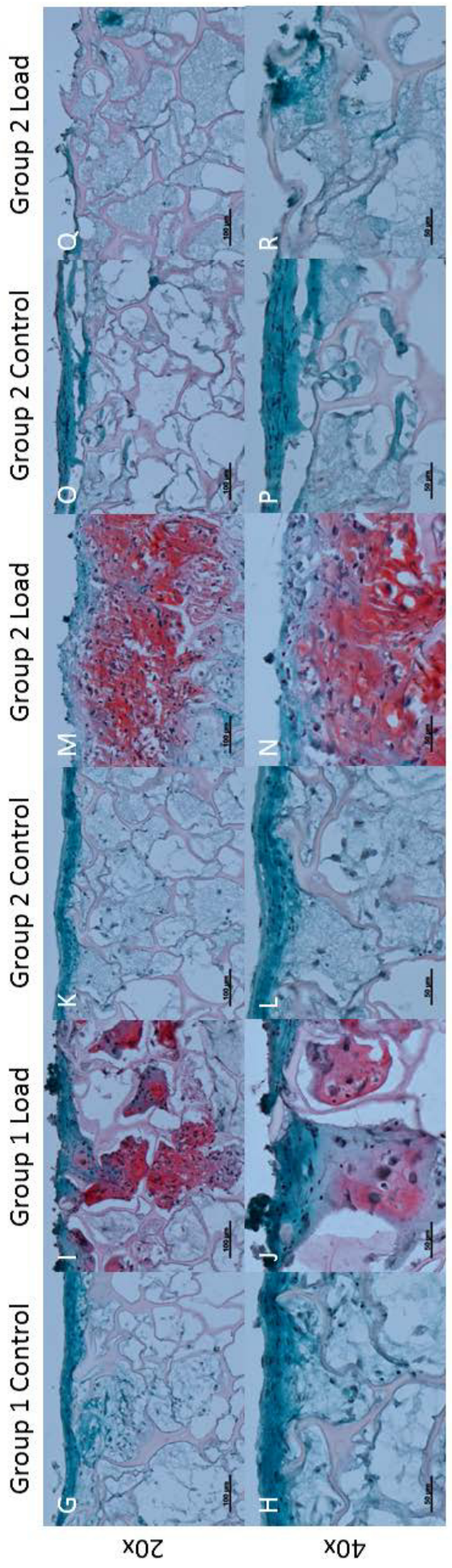
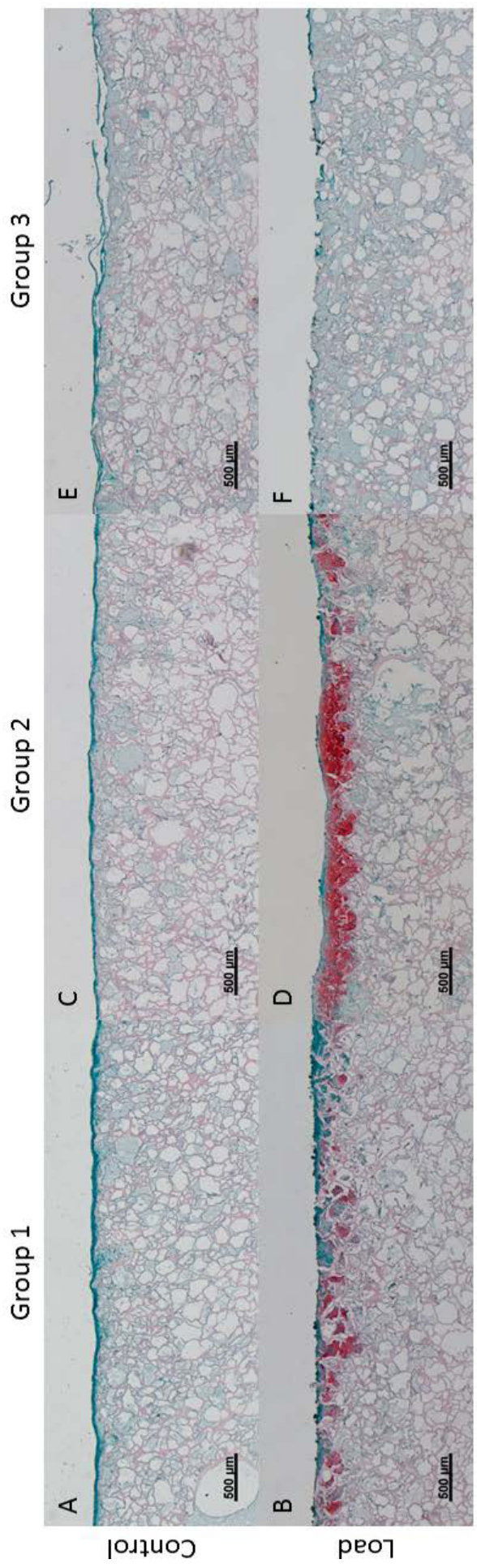
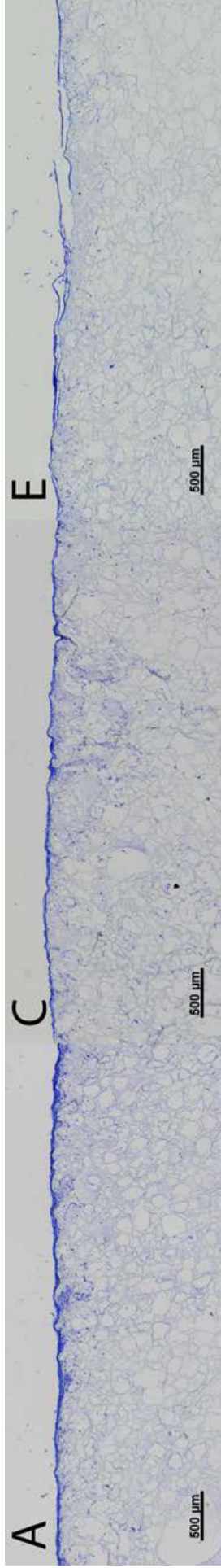


Figure 4.3

Representative images showing the surface of scaffolds stained with safranin O after four weeks of culture. Images A, C and E show control scaffolds from group 1, 2 and 3 respectively at 2.5x magnification whilst images B, D and F show loaded scaffolds at 2.5x magnification. Images G, I, K, M, O and Q show group 1 control, group 1 load, group 2 control, group 2 load, group 3 control and group 3 load respectively at 20x magnification. Images H, J, L, N, P and R show group 1 control, group 1 load, group 2 control, group 2 load, group 3 control and group 3 load respectively at 40x magnification.

Control



Group 1

Group 2

Group 3

Load

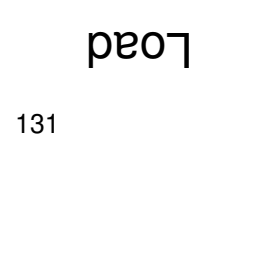
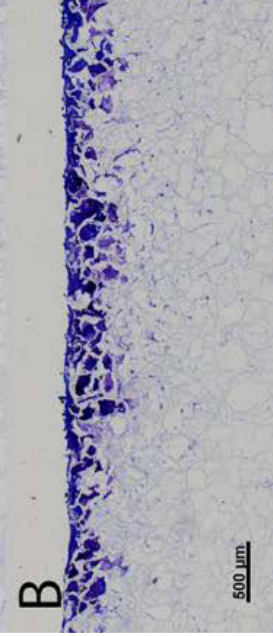
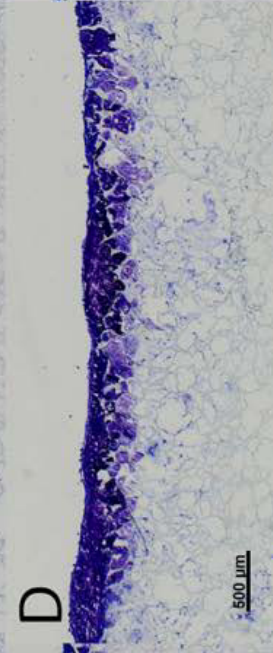
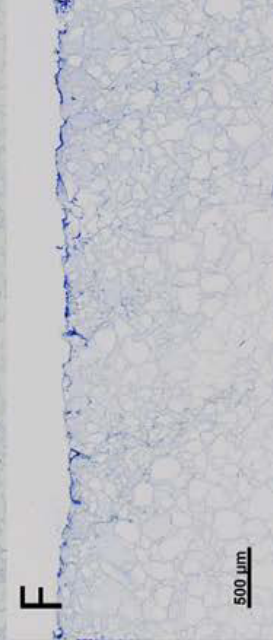
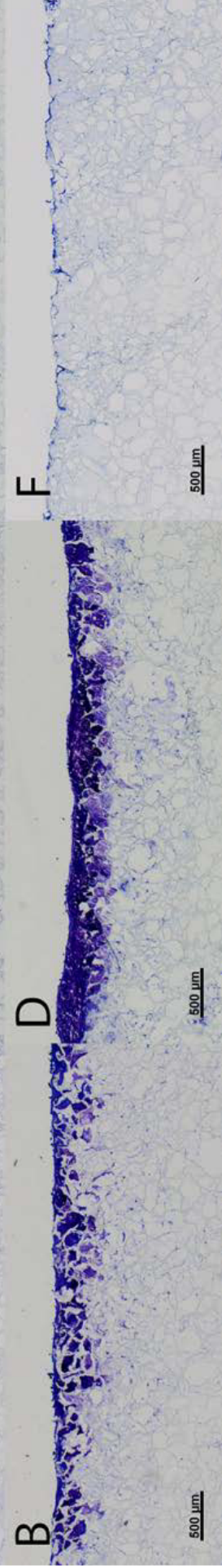


Figure 4.4

Representative images showing the surface of scaffolds stained with toluidine blue after four weeks of culture. Images A, C and E show control scaffolds from groups 1, 2 and 3 respectively, whilst images B, D and F show loaded scaffolds.

4.3.3 Immunohistochemical labelling of fibrin-poly(ester-urethane) scaffolds

All immunohistology was performed simultaneously on all groups within each biological repeat for all target epitopes.

Immunohistochemistry showed that collagen type II was not detected in any of the control scaffolds (Figure 4.5A, C, E). However, collagen type II was detected in group 1 (Figure 4.5B) and group 2 loaded scaffolds (Figure 4.5D); no staining was present in group 3 loaded scaffolds (Figure 4.5F). Positive labelling of collagen type II in group 1 and 2 loaded scaffolds was much less diffuse than the staining for collagen type I and is observed particularly on the loaded surface of the scaffolds, in areas that also stained positively with safranin O and toluidine blue. The staining was patchy in group 1 loaded scaffolds and stronger in group 2 loaded scaffolds, matching the positive staining detected with safranin O and toluidine blue.

Collagen type VI stained positively in both control and loaded scaffolds in all three groups (Figure 4.6) in a pattern very similar to that observed for collagen type I (Figure 4.8). Positive labelling was detected around the edges of group 1 (Figure 4.6A-B) and 2 scaffolds (Figure 4.6C-D) and increased with load (Figure 4.6B, D). Deposition of collagen type VI in group 3 was higher in control scaffolds (Figure 4.6E) compared to those that had been subjected to load (Figure 4.6F).

The antibody used for collagen type X staining reacted non-specifically with the fibrin component of the scaffolds meaning that positive staining occurred in both control and loaded scaffolds across all three groups (Figure 4.7). Sequence alignment of the Collagen type X alpha-1 chain with α , β and γ fibrinogen chains was determined using a protein BLAST search. The collagen type X alpha-1 chain share no sequence homology with the fibrinogen β or γ chains, however, two matches were found with the fibrinogen α chain, one of 12 residues with a 42% identity match and 75% similarity match and one of 18 residues with 44% identity match and 50% similarity match. These similarities in sequence may explain the non-specific staining of the fibrin component of the scaffold, however, no further investigation was performed. Areas in group 1 scaffolds (Figure 4.7B), and particularly group 2 loaded scaffolds that stained positively for sulphated GAG and collagen type II do not stain for collagen type X (as indicated in Figure 4.7D).

In contrast to collagen type II, collagen type I was deposited around the edges of both control and loaded scaffolds in all three groups (Figure 4.8), in a pattern that matched the distribution of cells within the scaffolds as observed when imaging fluorescently labelled cells (Figure 4.2). In both group 1 and 2 there was an increase in collagen type I deposition in response to mechanical load (Figure 4.8B, D). However, in group 3 the deposition of collagen type I was higher in control scaffolds (Figure 4.8E), as had been

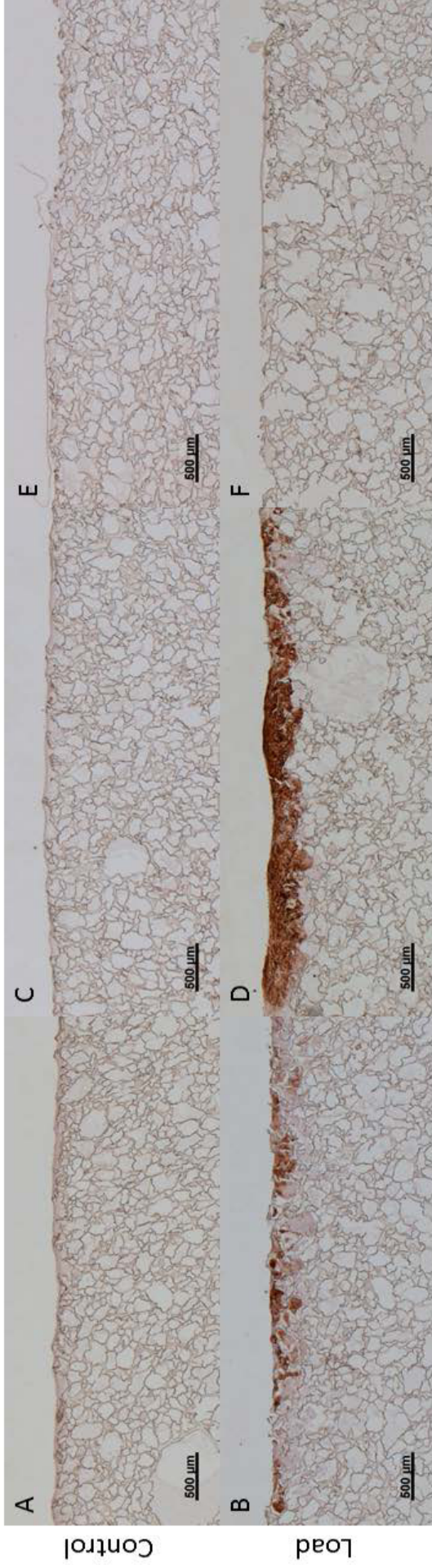
noted for type VI collagen deposition. Collagen type I expression was distributed in a similar manner in group 1 and 2 loaded scaffolds but the labelling intensity was higher in group 2 scaffolds.

Positive immunostaining for TGF- β 1 was not observed in any of the scaffold groups, but staining did occur in the bone region of the glenoid ligament enthesis section used as positive control tissue (data not shown).

Group 3

Group 2

Group 1



Group 2 Load

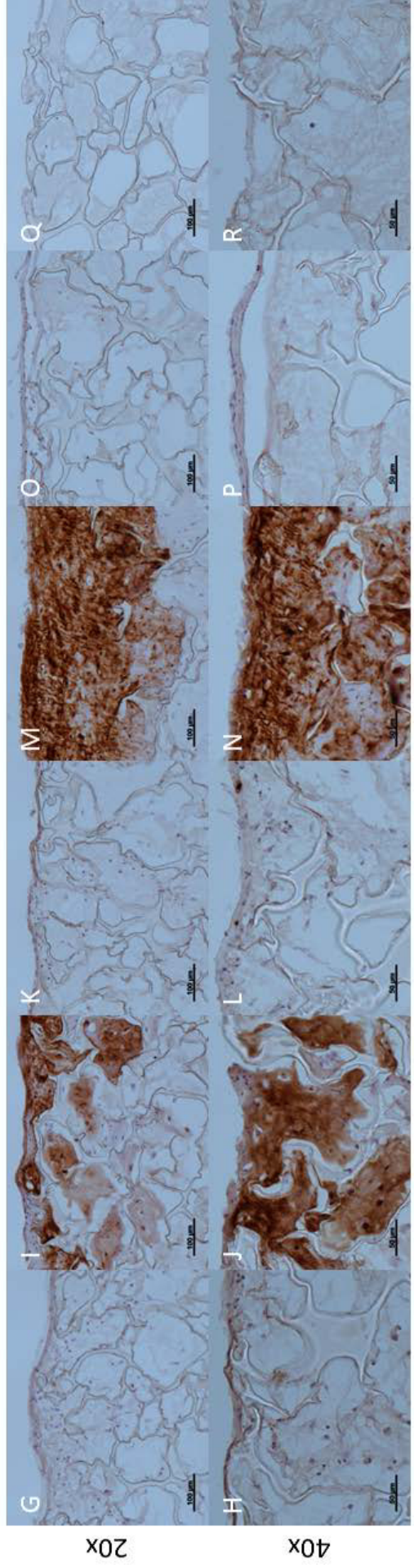
Group 2 Control

Group 2 Load

Group 2 Control

Group 1 Load

Group 1 Control



20x

40x

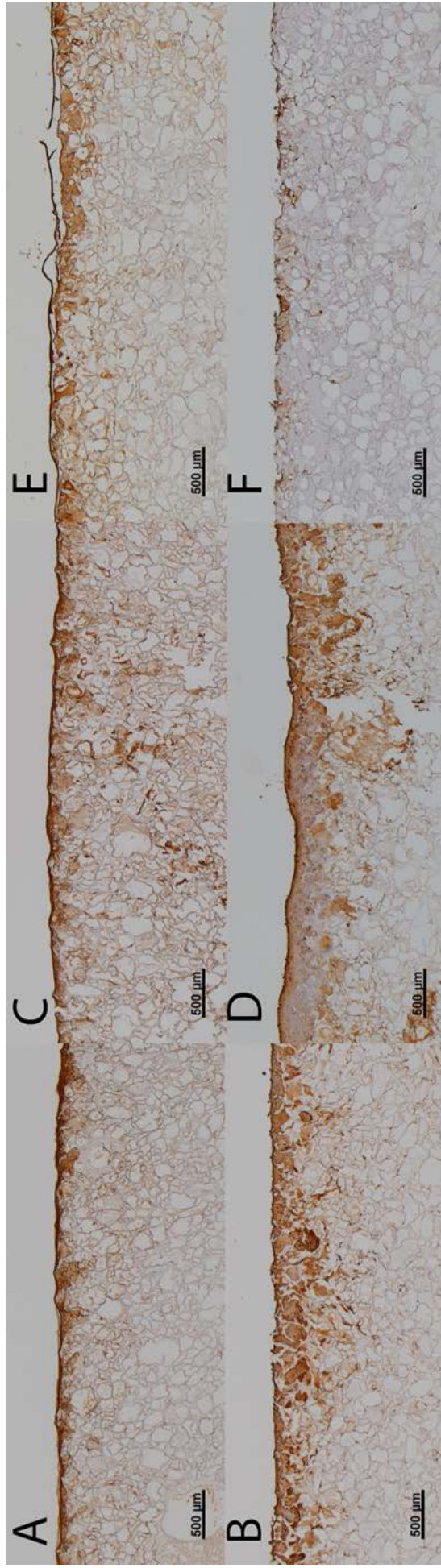
Figure 4.5

Representative images showing the surface of scaffolds stained immunohistochemically for collagen type II after four weeks of culture. Images A, C and E show control scaffolds from group 1, 2 and 3 respectively at 2.5x magnification whilst images B, D and F show loaded scaffolds at 2.5x magnification. Images G, I, K, M, O and Q show group 1 control, group 1 load, group 2 control, group 2 load, group 3 control and group 3 load respectively at 20x magnification. Images H, J, L, N, P and R show group 1 control, group 1 load, group 2 control, group 2 load, group 3 control and group 3 load respectively at 40x magnification. Labelling was performed using the ClICl anti-collagen type II IgG antibody made in mouse, the secondary antibody was a biotinylated anti-mouse IgG antibody made in horse and the labelling was detected using ImmPACT DAB.

Group 3

Group 2

Group 1



Control

Load

Figure 4.6

Representative images showing the surface of scaffolds stained immunohistochemically for collagen type VI after four weeks of culture. Images A, C and E show control scaffolds from group 1, 2 and 3 respectively whilst images B, D and F show loaded scaffolds. Labelling was performed using the 5C6 anti-collagen type VI IgG antibody made in mouse, the secondary antibody was a biotinylated anti-mouse IgG antibody made in horse and the labelling was detected using ImmPACT DAB.

Group 3

Group 2

Group 1



Control

Load

Figure 4.7

Representative images showing the surface of scaffolds stained immunohistochemically for collagen type X after four weeks of culture. Images A, C and E show control scaffolds from group 1, 2 and 3 respectively whilst images B, D and F show loaded scaffolds. Arrows indicate the area of the scaffold rich in cartilage-like matrix that appear negative for type X collagen. Labelling was performed using the C7974 anti-collagen type X IgM antibody made in mouse, the secondary antibody was a biotinylated anti-mouse IgM antibody made in goat and the labelling was detected using ImmPACT DAB.

Control

Load

Group 1

Group 2

Group 3

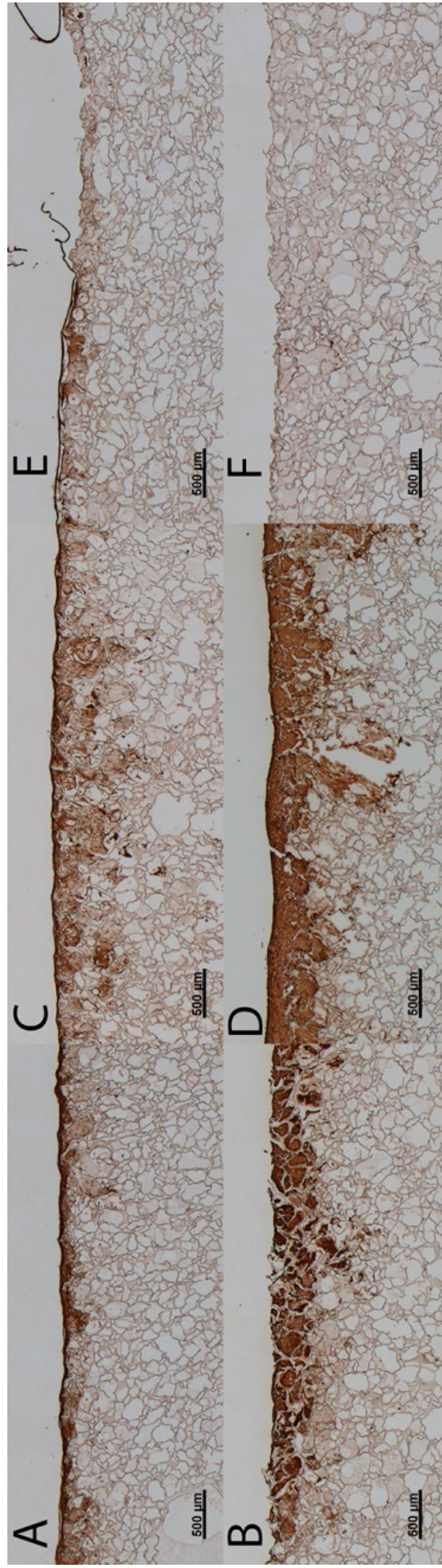


Figure 4.8

Representative images showing the surface of scaffolds stained immunohistochemically for collagen type I after four weeks of culture. Images A, C and E show control scaffolds from group 1, 2 and 3 respectively whilst images B, D and F show loaded scaffolds. Labelling was performed using the COL-1 anti-collagen type I IgG antibody made in mouse, the secondary antibody was a biotinylated anti-mouse IgG antibody made in horse and the labelling was detected using ImmPACT DAB.

4.3.4 Quantification of GAG and DNA content in fibrin-poly(ester-urethane) scaffolds and release into culture media

The application of multiaxial load to MSC laden fibrin-poly(ester-urethane) scaffolds had no effect on the DNA content of scaffolds within individual groups, irrespective of cell seeding distribution, after the four week culture period (Figure 4.9A).

There were no significant differences detected in the production of GAG by embedded MSCs in response to mechanical load (Figure 4.9B), although, in group 1 and 2, there was a trend towards increased total GAG in response to load. There was a significant difference between the total GAG production of both control and loaded scaffolds in groups 1 and 2 compared to group 3 scaffolds, both control and loaded ($P < 0.05$) as would be expected given the different seeding patterns in the different groups.

Calculation of the GAG/DNA ratio of each group showed an increased ratio in group 1 and 2 loaded scaffolds but this was not significant (group 1 control mean value $0.038 \text{ mg}/\mu\text{g} \pm 0.013$; group 1 load $0.051 \text{ mg}/\mu\text{g} \pm 0.014$, group 2 control $0.052 \text{ mg}/\mu\text{g} \pm 0.022$ group 2 load $0.056 \text{ mg}/\mu\text{g} \pm 0.017$) (Figure 4.9C). However, the ratio of GAG/DNA of group 2 loaded scaffolds was significantly higher than the ratio in group 3 loaded scaffolds ($P = 0.038$).

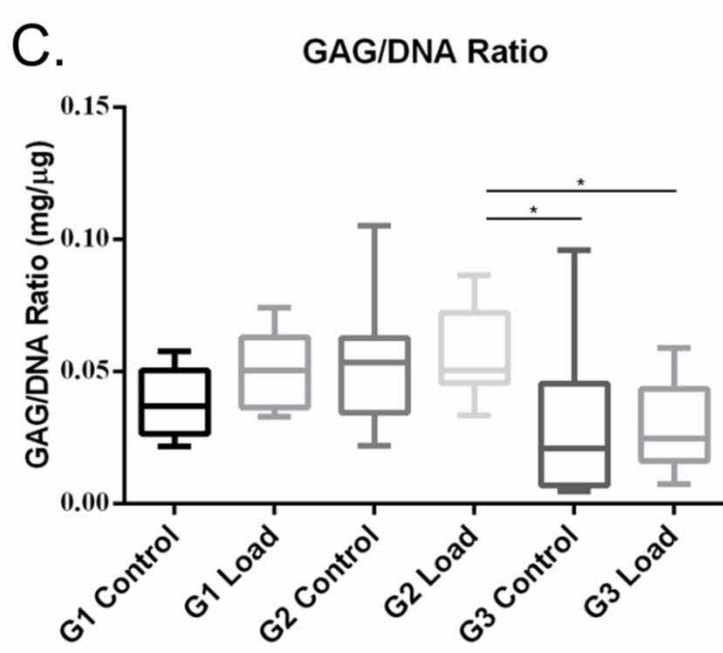
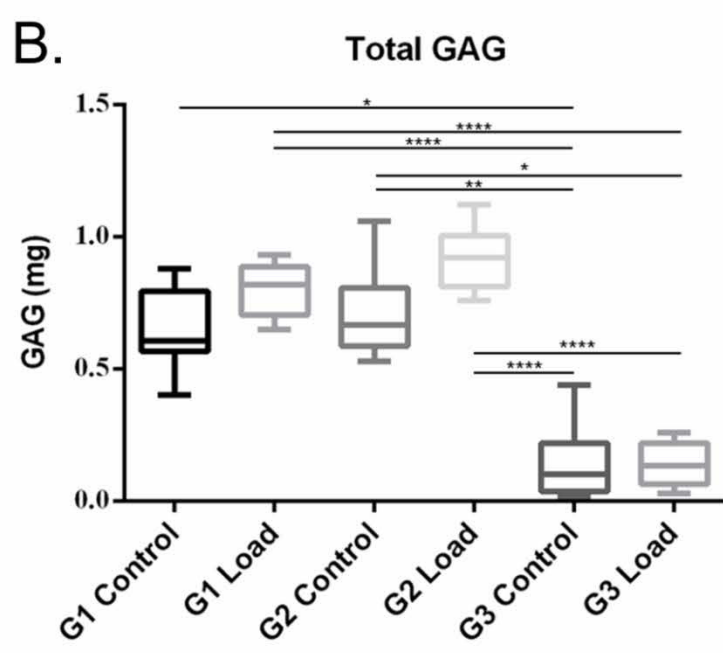
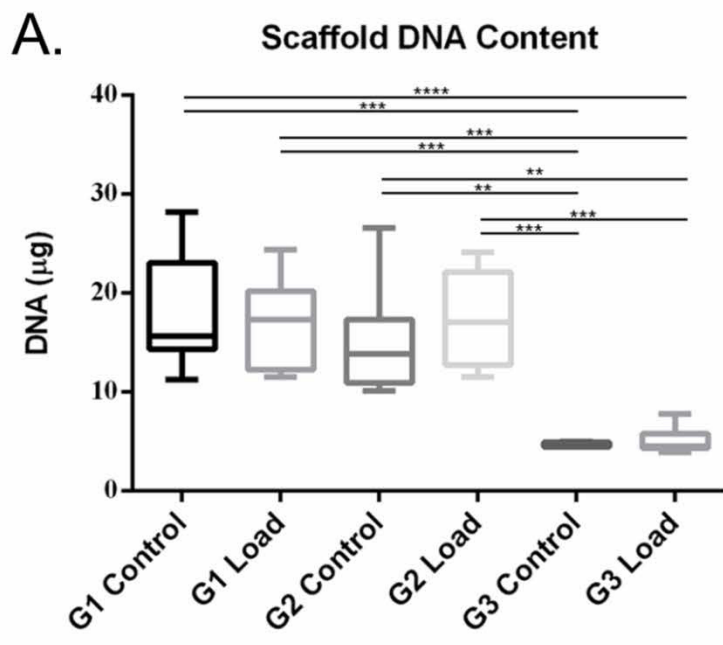


Figure 4.9

Biochemical analysis of MSCs seeded into fibrin-poly(ester-urethane) scaffolds after four weeks in culture. (A) Höchst 33528 dye was used to quantify the DNA in proteinase K digests of scaffolds. These data demonstrate the effect of even and asymmetric seeding patterns within scaffolds. (B) DMMB was used to determine the total amount of sulphated GAG produced by MSCs from both the collected culture media and proteinase K scaffold digests. (C) The GAG/DNA ratio was calculated from total DNA and GAG values to show the production of GAG relative to the MSCs present in each group. These figures represent data collected from four repeats of the experiment, each carried out in triplicate, with four different MSC donors. Statistical significance was defined as $P \leq 0.05$ and determined using the Kruskal-Wallis and Dunn's multiple comparison tests. * represents $P \leq 0.05$, ** represents $P \leq 0.001$ and *** represents $P \leq 0.0001$.

4.3.5 Gene expression profiles of MSCs seeded in to fibrin-poly(ester-urethane) scaffolds after seven days of culture

At day 7 real-time PCR was used to determine the relative gene expression levels of the chondrogenic marker Sox9, the marker of hypertrophy collagen type X and the fibroblast marker collagen type I. The results were normalised to the gene expression of cells on day 0 using the $\Delta\Delta C_t$ method, the housekeeping gene used was 18s.

There was no significant difference in the expression of Sox9 mRNA at day seven, with all groups demonstrating decreased expression compared to cells in monolayer at day 0 (Figure 4.10A), despite a trend towards an increase in group 1 loaded compared to control MSCs.

The expression of type X collagen was significantly increased in groups 1 and 3 loaded groups when compared to their respective control groups ($P < 0.05$; Figure 4.10B).

Quantitative analyses demonstrated that cells in control scaffolds expressed collagen type X at a lower level than monolayer cells (the mean fold-upregulation compared to day 0 for group 1, 2 and 3 respectively were 0.2504, 0.3567 and 1.4224), whilst loaded scaffolds showed a higher level of expression than monolayer cells (mean values 2.802, 1.864 and 3.011).

At day 7, there was no significant difference in collagen type I expression in response to load within any of the groups (Figure 4.10C). The mean fold-upregulation of collagen type I expression ranged from 0.7372 (a 1.35 fold-down regulation) to 2.133 compared to MSCs at day 0 showing only a slight variation in expression from monolayer cells. This suggests that the culture system used in this work has little effect on the production of collagen type I at day seven.

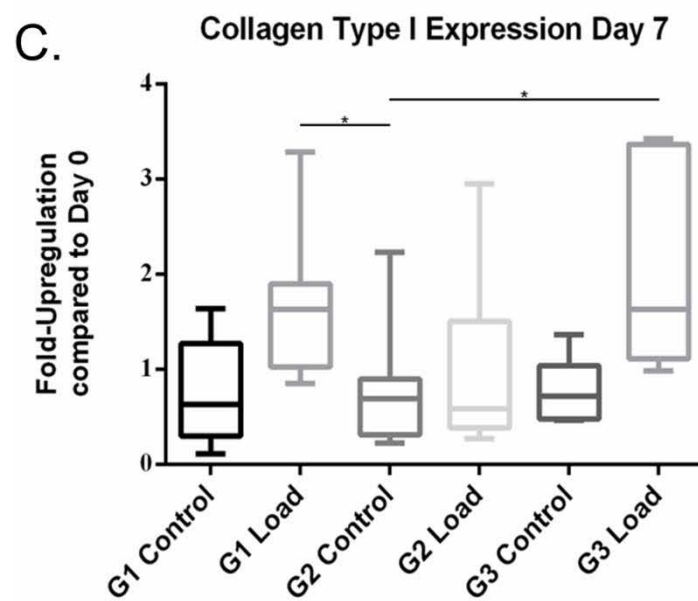
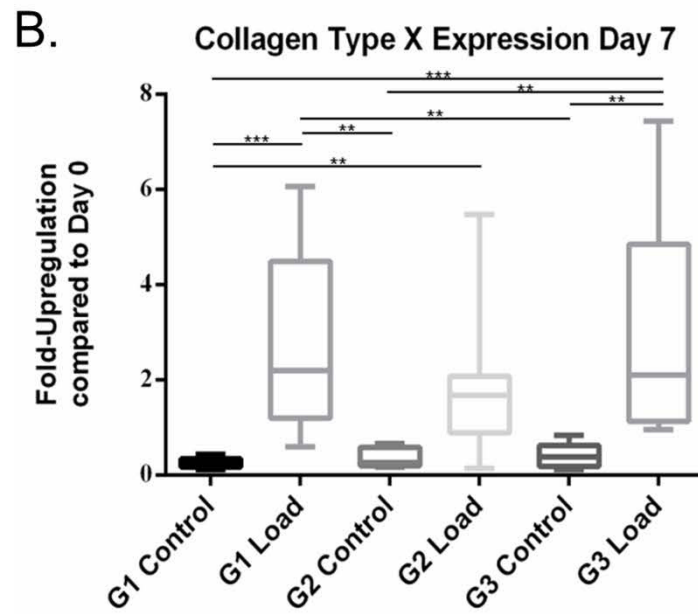
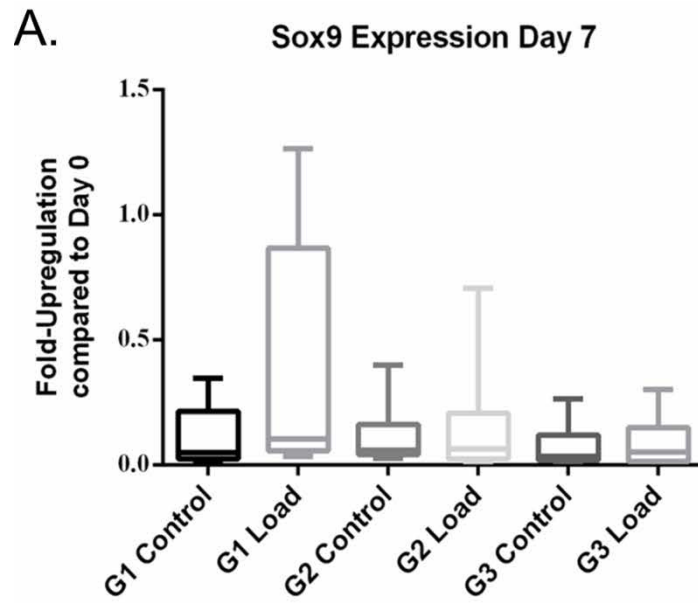


Figure 4.10

Analysis of chondrogenic marker transcript levels in MSCs at day 7 of culture, demonstrating the effect of even and asymmetric seeding patterns within scaffolds as determined by real-time PCR, (A) Sox9 (B) Collagen type X (C) Collagen type I. These figures represent data collected from three repeats of the experiment, each carried out in quadruplicate, with three different MSC donors. Statistical significance was defined as $P \leq 0.05$ and determined using the Kruskal-Wallis and Dunn's multiple comparison tests. * represents $P \leq 0.05$, ** represents $P \leq 0.001$ and *** represents $P \leq 0.0001$.

4.3.6 Gene expression profiles of MSCs seeded in to fibrin-poly(ester-urethane) scaffolds after twenty-eight days of culture

At day 28 real-time PCR was used to determine the relative gene expression levels of the chondrogenic markers aggrecan, Sox9 and collagen type II, the pericellular matrix marker collagen type VI, the fibroblast marker collagen type I, the marker of hypertrophy collagen type X and the osteoblast marker Runx2. The results were normalised to the gene expression of cells on day 0 using the $\Delta\Delta C_t$ method, the housekeeping gene used was 18s.

Mechanical load induced aggrecan transcription in group 1 loaded MSCs compared to the unloaded MSC scaffolds at day 28 ($P=0.018$; Figure 4.11A); a trend towards increased aggrecan expression was also observed in group 2 in response to load, but this did not reach statistical significance. Group 1 loaded scaffolds showed a significantly higher level of expression than group 1 control scaffolds ($P<0.018$). Aggrecan expression was down regulated when compared to cells at day 0 in all groups except group 1 and 2 load (the mean fold-upregulation values compared to day 0 were 15.86 and 3.960 respectively).

There was also a significant increase in Sox9 expression between group 1 and 2 loaded MSCs and their respective controls ($P<0.05$; Figure 4.11B). However, there was no increase in Sox9 transcription in MSCs subjected to load in group 3. Furthermore, there was no significant difference in Sox9 mRNA levels between loaded scaffolds in group 1 and group 2. Sox9 expression was similar to day 0 in all groups except loaded group 1 and 2 MSCs (the mean fold-upregulation values compared to day 0 were 5.053 and 3.520 respectively).

Collagen type II expression could not be detected in control groups, only in loaded groups. As a result, collagen type II expression was normalised to group 1 loaded scaffolds, as this represents the work previously carried out in this system and therefore was the most appropriate loaded group (collagen type II expressing group) to normalise to in the absence of expression in day zero or control scaffolds (Figure 4.11C). There was significantly higher expression in group 2 loaded scaffolds compared to group 3 loaded scaffolds ($P=0.004$, Figure 4.11C). The mean fold up-regulation of group 2 scaffolds was 1.163, which shows that the expression was similar to group 1 loaded scaffolds, group 3 loaded scaffolds, however, showed decreased expression compared to group 1 load (which represents a 4-fold decrease in expression).

The expression of collagen type VI was very similar in all groups and was also similar to the expression observed at day 0 (Figure 4.11D). There was a trend towards an increase in group 3 control and load compared to group 1 and 2, but significance was

only observed for the comparison between control MSCs in group 1 versus group 3 ($P=0.030$; Figure 4.11D). ($P=0.032$ and 0.030 respectively)

As at day 7, there was a trend towards increased collagen type I expression with load on day 28; however, there were no significant increases within groups when comparing load and control (Figure 4.11E). There were also no significant differences detected in collagen type I expression as a result of the different seeding patterns used in group 1-3 in loaded or control scaffolds. All groups showed a down-regulation of expression compared to day 0.

The pattern of expression of collagen type X was similar at both days 7 and day 28. There was a clear and significant increase in the expression of type X collagen in response to load within all three groups relative to their respective unloaded controls ($P<0.001$, $P<0.001$ and $P=0.031$ respectively; Figure 4.11F). The expression of collagen type X in control groups was similar to day 0 (the mean fold-upregulation values compared to day 0 were 1.299, 0.6267 (a 1.6-fold decrease) and 1.167 in group 1, 2 and 3 respectively), however, the expression was much higher in loaded scaffolds (the mean fold-upregulation values compared to day 0 were 299.9, 216.0 and 82.00 in group 1, 2 and 3 respectively). The fold change in mRNA expression observed for collagen type X in response to load is greater than the fold changes detected in the other collagens at day twenty-eight. However, within the loaded groups the mean Ct values of the other collagens, particularly collagens type I and II are lower than those of collagen type X suggesting greater abundance of mRNA for these other collagen types (Table 4.1).

As with the other genes analysed, there was an apparent trend of a load-induced increase in Runx2 mRNA expression, but this was not statistically significant (Figure 4.11G). However, the expression of Runx2 was increased in all groups compared to day 0 (mean-fold changes ranged between 2.154 and 3.870). When the Runx2/Sox9 mRNA ratio was calculated, a clear load-dependent reduction was visible in group 1, but this did not reach statistical significance (Figure 4.11H).

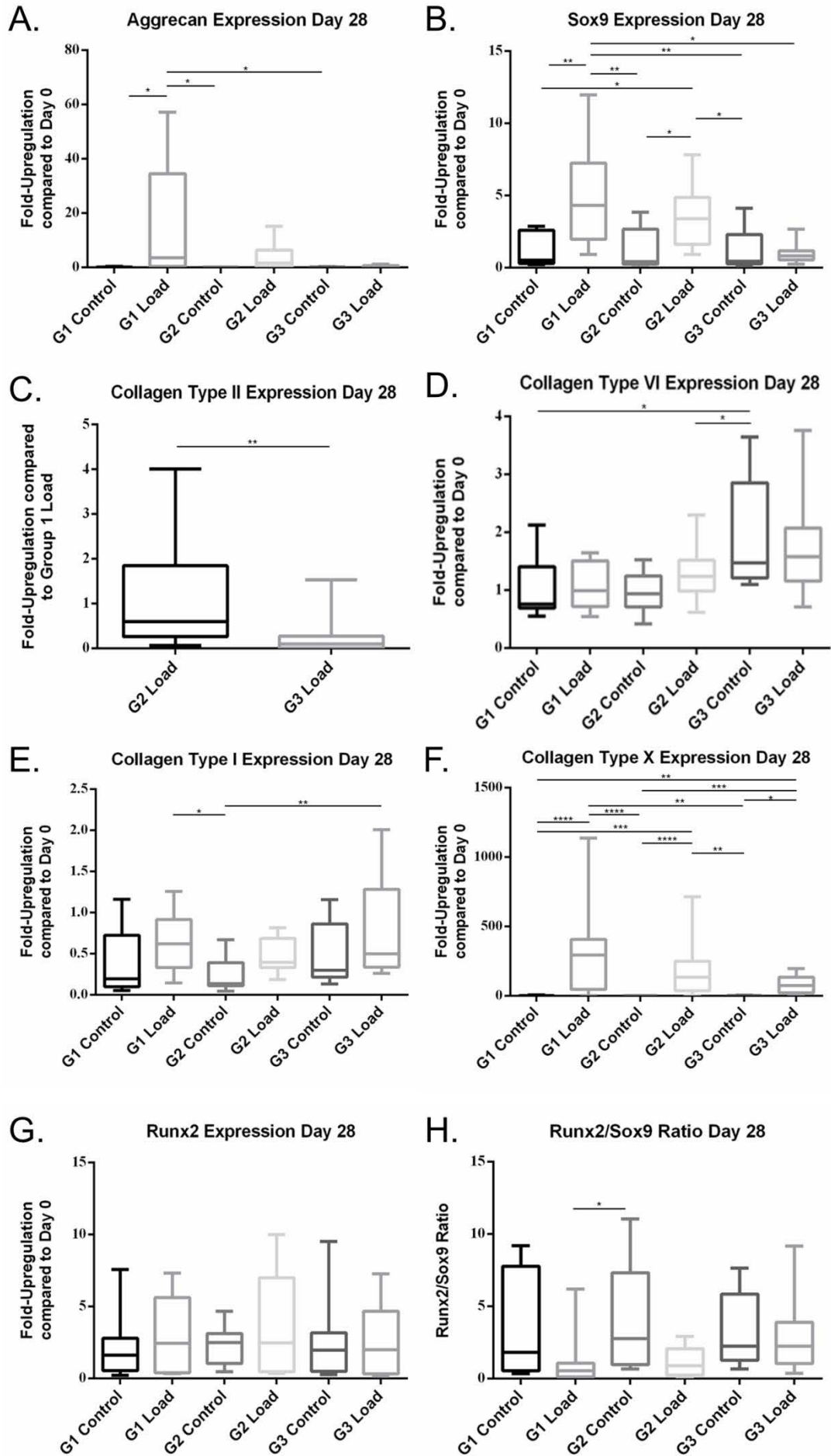


Figure 4.11

Relative quantification of gene expression measured at day 28 of culture by real-time PCR, demonstrating the effect of even and asymmetric MSC seeding patterns within fibrin-poly(ester-urethane) scaffolds. (A) Aggrecan (B) Sox9 (C) collagen type II (D) collagen type VI (E) collagen type I (F) collagen type X (G) Runx2 (H) Runx2/Sox9 ratio. These figures represent data collected from three repeats of the experiment, each carried out in quadruplicate, with three different MSC donors. Statistical significance was defined as $P \leq 0.05$ and determined using the Kruskal-Wallis and Dunn's multiple comparison tests. * represents $P \leq 0.05$, ** represents $P \leq 0.001$ and *** represents $P \leq 0.0001$.

	Mean Ct Value and Standard Deviation		
	Collagen Type I	Collagen Type II	Collagen Type X
Group 1 Load	21.86 ± 1.07	22.90 ± 5.53	26.90 ± 2.01
Group 2 Load	20.89 ± 0.73	21.94 ± 5.99	26.31 ± 2.53
Group 3 Load	23.52 ± 1.36	28.73 ± 4.24	30.07 ± 2.06

Table 4.1

The mean Ct values for collagen types I, II and X expressed by MSCs cultured for 28 days in fibrin-poly(ester-urethane) scaffolds and subjected to load. The lower Ct values for collagen type I and II suggest greater amounts of RNA for these two proteins were present in the MSCs analysis, despite the greater increase in collagen type X expression calculated by the $\Delta\Delta\text{Ct}$ analysis. The values represents data from three different biological repeats each consisting of three technical repeats.

4.3.7 Quantification of the total and active TGF- β 1 in collected culture media

The total and active TGF- β 1 was quantified in collected culture media using an ELISA. The data presented here represents the absolute concentration of total TGF- β 1 within the culture media in ng/ml and percentage of the total TGF- β 1 content that was in an active form .

During the first week of culture, there was a trend towards increased amounts of TGF- β 1 released into the media from both group 1 and group 2 MSC scaffolds subjected to load compared to their respective controls, this however was not significant (Figure 4.12A). Media collected in the second week of culture showed a significant increase in TGF- β 1 measured in loaded group 1 MSC scaffolds versus its respective control ($P=0.026$; Figure 4.12B). However, group 1 and group 2 loaded samples were not significantly different to each other. There was not a significant difference between group 2 control and group 2 loaded scaffolds, despite a trend towards an increase with load.

Week 3 media collected from group 1 loaded MSC scaffolds contained significantly more TGF- β 1 than group 1 control scaffolds ($P=0.050$; Figure 4.12C). There was also a clear trend towards increased TGF- β 1 in response to load in group 2, but due to large standard deviations this was not significant at the 5% level.

Analysis of week 4 media showed that there was increased release of TGF- β 1 into the media in group 1 and 2 in response to load, however, none of the intragroup changes reached significance at the 5% level ($P=0.104$, 0.370 and 0.999 for group 1, 2 and 3 respectively, Figure 4.12D).

As well as analysing the total amount of load-induced TGF- β 1 released into the media, the percentage of active TGF- β 1 was also quantified (Figure 4.12E). The results from week 1 media show that there was a significant increase in active TGF- β 1 detected in the media in group 1 and 3 loaded samples compared to their respective controls ($P<0.0001$ and 0.0032 respectively). There was also a trend towards an increase in group 2 loaded MSC scaffolds compared to control but this was not significant ($P=0.051$) due to the large standard deviation for the loaded values. In weeks 2, 3 and 4 media, the results show that the amount of active TGF- β 1 was significantly higher in all three of the loaded groups compared to their three respective control groups ($P<0.05$); a very low percentage of active TGF- β 1 was observed in the control MSC scaffolds over time in culture. There was no significant difference between loaded scaffolds across the three seeding groups or between control scaffolds across the three groups (Figure 4.12F-H).

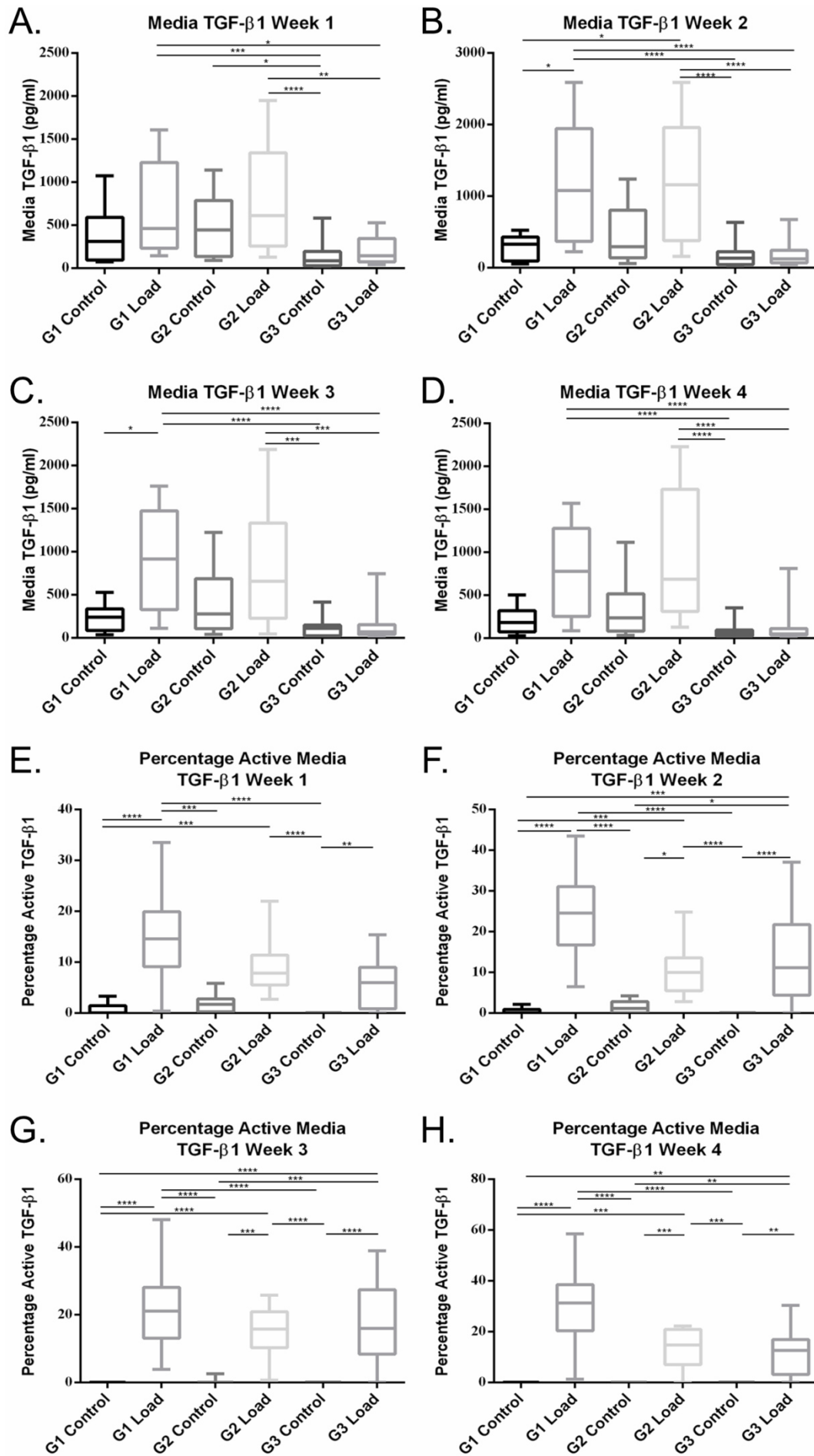


Figure 4.12

Quantification of the total and active TGF- β 1 content of media collected during each week of culture from MSCs in fibrin-poly(ester-urethane) scaffolds subjected to load; unloaded scaffolds served as controls. This data shows the effect of load on TGF- β 1 production and activation in groups with different cell seeding patterns. Media collected from each sample at the three media changes performed each week was pooled and frozen before ELISA quantification. (A-D) Total media TGF- β 1 content measured at each week during culture. (E-H) Active TGF- β 1 content of media collected over the course of culture. The data represents the absolute total and active TGF- β 1 content of the media that was analysed. Statistical significance was defined as $P \leq 0.05$ and determined using the Kruskal-Wallis and Dunn's multiple comparison tests. * represents $P \leq 0.05$, ** represents $P \leq 0.001$ and *** represents $P \leq 0.0001$.

4.4 Discussion

Previous work has shown that when multiaxial shear and compression loading was applied to MSCs seeded within fibrin-poly(ester-urethane) scaffolds, the cells undergo chondrogenesis in the absence of any exogenous growth factors, specifically TGF- β (Li et al., 2010a, Li et al., 2010b, Kupcsik et al., 2010, Neumann et al., 2013). This was characterised by the up-regulation of genes associated with chondrogenesis, such as collagen type II and aggrecan, as well as the deposition of cartilage like matrix (containing collagen type II and sulphated GAG) within the scaffolds themselves (Li et al., 2010a). This chondro-induction was shown to be driven by endogenously produced TGF- β 1 and could be blocked using the TGF- β receptor 1 (ALK5) inhibitor LY364947 (Li et al., 2010a). The induction of collagen II expression is a particularly powerful marker of chondrogenesis, as aggrecan and Sox9 were often found in monolayer expanded MSCs used as a day 0 controls for real-time PCR analysis, whilst collagen II was almost never detected under non-chondrogenic conditions. Further investigation showed that the shear component of the load was vital in the induction of chondrogenesis and this study set out to take advantage of that, and the potential signalling effects that were shown to occur between separate populations of MSCs in Chapter 3, to improve the amount of matrix deposition and chondro-induction in response to mechanical load. In order to do this, scaffolds were seeded asymmetrically with 10% of the total cell number on the loaded surface of the scaffolds in order to produce a population of cells that was directly exposed to the shear component of the load. Moreover, the absolute cell number remained the same for Groups 1 and 2. Alongside this, the media collected from samples during media changes was analysed for both total and active TGF- β 1 to investigate the effect of multiaxial mechanical load on TGF- β 1 activation.

Cells were labelled with a fluorescent dye and visualised after harvesting at 4 weeks in order to demonstrate that any effect observed was due to the MSCs seeded on the scaffold surface remaining there over the time in culture. MSCs seeded on the surface of control scaffolds are found to be distributed throughout the top 200-400 μ m of the scaffolds after four weeks of culture in both group 2 and 3, however in loaded group 2 scaffolds the cells appear to form a layer on the scaffold surface. The details of this phenomenon however not been studied further.

A reduction in cell number and decreased amounts of fibrin hydrogel was observed in the center of the scaffold compared to the outer areas of the scaffold after four weeks in culture. This is consistent with previous work and is not a result of heterogeneous seeding, as histology performed in the first weeks of culture indicated a homogenous distribution of cells and hydrogel throughout the scaffold (Zahedmanesh et al., 2014).

The changes in cell distribution appear instead to occur in the later stages of culture. These changes in distribution may result from the poor diffusion of nutrients and ϵ -aminocaproic acid (EACA) into the center of the scaffold leading to fibrin degradation and potentially cell death; however this has not been investigated.

The formation of a cell layer on top of loaded group 2 scaffolds suggests that the MSC cells on the surface potentially act as a separate population from the cells that are near, rather than on the surface of the scaffold, which despite their proximity remain in a 3D environment within the fibrin hydrogel. The distribution of the cells seeded on the surface of group 2 loaded scaffolds is restricted to the very surface of the scaffold. The position of the cells on the surface may have resulted in the increased polarisation of these cells compared to MSCs seeded within the hydrogel itself. The polarisation of the cells may lead to a change in their morphology and cytoskeletal organisation which may in turn affect the phenotype of the cells and their response to mechanical load, in particular shear (Benya and Shaffer, 1982, Wang et al., 1993). The distinction, and possible interaction between these two populations may explain the histological differences between groups 1 and 2.

Results of safranin O and toluidine blue staining shows that there is a clear increase in the deposition of sulphated GAG in group 1 and 2 loaded MSC scaffolds compared to their respective controls. Staining also improved in group 2 loaded scaffolds compared to group 1 loaded scaffolds. This was also true for collagen type II. Increased matrix deposition in group 2 loaded scaffolds compared to group 1 loaded scaffolds may result from interaction between the population of cells seeded on the scaffold surface with the cells seeded within the fibrin hydrogel. This is supported by the lack of staining in group 3 scaffolds, which demonstrates that the layer of surface cells alone is not able to deposit cartilage-like matrix in a manner similar to that seen in group 2, this therefore suggests that the interaction between the two populations is key in the increased deposition of matrix seen in group 2. The increased staining for GAG and collagen type II labelling was consistently seen using multiple donors. Collagen type X deposition also appears lower in areas of matrix that stain positively for sulphated GAG and collagen type II in group 2 compared to group 1. However, this interpretation requires caution due to the non-specific labelling of the fibrin scaffold by the primary antibody and may just appear clearer in group 2 loaded scaffolds due to the larger areas of matrix compared to group 1 loaded scaffolds. The labelling of collagens type I and VI also clearly increases with load in group 1 and 2, although more so in group 2, whilst expression is lower in group 3 load compared to its respective control. The increased expression of collagen type I has been previously described in response to load in this system (Li et al., 2010a, Schatti et al., 2011), collagen type I expression has also been noted in MSCs loaded in other systems (Huang et al., 2004, Angele et al.,

2004). These data show the clear chondrogenic effect of load on MSCs in this system and the improvement in matrix deposition that can be achieved with an asymmetric distribution of cells with the scaffolds.

Isoforms of TGF- β are commonly used to induce chondrogenesis in MSCs *in vitro* and have been shown to be responsible for the induction of chondrogenesis in mechanically stimulated cells (Huang et al., 2004, Li et al., 2012, Li et al., 2010a, Huang et al., 2005). Therefore, the amount of total TGF- β 1 and active TGF- β 1 was quantified in culture media to investigate possible correlation between the presence of this chondrogenic stimulus and any changes observed as a result of asymmetric seeding of scaffolds. The results of this work show that the application of load in group 1 and 2 scaffolds led to an increase in the amount of total TGF- β 1 within the culture media. Significant differences were observed in response to load within group 1 scaffolds. However, due to higher standard deviations in the amount of TGF- β 1 quantified in media collected from group 2 control scaffolds, significant differences were not observed within group 2 or when compared to TGF- β 1 levels in group 1. This increased variability, and the increased basal expression levels in group 2 control scaffolds compared to group 1 scaffolds may be due to better nutrition of surface seeded cells or increased paracrine signalling by the higher density monolayer model. This led to a smaller increase over basal expression levels in group 2, which was not investigated further. The production of TGF- β 1 by MSCs in response to mechanical load has previously been shown under similar conditions by this group as well as by others (Li et al., 2010a, Li et al., 2012, Huang et al., 2005).

Quantification of the percentage of active TGF- β 1 in each sample show that in groups 1, 2 and 3 the percentage of active TGF- β 1 in each loaded group was significantly upregulated compared to their respective control groups between weeks two and four of culture (fig.1 E-H). This clearly shows that the application of joint like load in this model system not only leads to an increase in the total production of TGF- β 1 but also induces the activation of the latent TGF- β released by the MSCs. The activation of TGF- β 1 has previously been shown in rat bone marrow derived MSCs in response to uniaxial compression (Li et al., 2012), however to the authors knowledge this is the first time joint like load has been demonstrated to be able to induce the activation of endogenous TGF- β 1 produced by human bone marrow derived MSCs in response to multiaxial mechanical load.

Over the course of the four weeks in culture no significant differences were observed in the percentage of active TGF- β 1 between the three loaded groups, despite the different distributions of cells within the scaffolds of these groups; this is interesting

because the scaffolds of the three groups contained different numbers of cells and the cells in the different scaffolds groups were at different stages of chondrogenic differentiation. This consistency in the degree of TGF- β 1 activation, despite the variation in the cell populations within the scaffolds, suggests that the increase in the amount of active TGF- β 1 in response to load results from a physical activation. This finding is similar to that observed by others in response to mechanical load, particularly shear, in the absence of cells rather than as a result of enzymatic activation due to activity of the cells within the scaffold (Ahamed et al., 2008, Albro et al., 2012). The inclusion of the protease inhibitor 6-aminocaproic acid, in order to prevent fibrin degradation during culture, in the media also suggests that this activation is unlikely to be due to the activity of plasmin, a known TGF- β activator, as it is one of the enzymes inhibited by 6-aminocaproic acid (Kupcsik et al., 2009).

This finding suggests that the multiaxial loading, applied to the MSCs embedded in scaffolds in this study, is activating latent TGF- β 1 present in the culture media. The increase in TGF- β 1 production and activation in response to mechanical load in this system affirms the importance of mechanical loading of cartilage implants at an early stage of culture if cartilage-like repair tissue is to be produced.

Analysis of gene expression at day 7 of culture showed that there was a trend towards the up-regulation of collagen type I and the hypertrophy marker collagen type X in response to load when compared to non-loaded controls, but no significant differences were observed between the loaded groups. No significant difference was detected in response to load in the expression of Sox9, although results suggested a trend towards an increase with load in groups 1 and 2. The expression of collagen type X is well associated with the chondrogenic induction of MSCs and has previously been shown to be induced by load in this system (Johnstone et al., 1998, Li et al., 2010a, Schatti et al., 2011, Neumann et al., 2013).

Results of gene expression analysis at day 28 showed that load increases the expression of collagen type I in all three groups, but these differences did not reach statistical significance. The lack of significance may relate to the high variability between cells from different human donors, as a result relevant trends in the data have also been discussed in this section. Collagen type II expression was only observed in MSCs subjected to load, and similar mRNA levels were detected in groups 1 and 2; levels were significantly higher in group 2 compared to group 3. This suggests that load-induced transcription of collagen type II mRNA in MSCs may be one of the more sensitive chondrogenic markers compared to, for example, aggrecan which can be detected consistently in monolayer expanded MSCs. The expression of collagen type VI, which is found in the pericellular matrix surrounding chondrocytes *in vivo*, did not

change with load in groups 1, 2 or 3. The expression of type VI collagen does however appear elevated (not significantly) in group 3 control and loaded scaffolds compared to scaffolds in the other two groups in a similar way to collagen type I. Collagen type X expression was significantly increased in all three loaded groups in comparison to their respective controls, but there was no significant difference between the individual groups. In all three groups, there was a trend toward increased aggrecan production with load; however, only in group 1 did load significantly increase aggrecan transcription compared to the unloaded control. Group 1 and 2 loaded scaffolds expressed significantly higher amounts of Sox9 than their respective control groups; furthermore, significantly more Sox9 mRNA was detected in group 1 subjected to load when compared to the equivalent group 3 cells. Increased Sox9 expression in MSCs has previously been reported in response to compressive load alone (Huang et al., 2005, Campbell et al., 2006) and to the multiaxial load applied by the bioreactor described in this chapter (Schatti et al., 2011). The Runx2/Sox9 ratio has been shown to be a predictive marker of osteogenesis (Loebel et al., 2014). A trend towards a decrease in the Runx2/Sox9 ratio, indicating chondrogenesis, was observed in response to load in groups 1 and 2.

These gene expression results show that load has a chondrogenic effect in both group 1 and 2, whilst MSCs in group 3 scaffolds adopt a more hypertrophic phenotype. However, there was no significant difference in the expression of any of the genes tested between group 1 and 2 loaded scaffolds, although, there was a trend towards greater aggrecan and Sox9 expression in group 1. The results of biochemical analyses of scaffolds and media also demonstrated no significant difference between the production of GAG in response to load or between cell seeding in group 1 and 2 scaffolds.

The results presented here indicate that seeding a layer of MSCs on the surface of a fibrin-poly(ester-urethane) scaffold can improve the deposition of matrix in the area directly exposed to mechanical load but does not appear to impact on the gene expression of the cells within the scaffold or the scaffolds GAG/DNA ratio. The lack of any significant changes observed in loaded scaffolds in groups 1 and 2 in PCR and GAG/DNA data may be linked to the use of cells from two donors which responded strongly (in terms of matrix deposition detectable through the use of histological staining) to chondrogenic induction (female 18 years-old and male 22 years-old) and two donors that responded weakly (female 49 years-old, male 48 years-old). This led to a reduction in the overall differences observed between groups when the results from the four donors were collated, therefore generating a greater standard deviation and

subsequent lack of significance at the 5% level. While this deviation can be reduced by selecting or pooling donors, the frequency with which a result is observed cannot be determined (Stoddart et al., 2012). Previous studies have consistently shown significant increases in GAG/DNA in response to load by MSCs in this system (Li et al., 2010a, Kupcsik et al., 2010). Results of collagen type II histology and safranin O/toluidine blue staining indicate that chondrogenesis occurs in highly localised areas as a result of mechanical stimulation. This means that cells in other areas of group 1 and 2 scaffolds (but not group 3 where all the cells are exposed to load) are not expressing markers of MSC chondrogenesis (collagen type II, type X, aggrecan and Sox9), but they do produce less specific markers such as collagen type I and type VI as demonstrated immunohistochemically. The proteinase K digest and RNA isolation procedures used in this study isolated DNA and RNA from one half of a scaffold cut vertically in two and, therefore, contains DNA and RNA from cells undergoing differentiation (on the upper surface of the scaffold) as well as the non-chondrogenic cells expressing more general markers in the rest of the scaffold. A large proportion of the cells being analysed in group 1 and 2 scaffolds, therefore, are not responding to the chondrogenic stimulus but still contributing 18s rRNA and DNA to the measures used to normalise the RT-PCR and GAG data respectively. The effect of this would be to 'dilute' or mask the apparent up-regulation of genes (e.g. collagen type II, type X, aggrecan and Sox9) that were expressed in the small proportion of the total population that was responding to load by undergoing chondrogenesis. This would not prevent significant differences from being detected in chondrogenic markers between loaded samples and control samples as there is a large difference in gene expression between loaded scaffolds which are receiving chondrogenic stimulation and control scaffolds which receive no chondrogenic stimulation. However, this would mask the, relatively, more subtle differences between group 1 and 2 loaded scaffolds, producing similar results for both groups. It is of note that the differences were slight for aggrecan, which is expressed in monolayer expanded MSCs, whilst collagen type II was only found in Group 1 and 2 MSCs subjected to load.

If this masking was occurring for chondrogenic genes, then it would not be expected for the more generally expressed proteins such as collagen type I and collagen type VI which are expressed throughout the scaffolds. Collagen type I mRNA levels show a trend (not significant) towards an increase in production with load in group 1 and 2 loaded scaffolds compared to controls, which was also observed by immunohistochemistry. No such trend is clear in collagen type VI gene expression, but again, increased deposition was detected immunohistochemically in group 1 and 2 loaded scaffolds, compared to controls. This lack of a clear difference in load and control samples at a gene expression level may seem to contradict the idea that gene

expression could be masked by non-stimulated cells. However, as genes, such as collagen type I and collagen type VI, are widely expressed in this system regardless of the application of load, it follows that there would be less clear differentiation between control and loaded scaffolds at a gene expression level (as with the lack of differentiation between chondrogenic markers in group 1 and 2 loaded scaffolds). This is more apparent when compared to markers of chondrogenesis (specifically type II collagen), which cannot be detected in non-loaded samples analysed using PCR or labelled immunohistochemically. The increased labelling of collagen type I and VI in loaded samples may simply be due to increased fluid flow and, therefore, nutrition as a result of loading leading to increased production of widely expressed molecules across the scaffolds, as opposed to a specific effect of the application of multiaxial load. The effect of loading on fluid flow throughout the scaffold could be investigated using fluorescently labelled dextran.

Analysis of the results of group 3 scaffolds provides some evidence for the masking of differences in group 1 and 2. The GAG/DNA ratio of group 3 scaffolds is only significantly different from group 2 loaded scaffolds, despite the dramatically increased production of GAG in both control and loaded scaffolds in group 1 and 2. Histology suggests that only a small population of cells near the surface of the scaffold produces GAG in groups 1 and 2, therefore, the expected GAG/DNA ratio of the cells producing GAG would be expected to be higher than group 3 scaffolds. However, the results show that the GAG/DNA ratio of group 3 scaffolds is higher than expected; this may be due to the better nutrition that these cells receive on the surface of the scaffold compared to cells seeded within the scaffold. Better access to nutrients may increase the basal expression of GAG by these cells. As the whole population would be exposed to this increased source of nutrients there are no cells to mask the result as in group 1 and 2 scaffolds, resulting in a higher than expected GAG/DNA ratio. However the smaller than expected difference between group 1/2 scaffolds and group 3 scaffolds may also be due to dilution of the newly synthesised GAGs with the DNA of cells in areas not producing GAG in group 1 and 2; this could contribute to the overall DNA measurement but not be reflective of the amount of GAG produced, therefore resulting in lower GAG/DNA ratio, in a similar manner to the masking described for the gene expression data. In order to investigate this masking effect for both PCR data and biochemical analysis mechanically loaded scaffold could be cut vertically in half at the end of culture. One half could then be cut again horizontally to isolate the top 25% of that half of the scaffold. The top 25% of the scaffold could then be analysed alongside the other full scaffold half. Should the non-chondrogenically stimulated cells mask any changes in gene expression or GAG/DNA quantification this should be apparent in the

results from the full half scaffold compared to the top 25% of the other half of the scaffold.

In group 3 scaffolds there were different patterns of gene expression and matrix deposition for types I and VI collagen compared to the scaffolds in other groups. In group 1 and 2, the gene expression is not significantly different in control or loaded samples (despite a trend in collagen type I) and there is positive immunohistochemical staining which is higher in loaded groups. However, in group 3 scaffolds, the expression of these genes is elevated compared to group 1 and 2 scaffolds, potentially due to the different environment (seeded as a layer on the surface of the scaffold) causing changes in the cell polarisation, morphology and phenotype, but immunohistochemical labelling for these two proteins is lower in loaded scaffolds than control scaffolds. This difference may be attributable to the increased loss of matrix components into the culture media, where collagen would not be detected, by the cells on the surface of group 3 loaded scaffolds. Fluorescent cell-tracking has shown that MSCs remain on the surface of loaded scaffolds compared to MSCs in free swelling constructs, which at day 28 are found throughout the upper layers of the scaffold. Analysis of the media GAG/scaffold GAG ratio for each sample demonstrated that group 3 scaffolds had the highest ratio of any of the three scaffold configurations, demonstrating the lowest ability to retain GAG and, potentially, other matrix components within the scaffold (data not shown). This may explain the difference in type I and VI collagen labelling between group 3 control and loaded scaffolds discussed above and warrants further investigation outside the scope of this thesis. Future experiments could address this suggestion by measuring the amount of collagen type I and VI released into the media, as opposed to deposition within the construct, using these antibodies for Western blotting.

The analysis of scaffolds using biochemical and RT-PCR techniques, and the analysis of latent and active TGF- β 1 in collected culture media provided no explanation for the changes observed histologically between the loaded groups 1 and 2. This emphasises the importance of histological analysis to demonstrate actual matrix deposition. In order to find an explanation for the differences observed between the data sets a number of different approaches, and additional analyses were performed, some of these are detailed below.

The formation of a layer of cells on the surface of the scaffolds was demonstrated in this chapter via fluorescent labelling. If the area covered by the MSCs seeded on the surface was great enough, then this layer may prevent the diffusion of matrix components from the scaffold into the media leading to an increase in scaffold matrix

deposition. However, if the media GAG/scaffold GAG ratio is calculated for group 1 and 2 loaded scaffolds, the ratio is almost identical when statistically tested $P > 0.999$ (data not shown). This analysis suggests that there is no difference in media GAG loss in the two groups demonstrating that the increase in staining observed in group scaffolds is unlikely to be due to increased retention within group 2 scaffolds.

The binding of TGF- β 1 to components of the matrix could increase the amount of growth factor available to cells within the scaffolds, but might not be detected by the TGF- β 1 ELISA measurement. Increased matrix deposition in group 2, could mean more TGF- β 1 creating a positive feedback loop for chondrogenesis that is not present in group 1 and, may explain the different staining between the two groups. In order to test this hypothesis the presence of bound TGF- β 1 was investigated immunohistochemically. The results of this investigation demonstrated that TGF- β 1 could not be detected in any scaffold despite labelling in the bone region of the glenoid ligament enthesis section used as positive control tissue (data not shown). As a result it is unlikely that it is a difference in the binding of TGF- β 1 to the scaffold and proteins within it causes the difference in matrix deposition between groups 1 and 2.

The difference could also be the result of a temporal effect, with the cells on the surface of the scaffold responding rapidly to load leading to earlier matrix deposition in group 2 compared to group 1. Histology has not been performed earlier than day 28, however, gene expression analysis on day 7 showed similar patterns of gene expression to day 28, suggesting this is unlikely. The results of day 7 gene expression analysis did however show an increase in the expression of collagen type I and X showing that the cells on the surface of group 3 scaffolds do respond to load, but only during the early stages of culture and not by day 28.

The seeding of cells on the surface of the scaffold could increase their access to nutrients in the media and, therefore, increase the amount of matrix deposited. However, in group 3 little matrix was produced or deposited suggesting that cross-talk between the surface cells and the cells seeded within the scaffold is required to see the changes in matrix deposition observed in group 2.

A potential factor that was not investigated is the effect of the high cell density created by seeding a layer of 400,000 cells on the surface of a scaffold 8 mm in diameter. High cell density is known to be important in chondrogenesis e.g. within developing bones and it may be that by seeding cells on the surface the increase in cell density contributes to the deposition of cartilage matrix molecules (Goldring et al., 2006). Recent work by Schrobback et al. has shown that blocking gap junctions or hemichannels can reduce the expression of chondrogenic markers in MSCs in pellet

and alginate culture (Schrobbach et al., 2015). One way to test the importance of cell-cell interaction in this system would be to repeat the experiment in the presence of a molecule that interferes with gap junction signalling such as 18- α glycyrrhetic acid, as used by Schrobbach et al., and observe the effects such an inhibitor has on chondrogenesis in response to load in this system.

4.5 Conclusions

The data presented here confirm previous results that have shown that multiaxial mechanical load can be used to induce chondrogenesis in MSCs in the absence of any exogenous growth factors, principally TGF- β . This work also shows that asymmetric seeding leads to clear differences in the deposition of matrix as demonstrated by the histological staining present in group 1 and group 2 scaffolds, particularly for sulphated proteoglycan and type II collagen. The results suggest that the change in matrix deposition is linked to interaction between the cells seeded on the surface of the scaffold and the cells seeded within the scaffold. However, these differences were not corroborated by changes in the gene expression or biochemical analysis, potentially for reasons that have been described. It may be that there are novel physical, chemical or biological factors that are involved in the relationship between these two populations of cells within these scaffolds that extend beyond the "classical markers" of chondrogenesis that have been investigated in this study, that are involved in this effect. This work also showed that the application of joint like mechanical load to cartilage tissue engineering constructs leads not only to the induction of latent TGF- β 1 release into the culture media, but also the activation of the secreted latent TGF- β 1. This activation appears to result from the physical forces applied to the system rather than enzymatic activation, however further work is required to confirm this mechanism of action.

Chapter 5 A Secretomic Comparison of the Induction of Chondrogenesis in Human Mesenchymal Stem Cells via TGF- β 1 and Mechanical Load

Aim

The aim of this research chapter was to investigate the different secretomic profiles of MSCs induced into chondrogenesis through TGF- β 1 stimulation and mechanical load in order to compare and contrast the effects of these two forms of induction. As mechanical load induces chondrogenesis via a TGF- β 1 dependent mechanism, this work was designed to investigate if the effect of load was analogous to the exogenous application of TGF- β 1 or if load has additional effects to TGF- β 1, and to identify potentially novel bioactive factors for use within MSC cartilage tissue engineering.

Sections of this Chapter have been submitted to European Cells and Materials in a manuscript entitled "Differences in human Mesenchymal stem cell secretomes during chondrogenic induction" which is currently under review.

5.1 Introduction

Currently, standard protocols for the induction of human mesenchymal stem cell (MSCs) chondrogenesis *in vitro* involve the culture of cells in a 3D environment (e.g. in a pellet/micromass culture or encapsulated within a hydrogel) coupled with the exogenous application of an isoform of TGF- β (Johnstone et al., 1998, Barry et al., 2001). This chondrogenic response is induced by TGF- β and mediated by SMAD signalling proteins which results in an increase in the expression of chondrogenic markers such as the transcription factor Sox9 and the matrix molecules aggrecan and type II collagen (Johnstone et al., 1998, Furumatsu et al., 2005, Hellingman et al., 2011). Following the induction of chondrogenesis both *in vitro* (Johnstone et al., 1998) and *in vivo* (Mueller and Tuan, 2008), MSCs progress towards hypertrophy, which is marked by the expression of molecules such as collagen type X and MMP13, eventually leading to cell death (D'Angelo et al., 2001, D'Angelo et al., 2000, Johansson et al., 1997). This progression of MSCs from a cartilage producing phenotype into a hypertrophic phenotype is reminiscent of the behaviour of mesenchymal progenitor cells during bone formation through endochondral ossification and presents a major barrier to the use of MSCs for the clinical repair of cartilage tissue (Mueller and Tuan, 2008, Goldring et al., 2006, Mackie et al., 2011, Sheehy et al., 2015).

Previous work has shown that the application of multiaxial load in our bioreactor system can induce chondrogenesis in human bone marrow derived MSCs in the absence of exogenous recombinant TGF- β (Li et al., 2010b, Schatti et al., 2011). Investigation into the mechanism behind the induction of chondrogenesis showed that the induction of chondrogenesis in this system is linked to the TGF- β signalling pathway (Li et al., 2010a). Blocking the TGF- β receptor 1 prevents this induction of chondrogenesis in response to multiaxial mechanical load (Li et al., 2010a). Work presented in Chapter 4 of this thesis shed further light on this mechanism by demonstrating that mechanical load can activate the latent TGF- β 1 produced by the cells in response to load; suggesting that load is required at the production and activation level in order to induce chondrogenesis in this system.

MSCs are known to respond to specific stimuli by synthesising matrix molecules and adopting cellular characteristics associated with tissues such as bone, cartilage and adipose. In addition to these matrix molecules, MSCs also secrete a large number of bioactive factors (Caplan, 2007, Czekanska et al., 2014). These factors provide information on the phenotype of the cells producing them, as well as mechanistic information about changes that occur in cells in response to certain stimuli, and what effect the cells might have on host cells around them in a clinical situation. The

secretomes of MSCs are increasingly being studied (Stoddart et al., 2015) and the secretomes of MSCs from a range of tissues including bone marrow, umbilical cord blood and adipose tissue, as well as MSCs derived from embryonic stem cells, have been investigated in a number of culture conditions in recent years (Haynesworth et al., 1996, Liu and Hwang, 2005, Kinnaird et al., 2004, Sze et al., 2007, Rehman et al., 2004). These studies have focused on different aspects of MSC biology including; angiogenesis, myogenesis and osteogenesis (De Lisio et al., 2014, Hoch et al., 2012, Oskowitz et al., 2011). A number of papers have also investigated the effects of chondrogenic stimulation on the MSC secretome (Grassel et al., 2009, Arufe et al., 2011, Rocha et al., 2014, Bara et al., 2014, Rodriguez et al., 2015). These studies have demonstrated the upregulation of factors such as VEGF, MMP13 and TIMP1 and 2. To the best of my knowledge no investigations have been made into the secretome of MSCs undergoing chondrogenesis in response to multiaxial load; furthermore, there is nothing reported in the literature comparing the secretomes of MSCs following mechanical load and TGF- β 1 stimulation.

The work presented in chapter 4 of this thesis showed that it was not possible to confirm the changes in matrix deposition detected histologically between group 1 compared to group 2 using "classic" markers of MSC chondrogenesis (e.g. GAG/DNA quantification and the determination of gene expression for a small number of genes). This may be a reflection of the narrow band of markers commonly used in this type of work, but also the high degree of complexity within the systems that are being studied. The work in this chapter was performed in order to broaden our understanding of the effect that inducing chondrogenesis has on MSCs, specifically on the soluble factors that they release into their environment.

The aim of this study was to compare the secretomes of unstimulated hBMSCs, hBMSCs cultured with TGF- β 1 in order to induce chondrogenesis, and hBMSCs cultured in the absence of TGF- β 1 but with multiaxial mechanical load in order to induce chondrogenesis. It was hypothesised that by analysing the secretomes of MSCs cultured in different chondrogenic culture conditions and in different mechanical environments it would be possible to compare the individual effects of mechanical load and TGF- β 1 stimulation on the chondrogenic induction of hBMSCs in order to gain greater understanding of the response of cells to these two different stimuli. This knowledge could be used to identify interesting and potentially novel bioactive factors for MSC based tissue engineering.

5.2 Materials and Methods

5.2.1 Donor Information

For this study, MSCs were taken from marrow aspirates from the vertebral bodies (thoracic or lumbar) of three male donors (aged twenty-two, thirty-seven and seventy-seven years old).

5.2.2 Experimental Design

In order to determine the specific effects of TGF- β 1 and multiaxial mechanical load on MSC chondrogenesis, a study was performed on different human MSC donors using three experimental groups. All three groups consisted of three 2x8mm fibrin-poly(ester-urethane) seeded with two million MSCs: group 1 scaffolds were kept in free swelling culture and received chondro-permissive medium (as described in Section 2.4); group 2 scaffolds were kept in free swelling culture and received chondro-permissive medium supplemented with 1ng/ml TGF- β 1; group 3 scaffolds were cultured in chondro-permissive medium and received six cycles of mechanical load over the eight day culture period (as detailed below). An exogenous TGF- β 1 concentration of 1 ng/ml was chosen for group 2 as previous studies have shown that mechanically loading MSCs in this bioreactor system leads to a media TGF- β 1 concentration of 1ng/ml (Li et al., 2010a), therefore the aim was to keep the chondrogenic stimulus similar between TGF- β 1 stimulated and loaded scaffolds. The media was collected from scaffolds on days two, four, six and eight for TGF- β 1 quantification and secretome analysis. Experiments were repeated three times to confirm the observations.

Following the results of this work, the study was repeated in order to isolate RNA from samples on day 8 for reverse transcription and real-time PCR, with the intention of confirming changes seen in the secretome analysis at the transcriptional level in the first set of experiments. The experimental design was identical to the initial study, except that the groups contained four technical repeats rather than three. This second experiment consisted of three independent repeats using cells from the same MSC donors that were used in the first study, and ensuring that the cells were used at the same passage as the MSCs used in the first study. The culture media was also collected on day days two, four, six and eight for nitrite analysis.

5.2.3 Mechanical loading

Multiaxial shear ($\pm 25^\circ$ at 1Hz) and compression loading (10% compression superimposed on top of a 10% pre-strain at 1Hz) was applied for one hour a day, six times, over eight days (on days 2-7).

5.2.4 Sample collection

Culture media was collected on day 2, 4, 6 and 8 of culture. On day 8, scaffolds were homogenized in TRI reagent (Molecular Research Centre Inc., Cincinnati, OH, USA) and stored at -80°C for RNA isolation and real-time PCR as described in chapter 2.

5.2.5 Characterisation of cytokine profile using a RayBio Human Cytokine Antibody Array

A full list of proteins detected by this kit is provided in Appendix 1. The terms factor or bioactive factor are used as a generic terms in this chapter for proteins analysed using this array kit.

Media collected on day 8 of culture was further analysed to determine the presence of 174 different cytokines within each sample using the RayBio Human Cytokine Antibody Array G-Series 2000 protein array according to the manufacturer's instructions. Briefly, glass chamber assay slides were brought to room temperature and dried before being blocked for 30 minutes using 1x Blocking Buffer. After blocking, 100ul of sample was incubated in each well of the assay slides for 16 hours before the wells were washed three times with 1x Wash Buffer I and once with 1x Wash Buffer II. Slides were then incubated with 1x Biotin-conjugated Anti-cytokine (70ul/well) for two hours before being washed as described previously with 1x Wash Buffer I and II; 70ul of 1x Streptavidin-Fluor was then added to each well of the assay slides and incubated for two hours. The assay slides were once again washed with 1x Wash Buffer I and II before the glass slides were removed from the chamber assembly, washed twice with 1x Wash Buffer I, washed once with distilled water and then centrifuged at 1000 rpm for three minutes and left to dry in a laminar flow hood for twenty minutes. The slides were then sent to THP Medical Products Vertreibrs Gmbh (Vienna, Austria) for measurement.

5.2.6 Quantification of media nitrite (Griess Reaction)

The concentration of nitrite in collected culture media was measured using the Griess assay as an indirect method of determining the relative level of nitric oxide in the experimental media samples. Griess reagent (modified) was prepared by dissolving 1.75 g of powder into 44 ml of double distilled water. A standard curve was then prepared using sodium nitrite. A 500x stock was prepared by dissolving 0.345 g of sodium nitrite in 100 ml of double distilled water, a serial dilution in chondropermissive medium was then performed to generate a standard curve consisting of 100 μM , 50 μM , 25 μM , 12.5 μM , 6.25 μM and 3.125 μM standards. 100 μl of standards and samples were then pipetted in duplicate into a transparent 96 well plate, 100 μl of Griess reagent was then added to each well. The reaction was allowed to proceed for 15 minutes in the dark before absorbance was measured at 530 nm. The standard curve was extrapolated to quantify the amount of nitrite in the samples with the lowest concentrations.

5.2.7 Statistical analysis

The results of secretome analysis and TGF- β 1 quantification represent data from three experimental repeats each performed using MSCs from a different donor repeated in triplicate. Real-time PCR results represent data from three experimental repeats in quadruplicate. The data presented represents a combination of all the repeats performed.

The results of TGF- β 1 quantification, nitrite quantification and real-time PCR analysis were tested for normality using the D'Agostino-Pearson omnibus normality test. Statistical differences of media TGF- β 1, nitrite ions and all genes analysed by real-time PCR except leptin and MMP13 within samples was determined using the Kruskal-Wallis test and Dunn's multiple comparison test. Statistical significance for leptin and MMP13 was determined using the Mann-Whitney test as the expression of these genes was not detectable in day 0 samples so the expression of loaded and control groups were therefore normalised to the expression in control scaffolds. Therefore, as the comparison was only between two groups rather than three for these genes the Mann-Whitney test was performed instead of the Kruskal-Wallis test.

The fluorescent intensity levels recorded for each sample from the cytokine array were adjusted to remove background interference. Outliers were then removed using the ROUT method, normality was determined using the D'Agostino-Pearson omnibus normality test and the statistical difference was then determined between control and TGF- β 1 cultured groups, between load and control and between load and TGF- β 1 cultured groups using Kruskal-Wallis test and Dunn's multiple comparison test.

5.3 Results

Due to the use of primary human cells in this work there was a high degree of variability between biological repeats. Due to this high level of variation and the relatively low numbers of donors tested many of the analyses do not reach significance at the 5% level. As a result both significant changes and non-significant trends within the data collected are described and discussed, where relevant, in this chapter.

5.3.1 TGF- β 1 Quantification

On day 2 the amount of TGF- β 1 measured in the culture medium of TGF- β 1 stimulated cells was higher than both control and loaded groups ($p < 0.039$) (Figure 5.1A). This was expected as the TGF- β 1 cultured group was receiving TGF- β 1 in the media at this point whilst the other two groups were not. At day 4 of culture (Figure 5.1B), the amount of TGF- β 1 released into the media from TGF- β 1 stimulated scaffolds was significantly higher than that of the control group ($P = 0.002$); however, this was not significantly different from the loaded group indicating that mechanical load was also inducing endogenous production of TGF- β 1 by the cells by day 4. By day 6 of culture, multiaxial mechanical loading led to a significant increase in TGF- β 1 production compared to control scaffolds (Figure 5.1C); the level of TGF- β 1 released into the media by MSCs stimulated with load was comparable to cells treated with TGF- β 1 and was significantly higher than that of the control group ($P = 0.021$ and < 0.001 respectively). This trend was also observed on day eight, with cells stimulated by TGF- β 1 or load producing significantly more TGF- β 1 than untreated MSCs (Figure 5.1D; $p < 0.001$ and $= 0.006$ respectively).

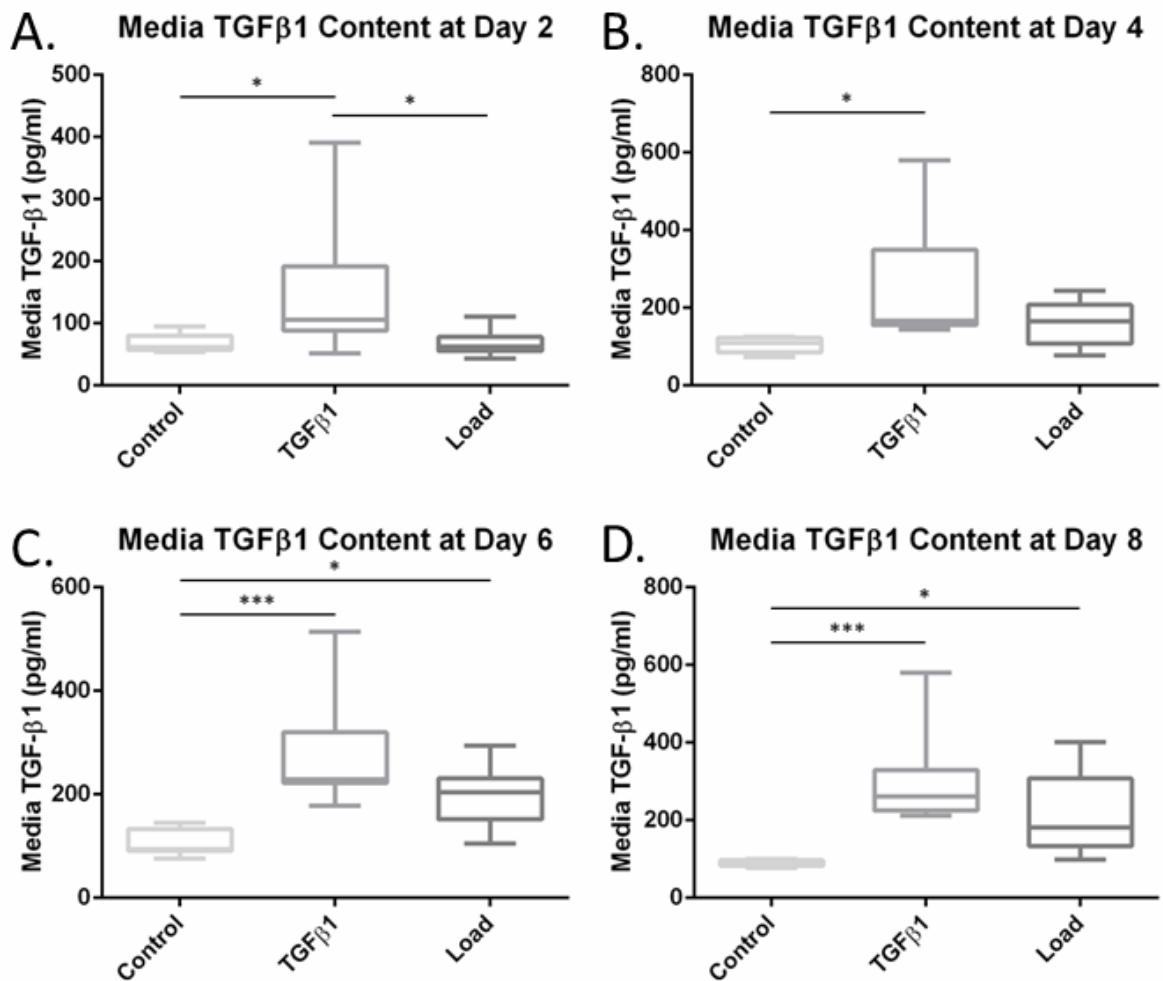


Figure 5.1

Quantification of TGF-β1 released into the culture media over the initial 8 day period of control (untreated), TGF-β1 stimulated or loaded MSCs embedded in scaffolds over four weeks of culture. This data represents the release of TGF-β1 over four two day windows and does not represent cumulative release. Statistical significance was defined as $P \leq 0.05$ and determined using the Kruskal-Wallis and Dunn's multiple comparison tests. * represents $P \leq 0.05$, ** represents $P \leq 0.001$ and *** represents $P \leq 0.0001$.

5.3.2 Characterisation of the production of bioactive factors in response to chondrogenic stimulation with TGF- β 1 and mechanical load

Media samples collected from control, TGF- β 1 stimulated and loaded constructs on day eight of culture were analysed for their protein content using a cytokine array. The samples were normalised to a positive control which was at the same concentration on each individual slide and the background fluorescence was removed. Full factor names are provided in Table 5.1.

All 174 factors were detected at varying levels of intensity in all experimental samples (Appendix 2). 55 factors had an average fluorescence intensity across the three groups of less than 100 including; leptin (93.79 ± 81.83) and MDC (54.81 ± 9.98). The average intensity of 94 factors was between 100 and 500 including; BLC (389.19 ± 557.10), MCP3 (201.30 ± 115.94), ALCAM (141.57 ± 37.54), uPAR (286.63 ± 109.39), leptin receptor (110.07 ± 29.35), MMP13 (422.77 ± 512.66) and PDGF α (171.75 ± 76.35). The intensity of 11 factors was between 500-1000 including; osteoprotegerin (986.16 ± 560.48) and VEGF (938.11 ± 397.31). 12 factors had an average intensity between 1000 and 4000 including; angiopoietin-2 (1341.87 ± 937.81), GRO (1326.36 ± 1011.64) and LAP (1302.15 ± 752.86). The intensity of two factors was above 4000; angiogenin (40273.68 ± 7037.95) and TIMP2 (6778.68 ± 2231.98). The factor with the highest recorded intensity, by a factor of 10, was angiogenin and the lowest was BMP6 (20.50 ± 18.21).

For each of the 174 factors analysed, three sets of comparisons were performed (i. between control (untreated) and TGF- β 1 stimulated scaffolds, ii. control and loaded scaffolds and iii. TGF- β 1 stimulated and loaded scaffolds) and the statistical significance between the groups determined. Analysis showed that 19 factors changed significantly in at least one of these comparisons (Figure 5.2). The three volcano plots in Figure 5.2 graphically represent the results of these three comparisons. These plots were produced by plotting the Log₁₀ of the fold change for a factor between one condition and another (e.g. control and TGF- β 1) on the X-axis against the -Log₁₀ of the p-value generated when testing the difference between the two conditions on the Y-axis. Therefore the further a factor is away from zero on the X-axis the greater the fold change up or down, and the further a factor is up the Y-axis the lower the p-value. A p-value of 0.05 equates to 1.30 on the Y-axis, therefore, any factors above this mark underwent a significant change.

Figure 5.2A shows a volcano plot generated based on the comparison between TGF- β 1 stimulated scaffolds and controls. Factors that appear on the left side of the Y-axis were detected at a higher level in control samples, and factors on the right hand side of the axis at a higher level in TGF- β 1 stimulated samples. Factors which are significantly different between untreated control and TGF- β 1 stimulated scaffolds have been

labelled. Figure 5.2B shows a volcano plot constructed in the same way as Figure 5.2A using data from the comparison between control scaffolds and loaded scaffolds, factors that appear on the left side of the Y-axis were detected in a higher level in control samples and factors on the right hand side of the axis at a higher level in loaded samples.

The volcano plots for control-TGF- β 1 scaffold and control-load scaffolds show both similarities and differences between the effects of load and TGF- β 1 stimulation compared to untreated control scaffolds. In both TGF- β 1 stimulated (Figure 5.2A) and loaded scaffolds (Figure 5.2B) there is a significant upregulation (found on the right hand side of the Y-axis and above 1.30 on the Y-axis) of BLC, MCP3, MIF, VEGF, MMP13 and PDGF α compared to control MSCs (Table 5.1). No factors were significantly down regulated in both TGF- β 1 stimulated and loaded MSC groups compared to controls. GRO was significantly down regulated in response to load but did not change significantly in response to TGF- β 1 stimulation ($P=0.075$, Table 5.1). Leptin, leptin receptor and MDC were upregulated in TGF- β 1 stimulated scaffolds compared to controls but did not change in loaded scaffolds compared to controls. MIP3 α , uPAR, LAP and angiogenin were significantly upregulated in response to load compared to control, but did not change in TGF- β 1 compared to controls.

The third volcano plot (Figure 5.2C) shows the results of a direct comparison between MSCs from TGF- β 1 stimulated scaffolds and loaded scaffolds. In this plot factors that were detected at a higher level in media collected from loaded scaffolds are found on the left hand side of the Y-axis and factors found at a higher level in media from TGF- β 1 stimulated scaffolds are found on the right hand side of the Y-axis. This plot clearly shows that the majority of factors measured were found at a higher level in the media of MSCs subjected to load. Angiopoietin 2, osteoprotegrin, ALCAM and DR6 were found at higher levels in media from loaded constructs than media from TGF- β 1 stimulated scaffolds whilst TGF- β 1 was found at higher levels in TGF- β 1 stimulated medium than loaded medium.

Further breakdown of these results showed that factors could be separated based on which comparisons demonstrated significant differences (Figure 5.3, 4 and 5).

5.3.3 Factors whose concentration was significantly different in media collected from loaded constructs compared to controls

The expression of four factors (MIP3 α , uPAR, LAP and angiogenin) was significantly increased in loaded scaffolds compared to control scaffolds ($P=0.032$, 0.036 , <0.001 and 0.002 respectively, Table 5.1, Figure 5.4C, D and E). GRO was significantly down regulated in response to load ($P=0.027$) and was decreased, but not significantly, in

response to TGF- β 1 stimulation ($P=0.075$) (Table 5.1, Figure 5.4B). Of all of the factors analysed GRO was the only factor to be downregulated significantly in response to either of the chondrogenic stimuli applied (TGF- β 1 or mechanical load).

5.3.4 Factors whose concentration was significantly different in media collected from TGF- β 1 stimulated constructs compared to controls

Leptin, leptin receptor and MDC were found to be significantly upregulated only between media from TGF- β 1 stimulated scaffolds and media from control scaffolds (Table 5.1, Figure 5.3C, D and E). No factors were down regulated in response to TGF- β 1 stimulation compared to controls. TGF- β 1 stimulation led to an increase in angiogenin production however this was not significant (for the TGF- β 1 to control comparison $P=0.053$, mean fluorescence intensity values - control: 33743 ± 6463 , TGF- β 1 stimulated: 42162 ± 3644 , loaded: 44916 ± 5449).

5.3.5 Factors whose concentration was significantly different in media collected from TGF- β 1 stimulated and loaded constructs

A total of three factors (Angiopoietin-2, osteoprotegerin and DR6) were found to be significantly upregulated in loaded samples compared to TGF- β 1 stimulated samples without significant changes between either group and controls (Table 5.1, Figure 5.3A, B and F).

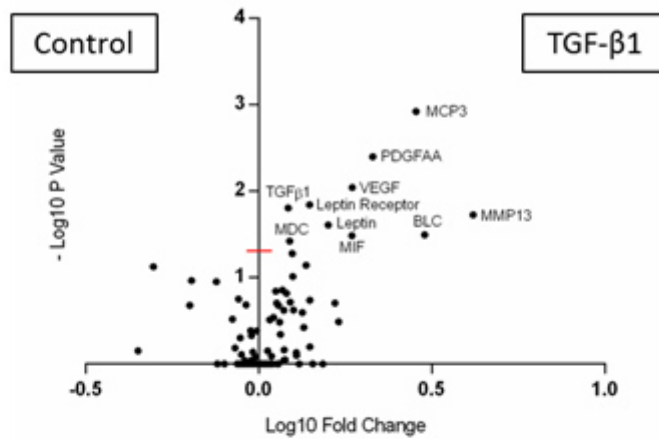
5.3.6 Factors whose concentration was significantly different in media collected from TGF- β 1 stimulated and loaded constructs compared to controls

Six factors (BLC, MCP3, MIF, VEGF, MMP13 and PDGF $\alpha\alpha$) were found to change significantly in both TGF- β 1 and loaded groups compared to controls. In all of these cases the factors were significantly higher in TGF- β 1 and loaded groups than controls (Table 5.1, Figure 5.5B, C, D, E, F and H). The similarities in the responses of these factors indicate similarities in the effects of these two forms of stimulation, potentially due to the effect of the TGF- β 1 signalling in both systems.

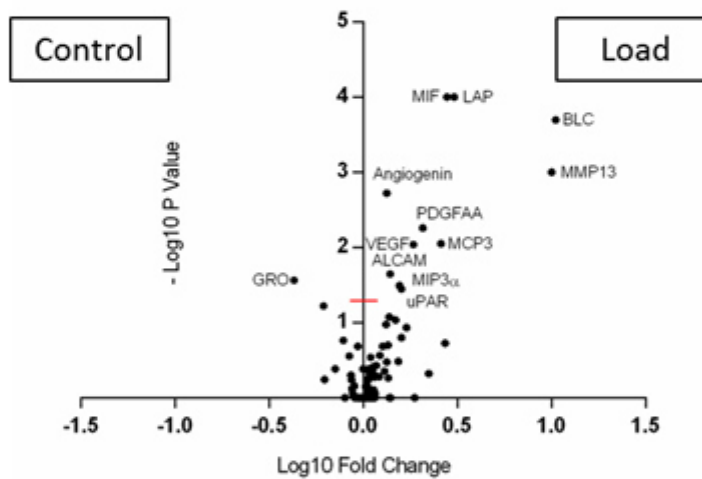
5.3.7 Factors whose concentration was significantly different in media collected from either TGF- β 1 stimulated or loaded constructs compared to controls

In contrast, ALCAM was significantly increased in the loaded group over both control ($P=0.023$) and TGF- β 1 stimulated groups ($P=0.038$) showing a clear difference in effect between load and TGF- β 1 stimulation (Table 5.1, Figure 5.5A).

A. Volcano Plot Showing the Comparison Between TGF β 1 Stimulated and Control Scaffolds



B. Volcano Plot Showing the Comparison Between Loaded and Control Scaffolds



C. Volcano Plot Showing the Comparison Between TGF β -1 Stimulated and Loaded Scaffolds

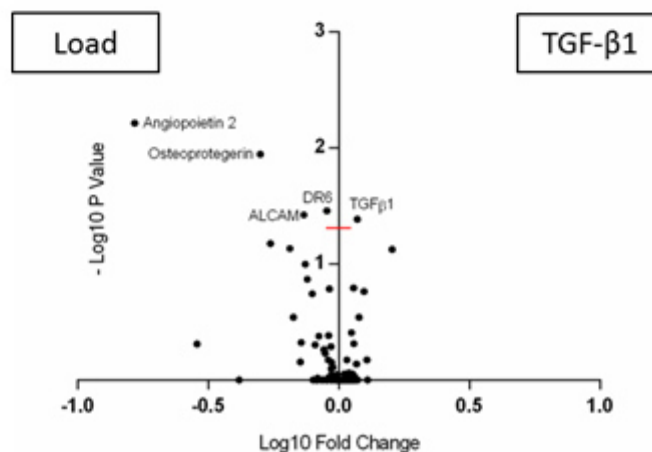


Figure 5.2

Volcano plots showing the results of the three sets of statistical comparisons made between groups made between the results of protein analysis of untreated control scaffolds, scaffolds stimulated with 1 ng/ml TGF- β 1 and mechanically loaded scaffolds. Protein analysis was performed using a RayBio Cytokine Array, These plots have – Log₁₀ p-value of the comparison on the Y-axis and Log₁₀ fold change of the comparison for each factor on the X-axis. As a result the greater the fold change the further a factor is away from zero on the X-axis and the lower the p-value of a comparison the further away from zero on the Y-axis. Factors that underwent a significant change have been labelled. The red line on the Y-axis represents a –Log₁₀ p-value of 1.3 this is equivalent to a p-value of 0.05, factors above this line underwent a significant change.

Plot A. represents a comparison of factors released into the media from MSCs stimulated with 1ng/ml TGF- β 1 versus untreated control cells. Factors on the left hand side of the Y-axis were higher in controls than TGF- β 1 stimulated samples and factors on the right hand side were higher in TGF- β 1 stimulated samples than controls.

Plot B. represents a comparison of factors released into the media from MSCs stimulated with mechanical load versus untreated controls. Factors on the left hand side of the Y-axis were higher in controls than loaded samples and factors on the right hand side were higher in loaded samples than controls.

Plot C. represents a comparison of factors released into the media from MSCs stimulated with 1ng/ml TGF- β 1 versus mechanically loaded samples. Factors on the left hand side of the Y-axis were higher in loaded samples than TGF- β 1 stimulated samples and factors on the right hand side were higher in TGF- β 1 stimulated samples than loaded samples.

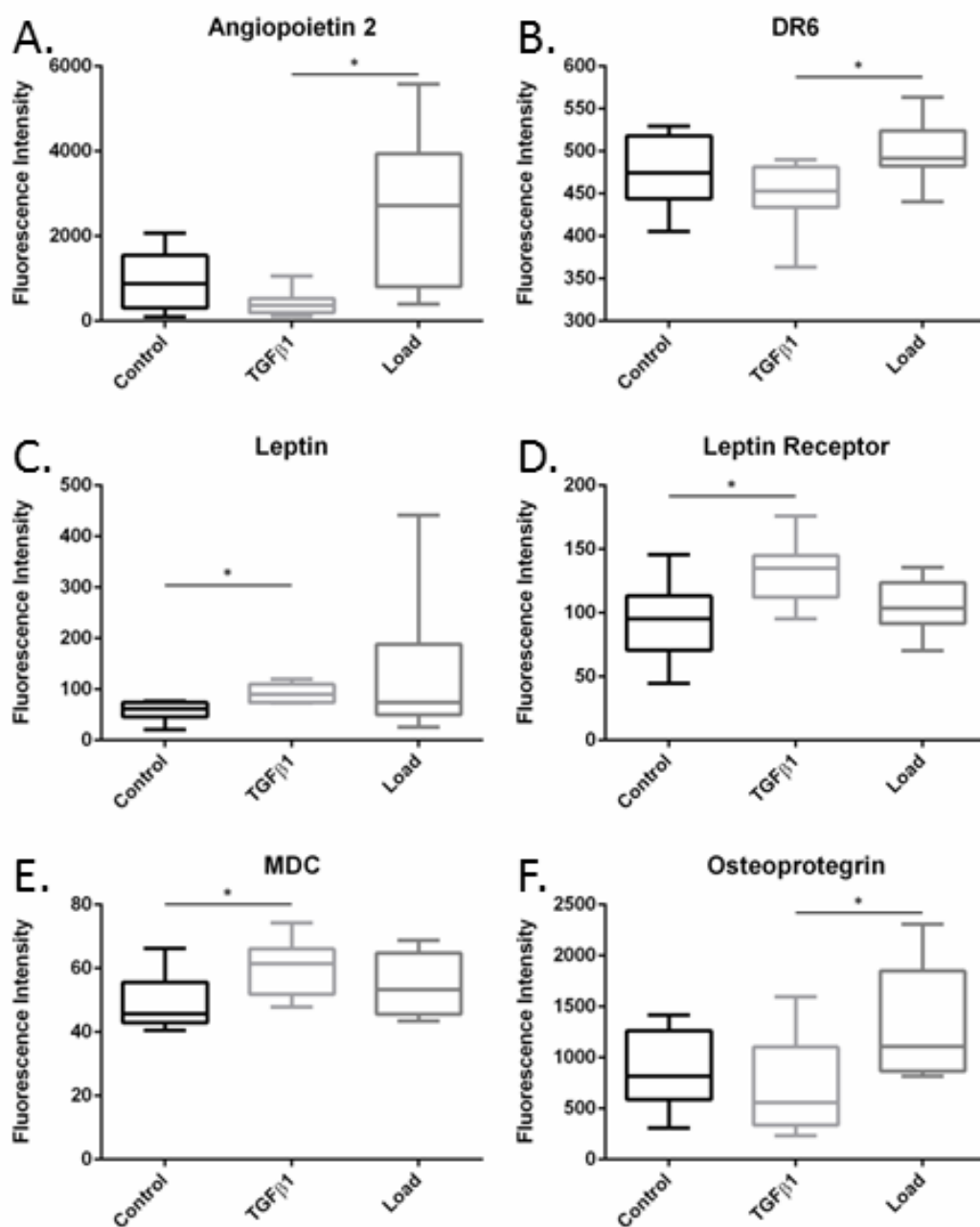


Figure 5.3

Box plots showing the factors whose medium concentrations were demonstrated by cytokine antibody array analysis to significantly change when medium collected from TGF-β1 stimulated constructs was compared to media from mechanically loaded or untreated controls. Statistical significance was defined as $P \leq 0.05$ and determined using the Kruskal-Wallis and Dunn's multiple comparison tests. * represents $P \leq 0.05$, ** represents $P \leq 0.001$ and *** represents $P \leq 0.0001$.

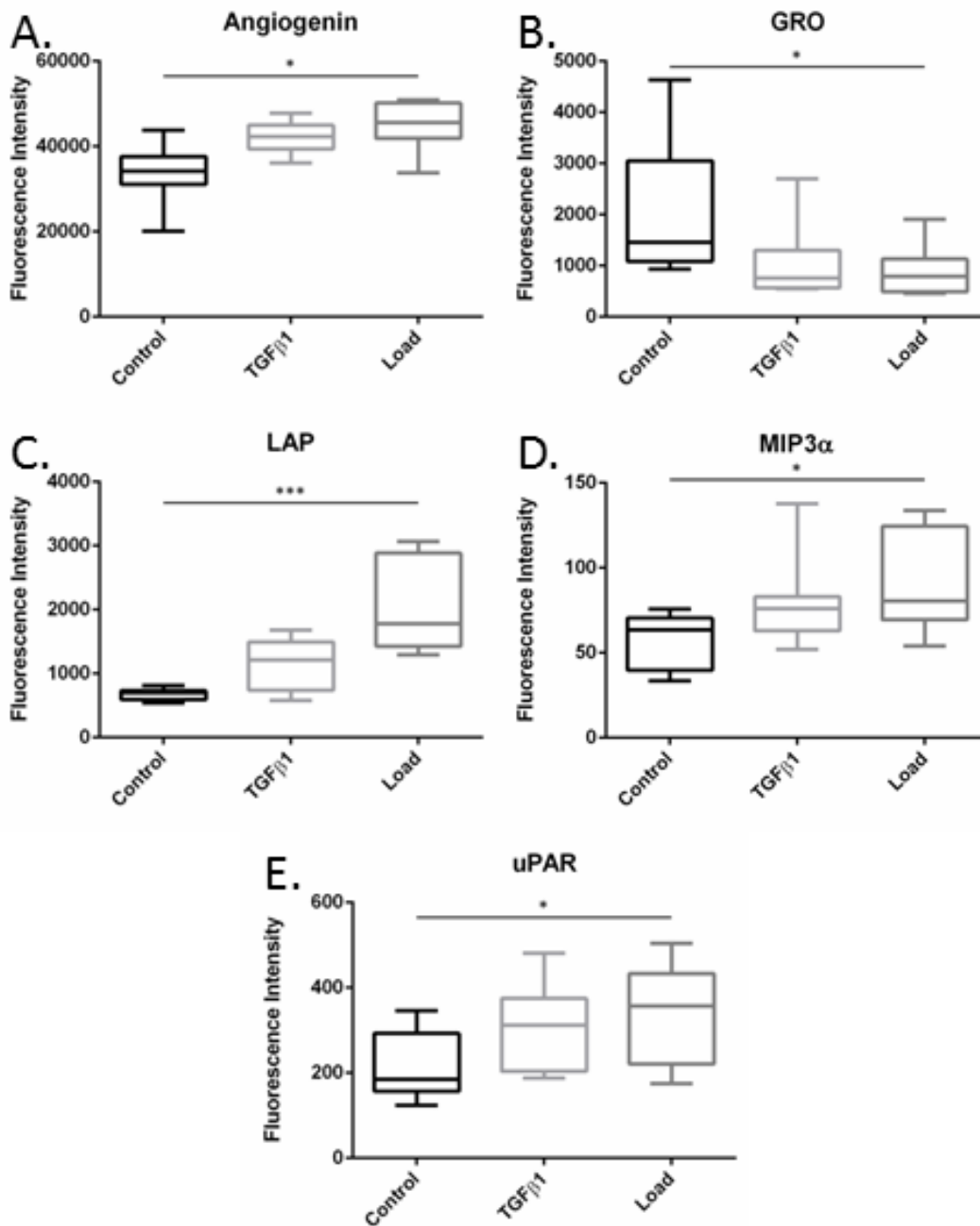


Figure 5.4

Box plots showing the factors whose medium concentrations were demonstrated by cytokine antibody array analysis to significantly change when to medium from mechanically loaded constructs was compared to media from TGF- β 1 stimulated and untreated control constructs. Statistical significance was defined as $P \leq 0.05$ and determined using the Kruskal-Wallis and Dunn's multiple comparison tests. * represents $P \leq 0.05$, ** represents $P \leq 0.001$ and *** represents $P \leq 0.0001$.

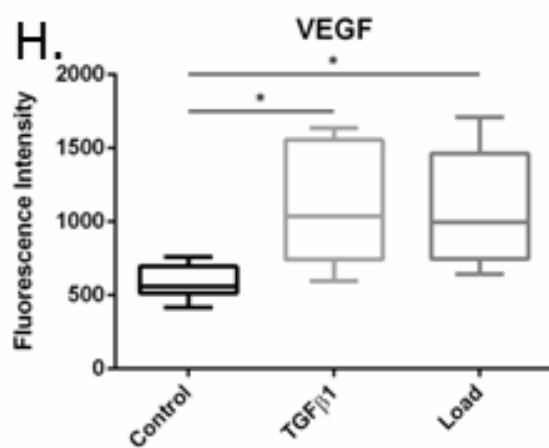
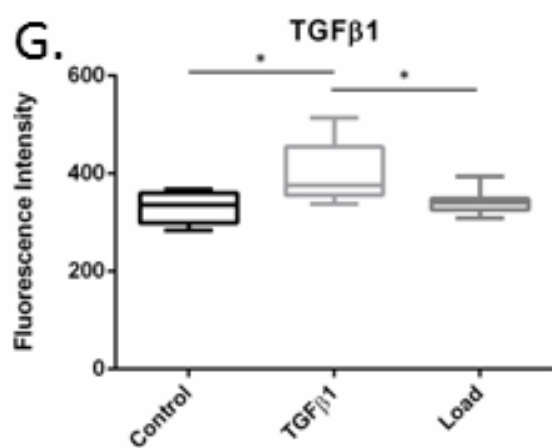
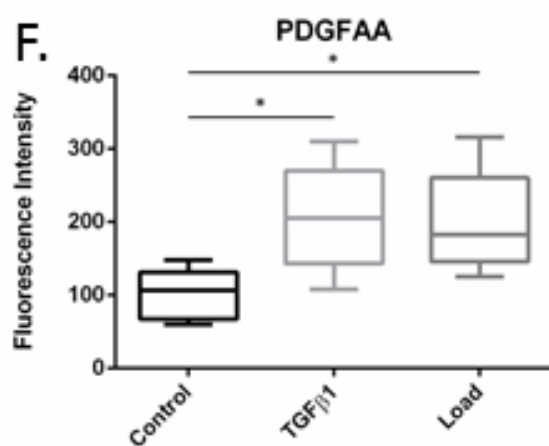
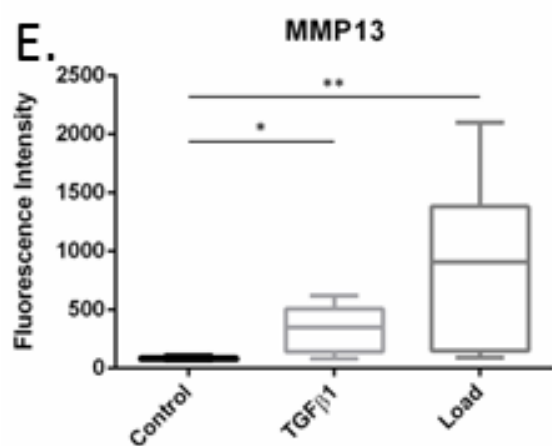
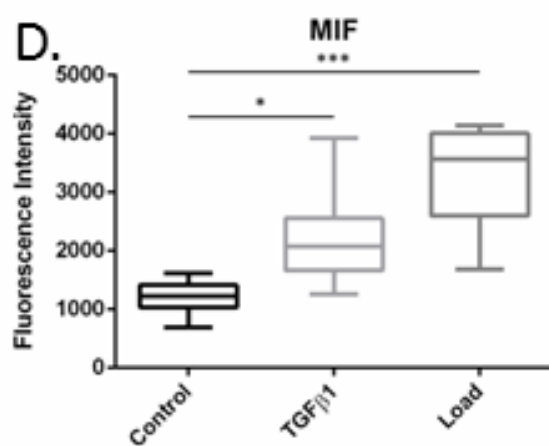
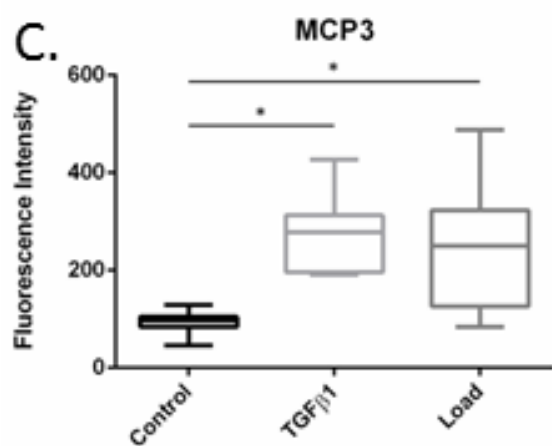
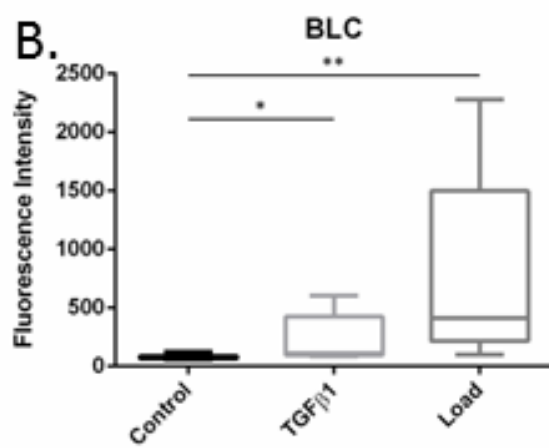
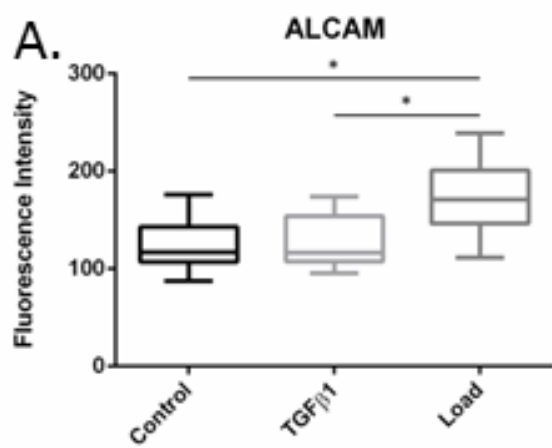


Figure 5.5

Box plots showing the factors whose medium concentrations were demonstrated by cytokine antibody array analysis to significantly change between two different groups (untreated control constructs, constructs stimulated with TGF- β 1 and mechanically loaded scaffolds). Statistical significance was defined as $P \leq 0.05$ and determined using the Kurskal-Wallis and Dunn's multiple comparison tests. * represents $P \leq 0.05$, ** represents $P \leq 0.001$ and *** represents $P \leq 0.0001$.

Factor name:	Abundance:	TGF-β1- Control p-value:	Load- Control p-value:	TGF-β1- Load p-value:
Factors with a Significant Difference in One Comparison				
Leptin	Low	0.0246	0.4757	0.6992
Leptin receptor	Low	0.0144	0.9999	0.1721
Macrophage derived chemokine (MDC)	Very Low	0.0378	0.3897	0.9813
Chemokine (CC motif) ligand 20 (CCL20), macrophage inflammatory protein 3 α (MIP3α)	Very Low	0.2550	0.032	0.9999
Chemokine (CXC motif) ligand 1,2 and 3 (CXCL1,2 and 3), Growth related oncogene α, β and γ (GROα, β and γ)	Moderate-High	0.0749	0.0272	0.9999
Urokinase receptor (uPAR)	Low	0.1820	0.0358	0.9999
Latency associated peptide (LAP)	Moderate	0.1968	0.0001	0.0667
Angiogenin	High-Very High	0.0526	0.0019	0.8959
Angiotensin 2 (ANG2)	High	0.7034	0.1869	0.0061
Osteoprotegrin (OPG)	Moderate-High	0.9999	0.1573	0.0113
Tumour necrosis factor receptor superfamily 21 (TNFRSF21), death receptor 6 (DR6)	Low-Moderate	0.4128	0.8959	0.0348
Factors with a Significant Difference in Two Comparisons				
Transforming growth factor β 1 (TGF-β1)	Low	0.0157	0.9999	0.0411
Cluster of differentiation 166 (CD166), activated leukocyte cell adhesion molecule (ALCAM)	Low	0.9999	0.0226	0.0378
Chemokine (CXC) motif ligand 13 (CXCL13), B lymphocyte chemoattractant (BLC)	Low-Moderate	0.032	0.0002	0.4884
Chemokine (CC motif) ligand 7 (CCL7), Monocyte specific chemokine 3 (MCP3)	Low	0.0012	0.0089	0.9999
Macrophage migration inhibitory factor (MIF)	High	0.0328	0.0001	0.2883
Vascular endothelial growth factor (VEGF)	Moderate-High	0.0091	0.0091	0.9999
Matrix metalloproteinase 13 (MMP13), collagenase 3	Low-Moderate	0.0189	0.0010	0.9999
Platelet derived growth factor AA (PDGFAA)	Low	0.004	0.0055	0.9999

Table 5.1

A table showing the results of statistical comparisons made between groups. All factors that showed at least one significant change are included. A p-value displayed in red represents a decrease in expression in the group the comparison was made to (e.g. a red p-value in the 'TGF- β 1-load' column indicates a decrease in the TGF- β 1 stimulated group compared to the loaded group) whilst green represents an increase in expression in the group the comparison was made to (e.g. a green p-value in the 'TGF- β 1-control' column indicates an increase in the TGF- β 1 stimulated group compared to the control group). Abundance demonstrates the overall amount of protein in the medium based on the strength of signal detected during measurement, this is only arbitrary and acts as a general indicator of the amount of a particular protein relative to the others detected (the units are arbitrary and represent fluorescence intensity, Very low <100, low 100-500, moderate 500-1000, high 1000-4000 and very high >4000).

5.3.8 Differential gene expression in MSCs chondrogenically stimulated with TGF- β 1 and mechanical load

Real-time PCR was performed in order to determine the relative gene expression levels of factors, synthesised by MSCs stimulated with TGF β 1 or multi-axial load on day 8 of culture. Target mRNA transcripts were chosen from the factors shown to undergo significant changes between groups in the initial secretome analysis (Figure 5.6). The results were analysed using the $\Delta\Delta$ Ct method using 18s as a housekeeping gene and normalising to expression at day 0. Of the 19 factors that underwent significant changes twelve were chosen for real-time PCR analysis: angiogenin, angiopoietin 2, BLC, GRO α , leptin, MCP3, MIF, MIP3 α , MMP13, OPG, PDGF α and VEGF. Aggrecan, collagen type X and Sox9 expression were also analysed to compare the chondrogenic effect of TGF- β 1 and load after seven days of stimulation. DR6 and MDC were not investigated due to the paucity of relevant information found in the literature and TGF- β 1 was not included due to the ELISA quantification of the protein present in the culture medium that had already been used to confirm the concentrations in media from different groups. Leptin receptor, ALCAM and uPAR were excluded on the basis that as cell surface proteins their detection in the media would bear greater relation to the induction of receptor cleavage than changes in gene expression; in a similar manner LAP was excluded as this is released during TGF- β 1 activation and its detection therefore is heavily dependent on post-translational processes.

Following the processing of the data generated by real-time PCR the results from BLC, MCP3 and MIP3 α were excluded after initial investigation due to extremely high standard deviations within sample duplicates which were not associated with a low level of expression of these factors compared to other factors. This was not a problem that was experienced with any of the other 9 genes analysed and was strictly limited to these three genes.

Of the nine factors presented (Figure 5.6), four exhibit gene expression patterns similar to the secretome profiles, two have a similar profile in one of the stimulated groups (TGF- β 1 or load) but not the other and three factors (MIF, VEGF and PDGF α) showed no change in either of the stimulated groups compared to the control group, despite showing changes in the secretome analysis.

Results of real-time PCR for angiopoietin 2 (mean fold-upregulation compared to day 0: control 6.69 ± 3.30 , TGF- β 1 2.53 ± 2.23 and load 7.70 ± 5.41 , $P=0.024$ when TGF- β 1 stimulated was compared to control and $P=0.013$ when compared to load) and osteoprotegrin (mean fold-upregulation compared to day 0: control 0.93 ± 0.33 , TGF- β 1 0.37 ± 0.33 and load 0.95 ± 0.39 , $P<0.001$ when TGF- β 1 stimulated was compared to control and $P=0.003$ when compared to load) show that, as in the secretome analysis,

the expression in the TGF- β 1 group is significantly lower than in either the loaded or control MSC groups (Figure 5.6B and G). The expression of MMP13 was normalised to the control group as the expression of MMP13 in cells at day 0 was donor dependent and only detected in two out of the three donors. Real-time PCR shows that the expression of MMP13 is similar in TGF- β 1 stimulated and loaded groups and that also the expression in both groups is much higher than control groups whose fold-upregulation can be considered to be 1 as the other two groups were normalised to it (Figure 5.6F). The expression of GRO was also lower than controls in both stimulated groups (Figure 5.6C), although not as strongly as suggested by the secretome data (Figure 5.2B) or previous results collected by this group (data not shown).

Secretome analysis showed that leptin was detected at a higher level in media from TGF- β 1 stimulated scaffolds compared to control MSC scaffolds (Figure 5.2B), whilst angiogenin was detected at a higher level in loaded samples than controls (Figure 5.2A). However, for both of these factors real-time PCR analysis showed no difference between stimulated groups in the case of leptin (Figure 5.6A, which as with MMP13 was normalised to the control groups due to donor dependent expression at day 0), or stimulated groups and controls in the case of angiogenin (Figure 5.6D).

The results of the secretome analysis showed that MIF, PDGF $\alpha\alpha$ and VEGF were all detected at higher levels in both TGF- β 1 stimulated and loaded groups than controls (Figure 5.2), whilst not being significantly different from each other. This was not reflected in gene expression analysis where neither stimulated group was found to be significantly different from the untreated controls.

Markers of MSC chondrogenesis, including aggrecan, SOX9 and collagen type X were also analysed at the gene expression level (Figure 5.7). Collagen type II was not included as previous work has shown its upregulation to be highly inconsistent between donors at day 7 in this system (data not shown). Potentially due to the early time point (day 8) that mRNA was collected at, significant differences were not observed in the expression of aggrecan (Figure 5.7A) and Sox 9 (Figure 5.7C). However a trend towards an increase in aggrecan expression is observable in TGF- β 1 stimulated and loaded groups and a trend towards an increase in Sox 9 expression is evident in response to load but not TGF- β 1. The expression of collagen type X was significantly higher in both groups than in controls (mean fold-upregulation compared to day 0: control 6.00 ± 3.67 , TGF- β 1 52.87 ± 42.87 and load 48.72 ± 32.95 , $P=0.024$ when compared to control and $P=0.013$ when compared to load, $P=0.001$ when TGF- β 1 stimulated was compared to control and $P=0.002$ when load was compared to control, Figure 5.7C).

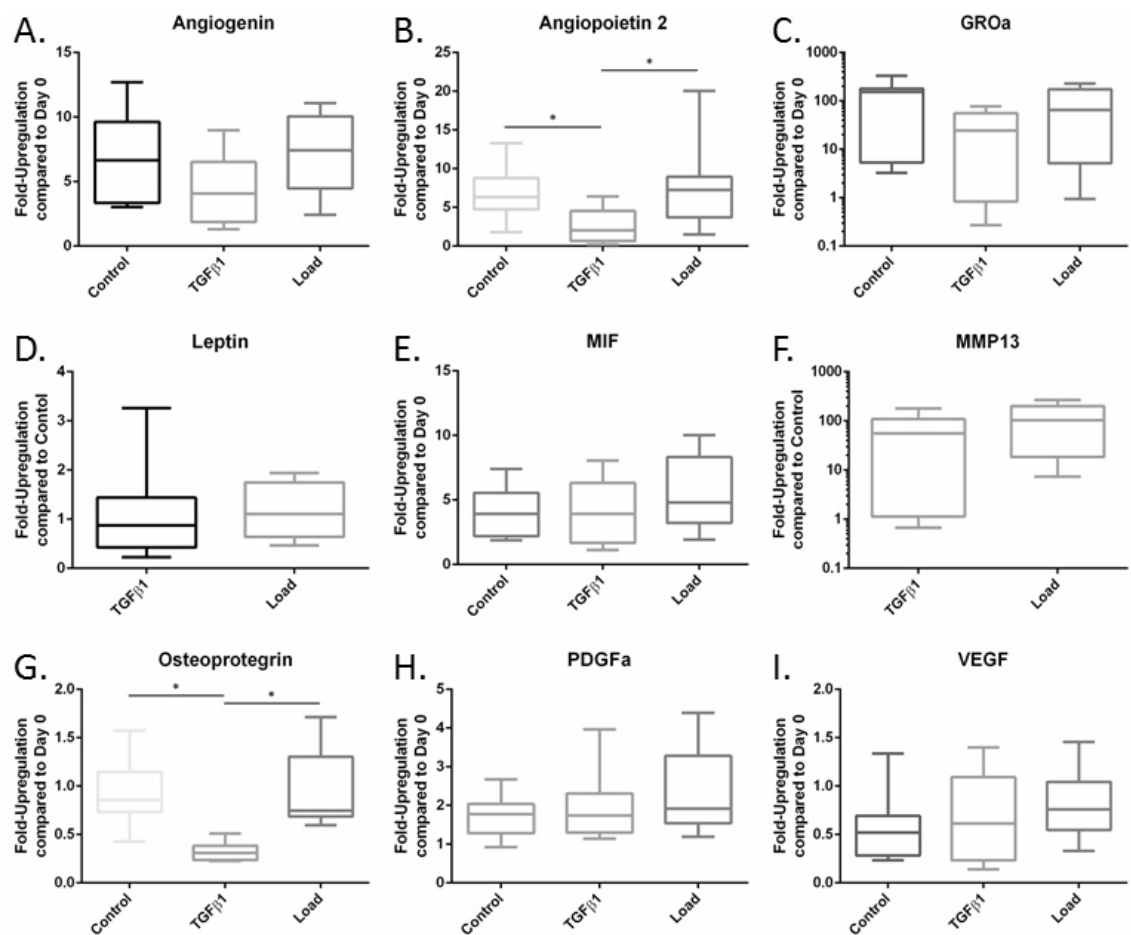


Figure 5.6

Box plots showing the results of real-time PCR analysis of TGF-β1 stimulated constructs, mechanically loaded constructs and untreated controls to confirm changes detected at a protein level at an mRNA level. This figure contains the results of the 12 factors investigated, each of which had showed a significant change between at least one comparison made between groups identified from the secretome analysis (the three comparisons were between control and TGF-β1, between load and controls and load and TGF-β1). The results were analysed using the $\Delta\Delta C_t$ method using 18s as a housekeeping gene and normalised to expression at day 0. Statistical significance was defined as $P \leq 0.05$ and determined using the Kruskal-Wallis and Dunn's multiple comparison tests. * represents $P \leq 0.05$, ** represents $P \leq 0.001$ and *** represents $P \leq 0.0001$.

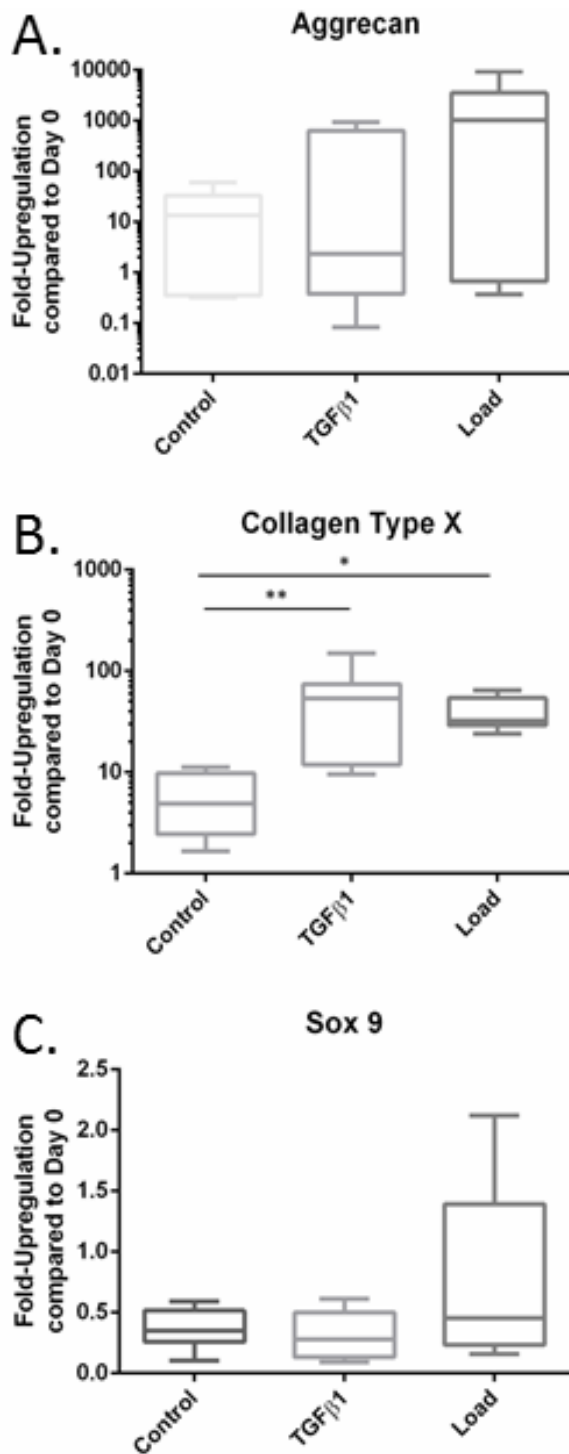


Figure 5.7

Box plots showing the results of real-time PCR gene expression analysis of the markers of MSC chondrogenesis: aggrecan, collagen type X and Sox 9 in response to TGF- β 1 stimulation and mechanical load. The results were analysed using the $\Delta\Delta C_t$ method using 18s as a housekeeping gene and normalised to expression at day 0. Statistical significance was defined as $P \leq 0.05$ and determined using the Kruskal-Wallis and Dunn's multiple comparison tests. * represents $P \leq 0.05$, ** represents $P \leq 0.001$ and *** represents $P \leq 0.0001$.

5.3.9 The production of nitrite by MSCs in fibrin-poly(ester-urethane) scaffolds exposed to exogenous TGF- β 1 and mechanical load

The presence of organic nitrites was detected in the culture media using the Griess reaction (Figure 8). Results showed that at all four time points (day two, four, six and eight) the level of nitrite detected in the media collected from loaded samples was significantly higher than the level of nitrite in the media from the TGF- β 1 stimulated group ($P=0.033$ at day 2 and $P<0.001$ at day 4, 6 and 8). There were also significantly higher levels of nitrites in the culture media of loaded scaffolds compared to control scaffolds on days four, six and eight ($P\leq 0.001$). No significant difference was detected between TGF- β 1 stimulated scaffolds and controls at any time point.

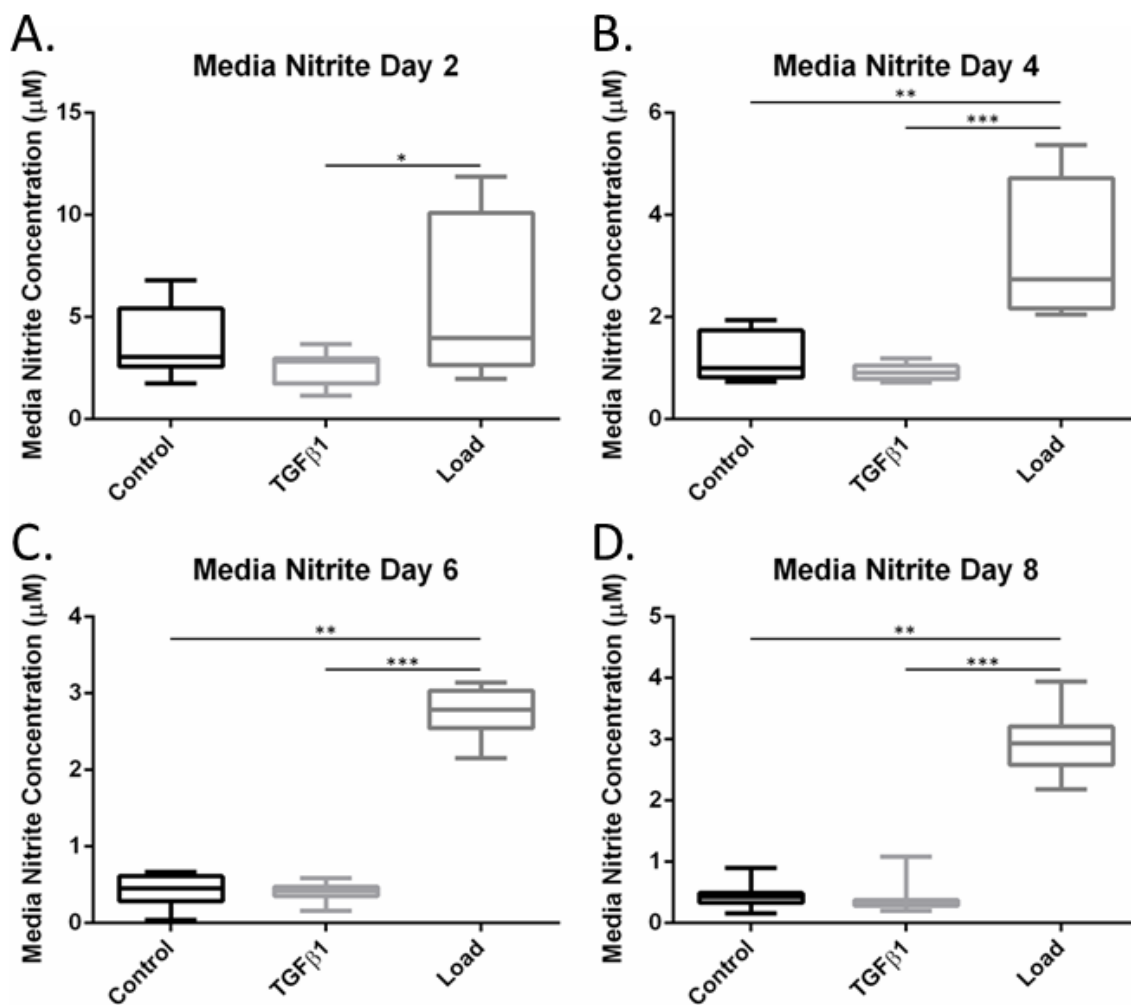


Figure 5.8

Quantification of nitrite (as an indirect measure of nitric oxide (NO)) in the culture media of MSCs cultured in scaffolds for four weeks and either subjected to TGF-β1 (1ng/ml) or multiaxial load; untreated MSCs served as controls, Statistical significance was defined as $P \leq 0.05$ and determined using the Kurskal-Wallis and Dunn's multiple comparison tests. * represents $P \leq 0.05$, ** represents $P \leq 0.001$ and *** represents $P \leq 0.0001$.

5.4 Discussion

Currently accepted methods for the induction of chondrogenesis in MSCs use a combination of 3D culture and the exogenous administration of an active recombinant isoform of TGF- β (Johnstone et al., 1998, Barry et al., 2001). Recent work has shown that chondrogenesis can be induced in MSCs using multiaxial mechanical load in the absence of exogenous TGF- β (Li et al., 2010a, Li et al., 2010b, Schatti et al., 2011). The purpose of this investigation was to compare the secretomes of MSCs stimulated either with exogenous TGF- β 1 or multiaxial load with each other and with unstimulated control scaffolds to identify similarities and differences between these two forms of chondrogenic induction and determine interesting or potentially novel factors for MSC based cartilage tissue engineering.

In both TGF- β 1 stimulated and loaded MSC groups secretome analysis showed that there was an increase in the level of BLC, MCP3, MIF, VEGF, MMP13 and PDGF compared to media collected from control scaffolds. These factors demonstrate similarities between the two forms of chondrogenic induction and may represent a core number of factors that are responsive to TGF- β 1, which is known to be involved in the induction of chondrogenesis in the loaded MSC scaffolds as well as the TGF- β 1 stimulated MSC scaffolds (Li et al., 2010a). MMP13 is associated with chondrocyte and MSC hypertrophy, whilst BLC secretion has previously been described at day seven of TGF- β driven chondrogenesis of MSCs. MCP3 has been shown to be induced by both TGF- β in murine dermal fibroblasts and mechanical stimulation in osteocytes and both PDGF $\alpha\alpha$ in osteoblasts and VEGF production in vascular smooth muscle cells and murine macrophages is known to be responsive to TGF- β (D'Angelo et al., 2000, Cristino et al., 2008, Ong et al., 2009, Tanabe et al., 2006, Jeon et al., 2007, Wang et al., 1997, Kitase et al., 2014). These factors therefore have clear links to TGF- β 1 or TGF- β 1 driven chondrogenesis and this may explain the similarities between loaded and TGF- β 1 stimulated MSC scaffolds when compared to control MSC scaffolds. The expression of all six of these factors has also previously been described in MSCs (Kinnaird et al., 2004, Rehman et al., 2004, Sze et al., 2007, Palumbo et al., 2014, Cristino et al., 2008, Hoch et al., 2012, Ribeiro et al., 2012, Grassel et al., 2009). Some of these factors have also previously been associated with similar responses to TGF- β in a range of cell types e.g. BLC in MSCs, MCP3 in murine dermal fibroblasts, VEGF in vascular smooth muscle cells, MMP13 in cartilage explants and PDGF $\alpha\alpha$ in osteoblasts (Ong et al., 2009, Wang et al., 1997, Tanabe et al., 2006, Jeon et al., 2007, Cristino et al., 2008, Fitzgerald et al., 2008).

As well as factors that responded similarly to both forms of chondrogenic induction there were a number that underwent opposing responses. Leptin, leptin receptor and

MDC were upregulated in TGF- β 1 stimulated scaffolds compared to controls, whilst there was no change in loaded scaffolds compared to controls. Leptin is associated with arthritic changes in cartilage and hypertrophic growth plate chondrocytes (Kume et al., 2002, Iliopoulos et al., 2007, Dumond et al., 2003). Work by Zeddou et al. on MSCs showed that TGF- β reduces leptin and leptin receptor expression, however, the use of a monolayer culture system and umbilical cord MSCs makes comparison between these results difficult (Zeddou et al., 2012). Increased levels of MDC in the synovial fluid and even serum has also been associated with arthritic joints (Flytlie et al., 2010).

In media from loaded scaffolds MIP3 α , uPAR, LAP and angiogenin were found at higher levels than controls, without a corresponding change in TGF- β 1 stimulated samples compared to controls. GRO was significantly down regulated in loaded samples compared to controls as well as in TGF- β 1 stimulated scaffolds (although not significantly in the case of exogenous TGF- β 1). MIP3 α and uPAR have both been associated with an increase in expression in response to mechanical loading, which correlates with the results presented here (Lee et al., 2012, Chu et al., 2006, Chen et al., 2013). LAP is released from the mature TGF- β 1 peptide during activation (Robertson and Rifkin, 2013). Mechanical loading, and in particular shear loading, has been shown to activate TGF- β , an effect also seen within the bioreactor culture system presented in this thesis (chapter 4) (Albro et al., 2012, Ahamed et al., 2008, Annes et al., 2004, Wipff et al., 2007). The increased presence of LAP in the culture media of loaded MSC scaffolds is therefore likely to be due to activation of endogenously produced pro-forms of TGF- β . GRO has been linked to arthritis, the induction of hypertrophic markers such as MMP13 and collagen type X, as well as mineralisation in chondrocytes (Merz et al., 2003, Endres et al., 2010, Olivotto et al., 2007). The application of endogenous TGF- β has also been shown to decrease GRO expression in epithelial cells (Lo et al., 2013).

Angiopoietin 2, osteoprotegrin and DR6 were detected in the culture media of loaded scaffolds at a significantly higher level than TGF- β 1 stimulated scaffolds, despite the fact that individually neither TGF- β 1 stimulated nor loaded groups were significantly different from controls. Significant differences induced between load and TGF- β 1, without either group changing with regards to the control group, results from low level down regulation in TGF- β 1 treated samples and/or slight upregulation in loaded scaffolds. Both angiopoietin-2 and osteoprotegrin have been shown to be induced by the application of shear forces which may be linked to their increased presence in media from loaded scaffolds over TGF- β 1 stimulated scaffolds (Goettsch et al., 2008, Li et al., 2014, Kim et al., 2006).

ALCAM was detected at a significantly higher level in media from loaded scaffolds than control and TGF- β 1 stimulated scaffolds, suggesting a strong link between its expression and mechanical load. ALCAM has been shown to be shed from the cell surface in response to TGF- β stimulation, although that was not evident in the secretome analysis; however, load led to a clear increase in receptor shedding as previously reported (Hansen et al., 2014). This effect of loading may result from a change in the expression of proteolytic enzymes e.g. ADAM17 which are known to cleave surface bound ALCAM (Hansen et al., 2014).

The amount of TGF- β 1 detected in the culture media by cytokine array was significantly higher at day eight in TGF- β 1 stimulated samples than both control and loaded samples. This is at odds with the result of the ELISA quantification of day 8 media, which showed that there was no significant difference in the media TGF- β 1 concentration of loaded and TGF- β 1 stimulated groups. The ELISA data also indicated that both TGF- β 1 stimulated groups and loaded groups were significantly higher than the control group on day eight. The results of the cytokine array also suggest that the overall fold change between TGF- β 1 and load was 1.17 and between TGF- β 1 and control 1.2 suggesting very little overall difference in the total amounts of TGF- β 1 present and is lower than the 1.5-fold change used by Rodriguez et al. to denote significance and the 2-fold change used to classify a factor as physiologically relevant by Grassel et al. (Grassel et al., 2009, Rodriguez et al., 2015). This result highlights the fact that this form of analysis is extremely valuable for identifying the presence of potential factors of interest within samples, but more precise techniques such as an ELISA should be performed for absolute quantification. For this reason, comparisons between TGF- β 1 stimulated scaffolds and controls and loaded groups and controls may provide clearer differences between groups than direct comparison between the TGF- β 1 stimulated and loaded groups.

Following the results of secretome analysis the work was repeated to confirm that changes observed at the protein level were also evident at the transcriptional level using real-time PCR. The results of this analysis confirmed some of the changes seen in the secretome data e.g for angiopoietin 2 and osteoprotegrin, but did not confirm the changes seen in all the factors e.g. VEGF and PDGF $\alpha\alpha$. Gene expression analysis was able to confirm the down regulation of the expression of angiopoietin 2 and osteoprotegrin in response to TGF- β 1 stimulation and the up regulation of MMP13 in response to both forms of stimuli. The results also suggested a down regulation of GRO in both stimulated groups as seen in the secretome data, but this was not significant. The detection of similar changes in both secretome and gene expression analysis suggest that the changes seen in these factors are robust; this highlights angiopoietin 2, osteoprotegrin, MMP13 and GRO α as factors of particular interest. The

changes seen in leptin and angiogenin, however, were not confirmed by real-time PCR. This may be due to the small fold changes seen in the secretome results for these factors (angiogenin: TGF- β 1-control 1.24-fold, load-control 1.33-fold, TGF- β 1-load 0.93-fold, leptin: TGF- β 1-control 1.58-fold, load-control 2.22-fold, TGF- β 1-load 0.71-fold). The low overall fold change in the detected levels of these factors may explain the lack of a significant change in gene expression levels despite a significant change in the amounts of protein detected.

The results of the secretome analysis show both clear similarities and differences between the effects of load and TGF- β 1 on the secretome of stimulated MCS, however, this was only backed up in some cases by gene expression analysis. There are several factors that may contribute to this mismatch in results. This may be linked, in part, to the use of different samples from different experimental repeats which may have behaved differently in response to the same stimuli. However, conversely this also supports the veracity of the changes in angiopoietin 2, GRO α , MMP13 and osteopontin, which were similar in both sets of results, despite the analysis of samples from completely different experimental repeats. These data may also be affected by the fact that the relationship between the level of a certain species of mRNA present within a cell, the amount of the corresponding protein translated by the cell and the amount of that protein released into the culture media by the cell are clouded by a plethora of processes that make direct comparisons hard to draw. This was also seen in Chapter 4 where the results of real-time PCR were ambiguous whilst the results of histology were clear cut. The timing of sample taking may also play a role in the mismatch of some of the mRNA and protein data. This is because protein and gene expression were determined at the same time point, day 8. As a result the mRNA expression may have already peaked and dropped earlier in the culture period which the protein was still detectable in the medium.

Specifically in the case of leptin and angiogenin the specificity and sensitivity of the different techniques used may play a role in producing different results where the overall change in the total amount of protein is not that large. Variation in the samples may play a role in influencing the perceived differences between results; this variation is observed both within samples from one donor and between donors (e.g. as seen in gene expression data for GRO α and MMP13), and may mask changes between the groups, repeating this work with more donors would be an option to counter this.

A more appropriate way of confirming the results of the cytokine array than RT-PCR would be to perform ELISAs for the factors identified on the culture media, however this was not possible due to high cost of such an approach and the limited quantity of samples available.

The gene expression analysis of the MSC chondrogenesis markers aggrecan, collagen type X and Sox 9 showed that there may be a stronger initial response to load than TGF- β 1 stimulation in aggrecan and Sox 9 expression, however the differences between the groups were not significant. Previous results comparing load and 1 ng/ml TGF- β 1 and multiaxial mechanical load reported by Li et al. (2010) showed a similar effect of TGF- β 1 stimulation and mechanical load on collagen type X expression but the authors did not look at Sox9 expression. The gene expression in this study was also only performed using the top 10% of the scaffold, removing 90% of the scaffold containing unstimulated cells. This may increase the gene expression changes by removing unstimulated cells as described in the discussion of Chapter 4.

Following the initial round of experiments, and processing of the secretome results, the literature was consulted for relevant work that was previously carried out on factors that were identified during secretome analysis as undergoing significant changes. Study of the literature highlighted similar responses to shear forces reported in other cells types in other model systems, For example; Kitase et al. demonstrated the upregulation of MCP3 in response to shear applied via fluid flow in osteoblasts, whilst angiogenin 2 has been shown to be upregulated by cyclic fluid shear in human aortic endothelial cells and cyclic shear generated by a cone and plate device in HUVECs (Tressel et al., 2007, Li et al., 2014). Palumbo et al. (2002) also used a cone and plate set up to show that shear stress increases the expression of PDGF in bovine aortic endothelial cells. The literature also highlighted the increase in nitric oxide (NO) production in response to shear loading in endothelial cells in response to mechanical shear forces (Bao et al., 1999, Goettsch et al., 2008). For this reason, the nitrite concentration in media collected during the second round of experiments was determined using the Griess reaction (as an indirect measure of media NO). The results of nitrite quantification showed that load in this system, as in those described in the literature, leads to higher levels of NO being released into the media compared to control and TGF- β 1 stimulated scaffolds over the first week in culture. NO has been long known as an aggravating factor in arthritis and nitric oxide synthase (NOS) suppression has even been shown to reduce symptoms of arthritis (McCartney-Francis et al., 1993). This suggests that reducing the presence of NO in systems subjected to load could improve the outcome of cartilage repair. However further work is required to identify the role of NO in response to load and the effect that it has on MSC chondrogenesis.

Mechanical load and TGF- β 1 are both known to produce a myriad of effects that are dependent on a huge range of factors. A wide search of the literature was performed for each factor highlighted by this work with regards to MSCs, chondrogenesis,

mechanical load etc. in order to gain an understanding for how the cytokines I analysed in this study respond to TGF- β 1 and various forms of mechanical load in previously published work, and how that matches the results presented here. Many of the factors discussed in this chapter have not been broadly studied with regards to MSC chondrogenesis, The aim of this literature search was therefore to assess whether the effects of these stimuli on other cells in other culture conditions broadly agreed or disagreed with the results presented in this study in the absence, in most cases, of a directly relevant body of literature.

Shear loading is a fundamental part of the multiaxial load applied in this system; shear load in the form of fluid shear or surface interaction has been shown to have similar positive effects to those that were seen in this system on factors such as MCP3 in osteocytes (Kitase et al., 2014), angiopoietin-2 in endothelial cells (Goettsch et al., 2008, Tressel et al., 2007, Li et al., 2014), osteoprotegrin in a bone marrow derived stromal cell line (Kim et al., 2006) and PDGF in endothelial cells (Bao et al., 1999, Palumbo et al., 2002). However there were some differences between these results and the literature e.g. it has been shown that shear can increase GRO production in osteoblasts (Govey et al., 2014), although the choice of cell type may also effect GRO production. Shear loading has been shown to reduce the activity of MMP13 in chondrocyte cultures, in this work no measurement was made of MMP13 activity but an increase in total MMP13 was seen in response to load (and TGF- β 1 stimulation) (Hamamura et al., 2013). In support of the results presented here MMP13 has been shown to be induced at an mRNA level in cartilage explants by shear and compression loading (Fitzgerald et al., 2008). High levels of shear have also been shown to reduce Angiopoietin 2 levels in HUVECs (Goettsch et al., 2008). Due to the importance of shear within the vascular system the majority of research into shear forces which overlap with these factors has been performed in endothelial cells and other vasculature derived cells. The results presented in this chapter, however, reflect the effects of shear loading in these systems despite their very different nature. This similarity in the effects of shear load on MSCs compared to other cell types in the secretome analysis was reflected in the increased presence of NO as determined indirectly via the Griess reaction which has previously been described in endothelial cells stimulated with shear forces (Goettsch et al., 2008, Bao et al., 1999).

Literature on other forms of mechanical load e.g. stretch and compression were also examined and corroborate the results of this study. Exposure to cyclic stretch increases the expression of MIF in gingival cells, MIP3 α in hTERT periodontal ligament cells, and angiopoietin-2 and PDGF in HUVECs (Wang et al., 1997, Lee et al., 2012, Hashimoto et al., 2002, Morimoto et al., 2003, Chang et al., 2003). Compression has been shown to induce the expression of MMP13 and uPAR correlating with the results

of this study (Chen et al., 2013, Chu et al., 2006, Fitzgerald et al., 2008). As with the effects of shear load in other systems it is impossible to draw direct comparisons with our system as these investigations used different types of cells and different types of load in different culture systems, however, the bulk of evidence reported corroborates the effects of mechanical load observed in our system.

Similarities and differences between the effects of TGF- β 1 stimulation on factors in this system and those reported in the literature can also be found. Previous work has shown that TGF- β stimulation induces MCP3 in murine fibroblasts, decreases GRO expression in human lung carcinoma epithelial cells, increases PDGF expression in an osteoclast cell line, induces VEGF and osteoprotegrin in vascular smooth muscle cells, upregulates uPAR expression in an epithelial cell line and stimulates BLC expression at seven days of hBMSC chondrogenesis (Matsukura et al., 2010, Ong et al., 2009, Lo et al., 2013, Wang et al., 1997, Tanabe et al., 2006, Yue et al., 2004, Toffoli et al., 2011, Cristino et al., 2008). These results corroborate the observations in this system and in the absence of further experiments to provide greater, more specific information. There were also effects of TGF- β 1 reported in the literature that do not fit the results produced by the model system used in this study. Cell associated leptin receptor and its ligand leptin were decreased in response to TGF- β 1 in BMSCs (Zeddou et al., 2012), however this does not necessarily reflect the effect of TGF- β 1 on soluble Leptin Receptors as observed in the work presented here. TGF- β 1 has been associated with the shedding of ALCAM from the cell's surface, however this was not observed in this system despite the clear increase in shedding in response to mechanical load. Recent work carried out by Rodriguez et al. investigated the secretome of adipose derived MSCs cultured in monolayer stimulated with 3ng/ml TGF- β 1 (Rodriguez et al., 2015). The results of this work bare some similarities to the data presented in this chapter e.g. a decrease in GRO α in response to TGF- β 1 stimulation. However, clear differences were also apparent to the work in this chapter e.g. no change in VEGF expression in response to TGF- β 1 and a decrease in leptin expression in response to TGF- β 1 stimulation. These differences are likely to result from a combination of the different tissue source used for the MSCs (adipose rather than bone marrow) and the different culture system (2D monolayer culture as opposed to 3D culture within a scaffold).

The results of this work not only bare similarities to work carried out in other systems but also to secretome studies previously carried out on MSCs. Factors such as GRO, VEGF, MIF, MIP3 α and OPG were detected in the media of MSCs cultured in high density monolayers by Liu et al. (Liu and Hwang, 2005). Furthermore, a number of factors found in the secretome of embryonic stem cell derived MSCs (the HuEs9.E1 MSC cell line) were also found in this investigation (ALCAM, Angiogenin, MCP3, GRO, BLC, MIF, MMP13 and VEGF) (Sze et al., 2007). With specific regard to studies

focusing on MSCs undergoing chondrogenesis MMP13 and VEGF have been shown to increase in chondrogenic conditions, as observed in both TGF- β 1 and load induced chondrogenesis in this system (Grassel et al., 2009, Arufe et al., 2011).

Each of the factors that underwent a significant change between groups was included in the review of the literature. The goal of this was to ascertain as much information as possible about the factors despite, in some cases, a lack of directly relevant literature. The relevant results of this literature search are presented here in a condensed form. The search terms used for each factor included the name of the factor and each of; cartilage, chondrogenesis, MSC, mechanical, shear, TGF- β and arthritis.

5.4.1 Leptin

Leptin has previously been described in the growth plate and may play a role in the process of endochondral ossification. Leptin has been localised to prehypertrophic chondrocytes and hypertrophic chondrocytes associated with invading blood vessels in murine growth plates (Kume et al., 2002, Kishida et al., 2005). Leptin knock out models show disruption at the growth plate e.g. disrupted column formation in the hypertrophic zone, however, the exact role of leptin during endochondral ossification has not been elucidated (Kishida et al., 2005). Leptin has, however, been shown to induce Wnt signalling in articular chondrocytes (Ohba et al., 2010).

As well as being localised to the growth plate leptin has also been localised immunohistochemically to arthritic cartilage, with the strength of immunohistochemical labelling shown to increase with OA severity, from very low levels in normal cartilage (Dumond et al., 2003). The expression of leptin at a gene level has also been shown to be higher in osteoarthritic chondrocytes compared to cells from normal joints (Iliopoulos et al., 2007).

Matrix metalloproteinases play a key role in cartilage degradation. Leptin has been associated with the induction of MMP 1, 2, 9 and 13 (Iliopoulos et al., 2007, Hui et al., 2012, Simopoulou et al., 2007). The knock down of leptin with siRNA has also been shown to lead to a down regulation of MMP13 production by osteoarthritic chondrocytes (Iliopoulos et al., 2007).

5.4.2 Leptin Receptor

The leptin receptor, like leptin has been localised to the murine growth plate, specifically in terminally differentiated hypertrophic chondrocytes rather than in cells at

an earlier stage of differentiation (Kishida et al., 2005). Leptin receptor has also been localised to perivascular cells in the bone marrow; leptin receptor positive cells play a key role in adult bone formation and turnover and were the main source of CFU-Fs in the samples tested (Zhou et al., 2014).

Glucocorticoids such as dexamethasone, which was used in both the chondropermissive and chondrogenic media used in this work, have been shown to induce the expression of leptin receptor in umbilical cord MSCs (Zeddou et al., 2012).

5.4.3 MDC

MDC is a chemoattractant produced by macrophages and dendritic cells; higher levels have been detected in the synovial fluid of those suffering from psoriatic arthritis and rheumatoid arthritis than those with osteoarthritis, however, comparisons were not made to controls from normal joints (Flytlie et al., 2010).

5.4.4 MIP3 α

Mechanical load has been associated with an increased level of MIP3 α production in both normal and osteoarthritic chondrocytes (Lee et al., 2012). This upregulation was linked to the presence of reactive oxygen species; its presence in loaded samples at a significantly higher level than controls may, therefore, be linked to the upregulated presence of NO in loaded samples. MIP3 α has also been shown to increase the expression of MMP13 in chondrocytes cultured *in vitro* (Mazzetti et al., 2004).

5.4.5 GRO

GRO as detected by the antibody array represents GRO α , β , and γ , not one specific isoform.

The expression of GRO is upregulated in osteoarthritic chondrocytes and is also found at higher levels in the synovial fluid of patients diagnosed with rheumatoid arthritis (Merz et al., 2003, Endres et al., 2010). GRO has also been shown to induce the expression of MMP13 and collagen type X in bovine chondrocytes cultured *in vitro* when applied at concentrations similar to those detected in the synovial fluid of inflamed joints (Merz et al., 2003). GRO has been described as having a hypertrophic effect by Olivotto et al. who showed that GRO induced both pro and active MMP13 and Runx2 in chondrocytes, whilst it down regulated aggrecan expression (Olivotto et al., 2007).

Work by Wenke et al. has showed that GRO can be induced by Sox9 signalling through the induction of the transcription factor AP2e, however in this experiment, chondrogenic differentiation seems to have the opposite effect on GRO expression (Wenke et al., 2011). This decrease may be linked to the presence of TGF- β 1 in both culture systems which has been shown to block VEGF induced GRO expression in a SMAD independent manner (Lo et al., 2013).

5.4.6 uPAR

A serine protease involved in the activation of the plasmin as well as other proteolytic enzymes such as MMPs (in particular MMP9), uPAR has been shown to be involved in matrix degradation and is found at higher levels in cartilage with increasing severity of osteoarthritis (Yue et al., 2004, Beaufort et al., 2004, Schwab et al., 2004). Mechanical load has been shown to upregulate the expression of multiple components of the uPA system whilst TGF- β 1 has been shown to induce the expression of uPAR in both transformed and non-transformed cells (Yue et al., 2004, Chu et al., 2006, Chen et al., 2013). The response of uPA to load and TGF- β 1 may explain the increases observed within this system. A number of enzymes including plasmin, MMPs, trypsin and uPAR itself can cleave membrane bound uPAR to produce a series of active or inactive cleavage products (Montuori et al., 2005, Sidenius et al., 2000). A change in expression of one of these enzymes in response to TGF- β 1 and mechanical load may, therefore be linked to the increase in response to both forms of chondrogenic stimulation observed. The identity of the uPAR fragment detected by the cytokine antibody array in the culture media has also not been characterised and therefore it is not possible to draw conclusions about uPAR shedding from the identity of the cleavage product.

5.4.7 LAP

The removal of the LAP from the TGF- β small latent complex is a key step in TGF- β activation and results in the release of the fully active TGF- β homodimer (Maeda et al., 2002). TGF- β activation can occur in a variety of ways including protease degradation (e.g. the serine protease plasmin or MMP13), mechanical stimulation (e.g. shear alone or the multiaxial load applied in this system), deglycosylation or the application of a number of physiochemical stimuli such as heat, extremes of pH and UV light (Robertson and Rifkin, 2013, Lyons et al., 1990, Maeda et al., 2002, Albro et al., 2012, D'Angelo et al., 2001). The increased level of LAP produced by MSCs subjected to load may result from the activation of endogenously produced TGF- β 1 in response to load, resulting in LAP release as opposed to the exogenously applied active

recombinant TGF- β 1 that was used to induce chondrogenesis in TGF- β 1 stimulated MSCs.

5.4.8 Angiogenin

As a potent angiogenic factor, angiogenin production is rarely looked at with regards to MSC chondrogenesis. Angiogenin has previously been found in the synovial fluid of patients suffering from arthritis, in particular inflammatory forms of arthritis such as rheumatoid arthritis and crystal induced arthritis (Liote et al., 2003).

5.4.9 Angiopoietin 2

In contrast to angiopoietin 1, angiopoietin 2 disrupts angiogenesis by acting as an antagonist for the Tie2 receptor tyrosine kinase through which angiopoietin 1 signals (Horner et al., 2001). Angiopoietin 2 is found in the proliferating and pre-hypertrophic regions of the growth plate whilst angiopoietin 1 is found lower in the growth plate, suggesting a protective effect against blood vessel invasion and the resulting induction of hypertrophy in these regions (Horner et al., 2001). The induction of angiopoietin 2 in response to shear forces has been demonstrated by several authors and has also been shown in response to mechanical stretch (Goettsch et al., 2008, Tressel et al., 2007, Li et al., 2014, Chang et al., 2003). The stimulatory effect of mechanical load on angiopoietin 2 production was also clearly observed in the results presented in this chapter.

5.4.10 Osteoprotegerin

Osteoprotegerin (OPG), along with RANK and RANKL can be found in the surface layers of normal articular cartilage; the presence of all three increases in osteoarthritis where they can also be found deeper within the mid-zone of cartilage (Komuro et al., 2001). The expression of RANK, RANKL and OPG can also be found in chondrocytes cultured *in vitro*, however, RANKL has no clear effect on the chondrocyte phenotype *in vitro* (Komuro et al., 2001). *In vivo*, OPG has been shown to have some protective effects in arthritis models, however this may be due to a reduction in damage of the subchondral bone through the prevention of degradative changes in response to the induction of arthritis (Shimizu et al., 2007, Kadri et al., 2008).

5.4.11 DR6

A member of the Tumour Necrosis Factor receptor superfamily DR6 plays a role in cell survival, proliferation and apoptosis (Klima et al., 2009). DR6 is known to act through the NF κ B pathway to mediate its effects (Wang et al., 2014b).

5.4.12 TGF- β 1

Articular cartilage contains large amounts of TGF- β . Albro et al. showed that 4-6 week old bovine femoral condyle cartilage contained 68.5 ± 20.6 ng/mL of TGF- β 1, predominantly in the latent form (Albro et al., 2013). TGF- β 1 has been used since the late 1990's to induce the chondrogenesis of MSCs *in vitro* (Johnstone et al., 1998). All three isoforms of TGF- β (1, 2 and 3) have been used to induce chondrogenesis; differences in the effect of the three isoforms, however, have not been clearly established (Barry et al., 2001, Mueller et al., 2010). The application of TGF- β to MSCs induces a phenotype more akin to the growth plate chondrocytes which undergo hypertrophy rather than stable articular chondrocytes (Mueller and Tuan, 2008). Further studies by this group have shown that TGF- β 1 is crucial in the chondrogenic effects of multiaxial load, as TGF- β 1 is produced by MSCs that are exposed to load and that by blocking the TGF- β receptor 1, load induced chondrogenesis is inhibited (Li et al., 2010a).

5.4.13 ALCAM

A cell surface protein, ALCAM is a marker of cells that have tri-lineage potential and is considered a putative MSC marker, particularly when used in combination with endoglin (CD105) (Chang et al., 2013, Alsalameh et al., 2004, Arai et al., 2002). The increased production/release of ALCAM into the media from MSCs subjected to load compared to the other groups is likely due to increased receptor shedding. Previous work has shown that TGF- β can induce ALCAM shedding (Hansen et al., 2014). This is unlikely to be the cause in this model as the TGF- β 1 stimulated group did not change compared to controls. However the response observed by Hansen et al. was linked to an increase in the proteolytic enzyme ADAM17. The results presented in this work may therefore be due to the specific upregulation of ADAM17 or another proteolytic enzyme in response to load which are not TGF- β 1 dependent (Hansen et al., 2014), however the mechanism of action remains to be determined.

5.4.14 BLC (CXCL13)

MSCs have previously been shown to express BLC on day seven of chondrogenic induction by TGF- β 1 in a 3D hyaluronic acid hydrogel (Cristino et al., 2008). This observation corroborates the finding of this chapter and suggests that the change in BLC may be a response to TGF- β 1 stimulation. Neutralisation of BLC with intra-articular anti-BLC antibodies in an autoimmune arthritis model showed decreased levels of cellular invasion and cartilage/bone degeneration (Zheng et al., 2005). The authors of this work suggest that the protective effect of anti-BLC antibodies relies on changes in the immune response rather than changes within the cartilage itself (Zheng et al., 2005).

5.4.15 MCP3

The presence of MCP3, like GRO α has been quantified in the serum of patients suffering from rheumatoid arthritis and been shown to be higher than in the synovial fluid of non-diseased control joints (Endres et al., 2010). This may be due in part to the role that MCP3 plays in the recruitment of monocytes and even MSCs to sites of injury (Cheng et al., 2014). The expression of MCP3 has been linked both to the application of fluid shear forces and TGF- β , which it is induced by, as well as to autoinduction (Ong et al., 2009, Kitase et al., 2014).

5.4.16 MIF

MIF expression is correlated with the changes in synovial fluid associated with rheumatoid arthritis and neutralisation of MIF with intra-articular antibody treatment has been shown to prevent degradation in an autoimmune arthritis model (Santos et al., 2001, Morand and Leech, 2005). The increased secretion of MIF has been linked to glucocorticoids both *in vitro* and *in vivo*, whilst IL10 has been shown to decrease MIF production (Morand and Leech, 2005, Santos et al., 2001). The expression of MIF was detected by Fujihara et al. in both unstimulated chondrocytes cultured *in vitro* and chondrocytes cultured in poly-L-lactic acid scaffolds (Fujihara et al., 2010). Mechanical load, has been shown to induce production of MIF *in vitro* and *in vivo* (Morimoto et al., 2003). Increased MIF production in response to glucocorticoids may explain the high basal level of MIF expression detected in this work and the response to mechanical load would explain the changes observed in MSCs subjected to load. However changes were also evident in the TGF- β 1 stimulated group suggesting an effect of TGF- β 1 as well as load. A protective role for MIF against senescence has been suggested by Palumbo et al. who showed that MIF, in part, mediates the anti-senescence effects of hypoxia on MSCs (Palumbo et al., 2014).

5.4.17 VEGF

In a similar manner to angiogenin, VEGF is often not considered when assessing MSC chondrogenesis, however it was clearly upregulated in response to both load and TGF- β 1 stimulation. VEGF is expressed by hypertrophic chondrocytes in the growth plate as well as in chondrocytes cultured *in vitro* (Bluteau et al., 2007). The role of VEGF in the growth plate is associated with vascularisation and the terminal differentiation of growth plate chondrocytes. The knocking out of VEGF expression leads to the failure of endochondral ossification (Dai and Rabie, 2007). Given the role of VEGF in hypertrophy and terminal differentiation *in vivo* this may provide a research avenue into the hypertrophic differentiation of MSCs *in vitro*.

The production of VEGF has previously been linked to TGF- β by Tanabe et al. who showed that dexamethasone could reduce the TGF- β driven induction of VEGF in aortic smooth muscle cells, and by Jeon et al. who showed that TGF- β induces VEGF expression in murine macrophages (Tanabe et al., 2006, Jeon et al., 2007). Mechanical load, specifically shear has been shown to induce VEGF expression, which in turn leads to an increase in angiopoietin 2 via VEGFR2 (Goettsch et al., 2008). However, in this system the very similar levels of VEGF in loaded and TGF- β 1 stimulated groups do not reflect this.

5.4.18 MMP13

MMP13 is considered to be a marker of chondrocyte and MSC hypertrophy in the growth plate and in culture *in vitro* (Mueller and Tuan, 2008, D'Angelo et al., 2000, D'Angelo et al., 2001, Tchetina et al., 2007). Mechanical load (in the form of uniaxial shear or compression) has been shown to have an effect on the production and activity of MMP13 (Fitzgerald et al., 2008, Hamamura et al., 2013). The application of supraphysiological mechanical load to murine joints with surgically induced osteoarthritis led to a decrease in MMP13 activity and reduced joint degeneration (Hamamura et al., 2013). The application of shear load *in vitro* to human osteoarthritic chondrocytes also reduced MMP13 activity (Hamamura et al., 2013). In contrast, Fitzgerald et al. showed that shear and compression could induce matrix molecule expression and MMP13 expression in cartilage explants (Fitzgerald et al., 2008). MMP13 induction by nitric oxide has previously been demonstrated in bovine aortic endothelial cells (Zaragoza et al., 2002). The level of expression of these two factors follows a similar pattern to the work presented in this chapter suggesting NO may play a role in MMP13 induction in response to load (Zaragoza et al., 2002). Work by D'Angelo et al. showed that MMP13 and other matrix vesicle associated proteases

could activate latent TGF- β ; the activation of MMP13 under load therefore may be associated with the increased activation of TGF- β 1 under load (D'Angelo et al., 2001).

5.4.19 PDGF

The expression of PDGF has been linked to both mechanical load and TGF- β . The application of load using a Flexercell device (which causes biaxial cyclic distortion of the membrane that the cells are attached to) was shown by Wang et al. to induce PDGF expression in an osteoblast cell line, whilst cyclic shear was demonstrated by Bao et al. and Palumbo et al. to induce PDGF expression in endothelial cells (Bao et al., 1999, Palumbo et al., 2002). Bao et al. linked the production of PDGF to the induction of nitric oxide production by shear loading, however, in this work the very similar levels of PDGF in both loaded and TGF- β 1 stimulated groups does not reflect the difference in nitrite levels detected in the media (Bao et al., 1999). The induction in both of these groups may therefore result from TGF- β induction which has previously been described in mesenchymal cells (Takaishi et al., 1994).

The factors identified through the secretome analysis, and the subsequent research in to the literature surrounding these factors has opened a plethora of potential avenues for further work.

Leptin was shown by secretome analysis to be upregulated in TGF- β 1 stimulated groups compared to controls. The presence of leptin in hypertrophic growth plate chondrocytes (Kume et al., 2002), osteoarthritic compared to normal cartilage (Dumond et al., 2003), its upregulation by the ALK1 SMAD 1/5/8 pathway (Zeddou et al., 2012), the induction of NO production by leptin (Vuolteenaho et al., 2014), and its role in the induction and activation of MMPs, including MMP13 (Iliopoulos et al., 2007), suggest a link to terminal chondrocyte differentiation and hypertrophy. Previous work has already shown that it is possible through siRNA mediated knockdown of leptin to reduce the production of the hypertrophy marker MMP13 in osteoarthritic chondrocytes (Iliopoulos et al., 2007). Targeting leptin signalling using siRNA or by using antibodies to target soluble leptin in the medium, or the leptin receptor (which was also upregulated in TGF- β 1 stimulated groups), and then looking at the effect on the induction of chondrogenesis and progression towards hypertrophy would show the extent to which leptin signalling is involved in MSC hypertrophy, and whether modulating its effects could prevent the terminal differentiation of MSCs.

Another factor linked to chondrocyte hypertrophy, MMP13 induction and activation, type X collagen expression and osteoarthritis is GRO (Merz et al., 2003, Olivotto et al.,

2007, Wenke et al., 2011); in our multiaxial loading system and in response to 1 ng/ml TGF- β 1 GRO was shown to be down regulated in response to chondrogenic stimuli. This is contrary to other results in the literature (Wenke et al., 2011) and poses some interesting questions, e.g. the hypertrophic effect of GRO in chondrocytes is well documented, but does it have the same effect in MSCs, and if GRO does have a hypertrophic effect on MSCs, what is the mechanism behind it and can it be modulated to produce a more stable cartilage producing phenotype? The first of these questions could be investigated by administering exogenous recombinant GRO to chondrogenically differentiating chondrocytes and looking at standard chondrogenic and hypertrophic markers. The second would require more detailed investigation into the induction and production of GRO in response to chondrogenesis e.g. by examining the p38 MAPK pathway that has been linked to the induction of hypertrophy by GRO (Wenke et al., 2011), or investigating the link between GRO and the transcription factor AP-2e which has also been shown to be regulated by Sox9 in chondrocytes, although in a manner contradictory to the results seen here (Wenke et al., 2011, Wenke et al., 2009).

The effect of load on TGF- β 1 presented in chapter 4 shows the importance not just of growth factor production but also of activation in this model system. uPAR's involvement in the activation of plasmin, which is a known activator of TGF- β , and MMPs (Schwab et al., 2004) as well as its responsiveness to TGF- β and mechanical load (Yue et al., 2004, Chu et al., 2006) make it very interesting within this system, especially with regards to providing a mechanism for the load induced activation of TGF- β 1. In order to investigate this it would first be required to determine if uPAR activity is positively or negatively regulated by chondrogenic stimuli, as the current results show increased uPAR in the media which could be related to a number of factors e.g. proteolytic shedding from the cell surface. If the effect on uPAR activity was positive then the effect of load on TGF- β activation through plasmin could be investigated by loading scaffolds in the presence and absence of different concentrations of EACA, a plasmin inhibitor, which was provided in the culture media used during the work carried out in this thesis, to prevent fibrin degradation (Kupcsik et al., 2009). Another factor that has previously been shown to be responsive to load and has the ability to activate TGF- β is MMP13 (Fitzgerald et al., 2008, D'Angelo et al., 2001). This makes MMP13 another potential candidate for the activation of TGF- β 1 in response to load, and could be investigated through the use of an MMP blocker in the presence of mechanical stimulation.

Angiopoietin 2 may also be able to provide an insight into the effect of load in this system. Other work has shown that angiopoietin 2 is upregulated in response to shear load in different systems (Goettsch et al., 2008, Tressel et al., 2007, Li et al., 2014).

These investigations have shown that VEGF (Goettsch et al., 2008) and canonical Wnt signalling (Li et al., 2014) played a role upstream in the induction of angiopoietin 2 in response to shear loading. By blocking VEGF signalling or Wnt signalling in the presence of load it would be possible to determine whether these pathways also play a role in the response to load in this system by looking at any changes in chondrogenesis in response to load.

A factor not normally discussed with regard to MSC chondrogenesis is VEGF, yet in the secretome analysis it was significantly upregulated in response to TGF- β 1 stimulation and load. This may be due to the induction of VEGF by TGF- β 1 (Jeon et al., 2007, Tanabe et al., 2006), although shear has also been shown to induce VEGF expression (Goettsch et al., 2008). As described above VEGF may be involved in the response of cells to shear forces, but it also induces the expression of GRO (Lo et al., 2013). VEGF is also a potent angiogenic factor and the chondrocyte specific knock out of VEGF leads to failed endochondral ossification (Dai and Rabie, 2007). For these reasons the effect of blocking VEGF signalling during the induction of chondrogenesis in MSCs, and the progression of cells towards terminal differentiation may provide a valuable insight in to the full effect of TGF- β during chondrogenic differentiation, as the potential induction of VEGF by the main chondrogenic stimulus, TGF- β 1, may also be inducing terminal differentiation through GRO rather than stable chondrogenesis.

Nitric oxide (indirectly measured through nitrite release into the media) was not identified by the initial secretome analysis, but was identified as a factor upregulated by load in the second round of experiments that was designed to build on the secretome analysis data. NO has previously been shown to increase in response to shear loading (Goettsch et al., 2008, Bao et al., 1999). NO has also been linked to the production and activation of MMP13 (Zaragoza et al., 2002) and is associated with arthritis, as blocking NO synthase (NOS) has been shown to reduce the severity of disease in animal models of arthritis (McCartney-Francis et al., 1993). This makes NO a very interesting factor in this system particularly on the behaviour of MSCs cultured with higher NO levels in response to mechanical load; blocking NO synthesis by NOS may improve the response of cells cultured in this system by removing this chondro-inhibitory stimulus, which may also have an effect on hypertrophy (as suggested by its ability to induce MMP13 expression and activation). Load induced production of NO is also relevant in the rehabilitation after cartilage repair surgery e.g. microfracture, where the induction of NO in response to mechanical load may hinder the repair process, especially as the levels detected in culture media in this system in response to load were higher than those that have been detected in arthritic joints (Farrell et al., 1992).

5.5 Conclusions

The aim of this chapter was to characterise the secretomes of loaded and TGF- β 1 stimulated cells in order to compare the effects of these two forms of chondrogenic stimuli, which have been shown to be related through TGF- β 1, and in doing so identify potentially novel factors for investigation with regards to cartilage tissue engineering with MSCs.

The results of secretome analysis presented here show that there are clear similarities and differences between the secretomes of MSCs stimulated with multiaxial mechanical load or TGF- β 1. These results indicate that there were a number of factors that are, potentially, specifically associated with TGF- β 1 that share patterns of expression between these two chondrogenic conditions (BLC, MCP3, MIF, VEGF, MMP13 and PDGF, as well as GRO and angiogenin which showed similar changes in response to TGF- β 1 stimulation without reaching significance) whilst other factors are associated more specifically with TGF- β 1 stimulation or load e.g. leptin or angiopoietin-2. This indicates that despite the key role of TGF- β 1 in both systems multiaxial mechanical load and TGF- β 1 induced chondrogenesis are not analogous, but both have shared and differing effects on differentiating MSCs.

Nineteen factors were identified by secretome analysis as being significantly different in two or more groups. Of particular interest as markers for the process of MSC chondrogenesis are the factors that showed similar changes in response to both TGF- β 1 stimulation and mechanical load. In addition the three factors (angiopoietin 2, MMP13 and osteoprotegrin) whose changes in secretome profile between the groups were mirrored by their changes in gene expression as determined by real-time PCR. Alongside the identification of these factors in chondrogenically stimulated MSCs, this work, for the first time, also identified the increase in NO production in response to joint simulating mechanical load. These factors, and the manipulation of them, offer a multitude of potentially interesting avenues for further investigation with regards to cartilage tissue engineering.

Chapter 6 General Discussion

Mesenchymal stem cells hold great potential in the regeneration of musculoskeletal tissues such as bone and cartilage. However, the induction of chondrogenesis in MSCs with the growth factor TGF- β , or mechanical load, results in a hypertrophic phenotype which is closer to that of growth plate chondrocytes than stable articular chondrocytes, and hinders the clinical use of MSCs. The aims of this thesis were three-fold. The first aim, investigated in Chapters 3 and 4, was to produce MSC containing fibrin poly(ester-urethane) constructs whose cell populations were spatially and temporally (in terms of the stage of chondrogenic differentiation) heterogeneous, and determine the effect of signalling between different populations within the constructs on chondrogenic differentiation and hypertrophy. The second aim of this thesis was to investigate potentially novel markers for chondrogenesis in MSCs (Chapter 5). The need for potentially novel markers was highlighted by the inconsistency between the results of the different forms of analysis used in Chapter 4. The third and final aim was to compare the effect of chondrogenic induction with TGF- β 1 or mechanical load on MSCs by analysing the profile of the proteins secreted by stimulated cells into the culture medium (Chapter 5).

The approach taken in this work was not that of a classical tissue engineering application, whereby steps are taken in order to optimise a culture system *in vitro* for the generation of tissue as similar to that of the tissue targeted for repair *in vivo* as possible. Instead the approach in this project was to use a bioreactor capable of applying joint-like multiaxial load in order to model the effect of the loading environment of a diarthrodial joint on MSC containing fibrin-poly(ester-urethane) scaffolds in order to investigate the effects of joint loading on regenerative medicine constructs. With this in mind, the decision was made to use MSCs, as these are widely considered to be candidate cells for regenerative medicine based therapies for a wide range of indications, and to use a fibrin scaffold, as this is already in daily clinical use around the world. The combination of bone marrow derived MSCs and fibrin scaffold is also similar in nature to the repair tissue generated through microfracture, a commonly used marrow stimulation technique for cartilage repair. Therefore, the results obtained could be informative for rehabilitation protocols.

6.1 The effect of temporally and spatially structured scaffolds on MSC chondrogenesis and hypertrophy

It has been hypothesised that the loss of the strict spatial relationships between cells at different stages of differentiation that govern the formation of cartilage and bone *in vivo* plays a role in the progression of MSCs towards a hypertrophic phenotype when they are chondrogenically differentiated (Pelttari et al., 2006). Currently, well accepted models of MSC chondrogenesis, such as the pellet culture, involve the synchronous differentiation of a single population of MSCs, driven by the exogenous application of 10 ng/ml TGF- β 1 (Johnstone et al., 1998). Within such a model there is no potential for separate populations of cells to interact with each other. Drawing inspiration from the growth plate, whilst not aiming to mimic or replicate its processes directly, I aimed to both re-introduce a degree of structural organisation, and to expose MSCs to populations of cells at different stages of chondrogenic differentiation, to determine the effect of such a culture system on the induction of chondrogenesis and hypertrophy in mechanically stimulated MSCs (presented in Chapter 3). A degree of structural organisation was introduced into scaffolds by stacking 2x8 mm fibrin-poly(ester-urethane) scaffolds seeded with populations of MSCs at different stages of chondrogenic differentiation, one on top of the other. In order to expose MSCs to cells at a different stage of chondrogenic differentiation some of the MSC containing 2x8 mm scaffolds were exposed to chondrogenic medium containing 10 ng/ml TGF- β 1 for one or two weeks (group 3 and 4 bottom scaffolds respectively). This allowed for naïve MSCs to be cultured in the proximity of scaffolds containing other naïve MSCs or MSCs at two different points along the chondrogenic differentiation pathway. The effect of this culture system on the chondrogenic response of embedded cells to load was then determined biochemically by analysing the DNA content of the scaffolds, the GAG content of scaffolds and collected culture media as well as quantifying the the TGF- β 1 content of collected culture media, and performing histological and gene expression analysis.

The results of this work were highly variable. This is common when working with primary human cells, particularly in this work where there were clear differences in the ability of cells isolated from different bone marrow preparations to undergo chondrogenesis. This was evidenced, for example, by the very low or non-detectable amounts of collagen type II and aggrecan mRNA that could be isolated from one of the donors cells after being cultured under certain experimental conditions, specifically control groups and group 1 and 2 scaffolds. Although trends were apparent in many situations, these differences did not reach significance at the 5% level, therefore the outcome of the study may have been improved by carrying out more repeats of the experiment with different donors. It may have also been beneficial to use cells from

marrow preparations that have already been shown to be chondrogenic or to use another metric e.g. the speed of cell growth during the expansion phase (Glueck et al., 2015) to select, or more importantly reject certain donors for use in experiments.

Gene expression analysis demonstrated that load had a significant and positive effect on the expression of Sox9 and collagen type X across all of the groups. These data suggest that the application of load has a positive chondrogenic effect. Under these conditions I could find little evidence that interaction between cells at different stages of differentiation affected the progression of cells towards hypertrophy. However, high degrees of variation meant that little statistical significance could be shown in the gene expression data. The lack of significance in the presence of clear trends within the data (e.g. the upregulation of aggrecan gene expression in group 1 in response to load) may be related to the low 'n-number' of some groups. A low 'n-number' reduces the number of degrees of freedom and therefore increases the threshold for a significant result to be recorded. The analysis may also be affected by the large number of groups (14) which were being statistically compared using non-parametric tests; this relatively large number of comparisons (e.g. compared to work presented in Chapter 4 where 6 groups were compared or Chapter 5 where 3 groups were compared) and as a result a lower p-value is required in order for a result to pass as significant during a multiple comparisons correction (in this case Dunn's Multiple comparison test). In order to reduce the number of groups and remove some of the variability in the results the gene expression data from control groups alone was also analysed. This highlighted a significant increase in collagen type X expression in group 4 top control scaffolds (naïve MSCs, placed on top of scaffolds containing MSCs that had undergone two weeks of predifferentiation) compared to group 1 (naïve MSCs in both top and bottom scaffolds) top control scaffolds and a trend towards a similar increase in Sox9 and aggrecan mRNA expression. This increase in the expression of markers associated with MSC chondrogenesis suggests that the predifferentiated group 4 bottom scaffolds have a chondrogenic effect on group 4 top control scaffolds which is not replicated in group 1, where both top and bottom scaffolds contain naïve MSCs. Interestingly, this data indicates that the preculture of bottom scaffolds may have a chondrogenic effect on top scaffolds, even in the absence of other chondrogenic stimuli such as mechanical load or TGF- β 1. This potential chondrogenic effect of predifferentiated bottom scaffolds was also evidenced in the results of histological staining for sulphated GAG with Safranin O. The results of Safranin O staining of group 3 and 4 control scaffolds demonstrated the deposition of sulphated GAG in control top scaffolds, despite the application of no other chondrogenic stimulus than the predifferentiated bottom scaffolds. This GAG deposition may result from the diffusion of GAG from the underlying scaffold into the top scaffold or it may be due to a chondrogenic effect of the

bottom scaffold on the top scaffold. The distribution of the GAG within the top scaffolds matches that detected in chondrogenically differentiated scaffolds, suggesting that the GAG may have been deposited by chondrogenically differentiating cells in the top scaffold. It would be possible to investigate whether this deposition is due to diffusion of GAG into the upper scaffold by culturing an acellular fibrin filled scaffold on top of a predifferentiated scaffold and then performing histology on the upper scaffold to determine the degree of GAG diffusion into the top scaffold. However, this would remove any effect of cross talk between the two scaffolds and may therefore, change the level of GAG production. In order to test the potential for direct chondrogenic stimulation from the bottom scaffold, the bottom scaffold could be predifferentiated as described and then transferred to chondropermissive media as in this work. The culture media could then be collected and analysed for its TGF- β 1 content and also applied to naïve MSCs in a conditioned media study to ascertain if the bottom scaffolds release soluble factors that can stimulate chondrogenesis in an indirect co-culture model.

In retrospect, a major flaw with the analysis performed in this chapter is the inability to account for the relative contribution of each scaffold (top and bottom) to the GAG and TGF- β 1 quantified in the culture media. This problem arose through the alteration in construct design in my work from the solid, one piece 4x8 mm scaffold used in previous studies (Li et al., 2010a, Schatti et al., 2011, Neumann et al., 2013) and in the other chapters of my thesis, to two 2x8 mm scaffolds stacked on top of each other. This alteration in construct design, and my focus on the relative behaviour of the populations in the two different scaffolds means that whilst it was previously possible to attribute all of the factors released into the medium to one population of cells, this was not possible in my work. It is clear from my work that both scaffolds can contribute to the molecules released into the culture media (e.g. the media GAG content is clearly affected by the bottom scaffolds, particularly in response to load). As a result it was not possible to produce a suitable GAG/DNA ratio for the scaffolds, which has previously been an important outcome measure for this type of study (Li et al., 2010a, Schatti et al., 2011, Neumann et al., 2013). The quantification of GAG and TGF- β 1 in the culture media also needs to be interpreted with caution as the results represent biosynthesis occurring in the scaffold as a complete unit but provides no resolution between the two scaffolds. As previously discussed (Section 3.4), tracking the origin of the GAG produced in the two scaffolds provides a significant hurdle and is not easily solved. Another approach to the analysis of the effect of this system on chondrogenesis would be to perform a much more detailed histological analysis, rather than focus on the biochemical results. The first step in this process would be to maintain the relative orientations of the top and bottom scaffolds as they were during culture throughout the histological processing. This would be possible as mentioned in the discussion of

Chapter 3 through the snap freezing of scaffolds within a mould. As presented here, the only histological analysis performed was Safranin O staining for sulphated GAG. Expanded histological analysis could include the immunohistochemical labelling of collagen type II, collagen type X (should a suitable antibody that does not cross react with fibrin be found) and aggrecan to show the deposition of cartilage extracellular matrix components within the scaffolds, relative to the two scaffolds in the system and the loaded surface of the top scaffold. In addition to this, fluorescence in-situ hybridisation (FISH) could be used to determine where in the scaffolds the mRNA for proteins such as collagen type II, collagen type X, Sox9 and aggrecan are being produced. This would be extremely useful in determining the relative effect of the top and bottom scaffolds on each other e.g. through the relative amounts of matrix deposition/mRNA expression along the border of the two scaffolds. This form of analysis, in particular FISH, could also be extremely useful for tracking the changes in matrix deposition and the expression of different mRNA transcripts both spatially and temporally by performing analysis at multiple time points. In the study presented in Chapter 3 all gene expression analysis, histology and biochemical analysis of the scaffolds were performed at the end of the culture period. By observing the changes in matrix deposition/mRNA expression more closely over time it may be possible to better compare the effect of this culture system and the MSCs cultured within it on chondrogenesis and hypertrophy.

The investigation performed into the effect of temporally and spatially structured scaffolds on MSC chondrogenesis and hypertrophy demonstrated that despite some evidence that MSCs at different stages of chondrogenic differentiation could interact and, potentially, induce a chondrogenic phenotype in cells that received no other chondrogenic stimulation, no clear evidence was obtained that the co-culture model system had an effect on the induction of chondrogenesis or hypertrophy in MSCs in the presence or absence of load. This lack of evidence was, in part, related to the complexity of the model system which involved using cells at different stages of differentiation, in multilayer constructs in either the presence or absence of mechanical load. As a result, the focus of my work presented in Chapter 4 turned to producing a simpler model to investigate the potential effects hinted at by the results presented in Chapter 3.

6.2 The effect of asymmetrically seeding scaffolds on MSC chondrogenesis and hypertrophy

The work presented in Chapter 4 aimed to investigate the combined observations of two previous studies. The first of these observations was the potential for separate populations of MSCs to affect their respective chondrogenic differentiation when cultured in the same fibrin-poly(ester-urethane) construct (as described in Chapter 3). The second observation was that in the loading system described in this thesis the application of dynamic mechanical shear alone had a greater chondrogenic effect on MSCs than the application of cyclic compression alone (Schatti et al., 2011). The initial aim of the work in Chapter 4 was therefore to seed fibrin-poly(ester-urethane) scaffolds with two populations of cells; one population seeded inside the scaffold as described previously, and a second population on the loaded surface of the scaffold, which would be directly exposed to the shear component of the load. The effect of this seeding pattern on the chondrogenesis and hypertrophy of the MSCs was then investigated in response to multiaxial mechanical load.

In order to confirm the presence of cells on the surface of scaffolds a number of fibrin-poly(ester-urethane) constructs were seeded with cells that had been labelled with the red fluorescent dye PKH26. Imaging after four weeks of culture demonstrated that the cells seeded on the surface appear to remain on/around the surface of the scaffold. Furthermore, in group 2 the cells in the control scaffolds appear widely distributed throughout the uppermost regions of the scaffold whilst cells seeded on the surface of loaded scaffolds surface of the scaffold remained localised only to the very surface of the scaffold. The results of this staining therefore suggest that the cells do remain on the surface of the scaffold over four weeks of culture. However, the presence of the dye does not indicate the viability or activity of the cells. No direct observations were made in order to study the nature of the cells seeded on the surface of the scaffolds. However, the DNA content of group 3 control and loaded scaffolds after four weeks of culture suggests approximately the same number of cells that were seeded onto the scaffolds are also found after four weeks of culture. Over the course of processing for analysis it was also possible to extract mRNA from group 3 scaffolds. These two facts do not directly confirm, but indirectly suggest that there is a viable population of cells on the surface of the scaffold after four weeks in culture in group 3, which suggests that cells seeded onto the scaffold surface can survive the culture period, and mechanical loading, and remain viable and active. Further investigation into the activity of cells seeded onto the scaffold surface could be performed on scaffolds seeded in the manner of group 3 using a metabolic activity assay such as almar blue or MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Scaffolds could also be fixed and processed for histology after an MTT assay had been performed in order to show

the location and distribution of metabolically active cells. The fluorescent tracking of the cells was performed only once ($n=1$ for each group), at an early stage of the project, as an indication of cell location after four weeks in culture. Due to the subsequent lack of clear differences in scaffold behaviour that could be detected biochemically or at the transcriptional level, the specific location, phenotype, and interaction of cells seeded on the scaffold surface is of greater interest now than at the time this component of the study was performed. As a result, repetition of this experiment and expansion to include investigation of cell viability, metabolic activity, proliferation and cell-cell interactions (e.g. by labelling for the presence and functionality of connexins in the scaffolds) may provide more information on the differences between group 1 and group 2 scaffolds.

Histological and immunohistochemical analysis of the constructs showed that asymmetrically seeded scaffolds (group 2) demonstrated increased deposition of cartilage-like matrix in response to load when compared to evenly seeded scaffolds (group 1). This analysis also demonstrated that no histologically detectable matrix was deposited in group 3 scaffolds. As a result it is likely that the increased staining in group 2 scaffolds compared to group 1 scaffold is not due to the activity of the cells seeded on the surface themselves but instead the interaction between the cells seeded on the surface and the cells seeded within the scaffold. The increased deposition could be caused by increased matrix retention in response to the seeding of the cells on the surface of the scaffold; however, analysis of total GAG content measured in both the scaffolds and culture medium suggests that this is not the case. Interaction between a layer of cells on the surface of a cartilage tissue engineering scaffold and cells seeded within the scaffold has been recently shown by Mesallati et al. (2015). This study investigated the effect of seeding a layer of MSCs (derived from either bone marrow or intra-patellar fat pad) on top of an agarose hydrogel containing articular chondrocytes (Mesallati et al., 2015). The results showed that the application of a layer of either bone marrow or fat pad derived MSCs onto the surface of the scaffold led to increased proliferation of the chondrocytes within the scaffold and the increased deposition of sulphated GAG (Mesallati et al., 2015). These data support the results reported in Chapter 4 that suggest the potential for increased matrix deposition in response to structuring the scaffold in this way, despite the use of a different scaffold system, cell sources and chondrogenic stimulation. Proliferation of the cells within the scaffolds was identified by Mesallati et al. (2015), however the proliferation of cells within, or on top of the scaffold was not characterised in the system presented in Chapter 4. The proliferation of cells in different locations within the scaffold could be assessed using bromodeoxyuridine (BrdU) incorporation assay and histological processing.

Despite the differences in matrix deposition that were detected histologically, no clear differences were detected between group 1 and group 2 scaffolds in the analysis of media and scaffold GAG contents, the degree of GAG retention within the scaffolds, the relative gene expression levels within scaffolds at day 7 or 28 of culture, the deposition of collagen type VI, the total production of TGF- β 1, the activation of TGF- β 1 or the retention of TGF- β 1 in the deposited extracellular matrix. The exhaustive investigation of the "standard" or "classical" markers of MSC chondrogenesis that are routinely used to characterise the chondrogenesis of MSCs *in vitro* yielded no further explanation or confirmation of the matrix deposition effects observed at a histological level. This suggests that there may be other markers which are not currently being investigated that provide useful information about the differentiation state of MSCs. Therefore, in order to explore potentially new markers of MSC chondrogenesis the work described in Chapter 5 analysed a panel of soluble proteins released by MSCs in response to chondrogenic stimulation with TGF- β 1 or mechanical load.

The second aim of the work described in Chapter 4 was to investigate the effect of multiaxial mechanical load on the activation of TGF- β 1. Previous work by Li et al. (2009) has shown that the application of multiaxial load in our bioreactor system leads to the production and release of TGF- β 1 into the culture medium surrounding the scaffolds, which then drives the chondrogenic response observed in mechanically stimulated MSCs (Li et al., 2009). Latent TGF- β 1 is known to be activated by a number of forms of mechanical force including fluid shear and cell generated traction forces (Albro et al., 2012, Annes et al., 2004, Wipff et al., 2007), hence the work presented in Chapter 4 aimed to determine if the multiaxial load applied using our bioreactor system can also activate latent TGF- β 1. The percentage of active TGF- β 1 was determined in the culture medium using an ELISA kit. The results demonstrate that the percentage of active TGF- β 1 was significantly increased in all three loaded groups compared to their respective free swelling controls. The degree of TGF- β 1 activation was also similar in all three loaded groups at all four time points (week 1, 2, 3 and 4) despite the scaffolds containing different numbers of cells and cells at different stages of differentiation. The activation of TGF- β 1 has previously been well characterised in response to shear forces generated through fluid flow or the stirring of a fluid (Albro et al., 2012, Ahamed et al., 2008). Work by Li et al. (2012) previously investigated the effect of mechanical load on the production and activation of TGF- β 1 by rat bone marrow derived MSCs in a chondrogenic environment. Uniaxial compression was applied to rat MSCs in alginate scaffolds after a seven day preculture period in chondrogenic medium containing TGF- β 1; the results showed that the application of load increased both the production and activation of TGF- β 1 in response to mechanical load (Li et al., 2012). The increase in both the production and activation of TGF- β 1 by mechanically stimulated MSCs is very

similar to the results described in Chapter 4. The total TGF- β 1 content described by Li et al. (2012) is similar to but higher than that observed in response to multiaxial load (Chapter 4), however, the percentage of active TGF- β 1 reported in response to application of uniaxial compression is similar to, but lower than, the percentage of active TGF- β 1 detected in response to multiaxial load in Chapter 4 (Li et al., 2012). Uniaxial compression and shear loading have both been shown to activate TGF- β 1 in different models (Albro et al., 2012, Li et al., 2012); however, the system described in Chapter 4 applied a combination of both shear and compression. The importance of the shear component of the multiaxial load, above that of compression alone, on inducing chondrogenesis in MSCs in our bioreactor system has already been described (Section 4.1). However, the investigation described in Chapter 4 did not extend to the characterisation of the effects of the separate components of the load on TGF- β 1 activation, rather the application of multiaxial load. This is discussed further in the Future Work section of this chapter. Further work could also investigate the effect of load on TGF- β 2 and 3, as well as TGF- β receptor expression. Previous work has shown TGF- β 1 to be the most important isoform in this system (Li et al., 2010a); however, the changes in cell distribution within the scaffold may also affect other parts of the TGF- β pathway.

Until the work in Chapter 4 was performed, the established hypothesis for the induction of chondrogenesis in our bioreactor system was that the application of mechanical load leads to the production of endogenous TGF- β 1 by the loaded cells (Li et al., 2010a). Endogenously produced TGF- β 1 then induces the changes observed in the gene expression of chondrogenic markers like collagen type II and aggrecan in MSCs within the scaffolds and the associated deposition of cartilage-like extracellular matrix proteins (Li et al., 2010a). However, the novel results presented in Chapter 4 suggest that the process is actually more complex; the application of multiaxial mechanical load induces the endogenous production of latent TGF- β 1 by loaded cells, the latent TGF- β 1 is then activated by the application of further mechanical load which then has a chondrogenic effect on the cells within the scaffold. This information provides another level of mechanistic understanding of our system and provides a staging post going forwards for further study of the effect of joint-like load on MSCs.

A weakness of the Chapter 4 study is the lack of parallel groups for group 1-3 that are stimulated with TGF- β 1 rather than mechanical load. Exclusion of these groups means that it is not possible with the current data set to determine if the increased matrix deposition detected histologically in group 2 loaded scaffolds compared to group 1 loaded scaffolds is due to the specific stimulation of the surface population with the shear component of the mechanical load, or simply due to the presence of the population itself, regardless of the chondrogenic stimulus applied. Repeating the work

with groups stimulated with TGF- β 1 would allow for characterisation of the response of cells in the scaffolds to a chondrogenic stimulus that does not directly target the surface population, in the way that the mechanical load applied in this work did. Should the results in TGF- β 1 stimulated scaffolds match that of the mechanically loaded scaffolds then that would suggest that the increased matrix deposition observed in group 2 scaffolds is due to the presence of the population of cells on the surface of the scaffold interacting with the cells seeded inside the scaffold, but independently of the effect of mechanical load, specifically shear, on the cells seeded on the scaffold surface.

Another weakness of the study performed in Chapter 4 (and the study described in Chapter 3) is the limited characterisation of the effect of this culture system on hypertrophy. Collagen type X transcript levels were determined in all groups and showed no differences between groups 1 and 2. Immunohistochemistry was also performed to label for the presence of collagen type X deposited within the scaffolds themselves. Given the importance of histological and immunohistochemical characterisation of the molecules deposited in the scaffolds presented in Chapter 4 the specific location and relative amount of collagen type X inside the scaffolds was of singular interest. However, due to the cross reactivity of the anti-collagen type X antibody with the fibrin component of the scaffold this was not possible. The results of the collagen type X labelling do suggest that areas that stain positively with safranin O and toluidine blue for sulphated GAG and collagen type II, which indicated an area undergoing a chondrogenic response, do not stain positively with collagen type X. The absence of collagen type X in these areas could not be confirmed due to the background staining detected in these sections. Localised detection of collagen type X would provide extremely interesting information on the chondrogenic effect of load in this system and the potential stability of the cartilage-like tissue that can be generated. In the absence of a suitable collagen type X antibody, an in-situ hybridisation approach to detect collagen type X mRNA would also provide important information about the potential localisation of collagen type X production within the scaffolds, allowing for further comparison between groups 1 and 2 and a better understanding on the chondrogenic phenotype that is induced by the multiaxial, joint-like mechanical load applied in this system.

6.3 Using the secretome of chondrogenically stimulated cells to investigate potentially novel markers of MSC chondrogenesis and compare the effects of TGF- β 1 stimulation and multiaxial mechanical load on MSCs

Analysis of the bioactive factors released by MSCs seeded into fibrin-poly(ester-urethane) scaffolds was performed in Chapter 5 with two aims in mind. The first aim was to compare the effect of two different chondrogenic stimuli, namely the exogenous application of TGF- β 1 and mechanical load, on the secretomes of stimulated cells to determine if the two forms of stimulation are analogous or different in the responses that they elicit. As previously described in this thesis, the chondrogenic effect of multiaxial load is heavily reliant on TGF- β 1 as load induces both the production and activation of this key chondrogenic stimulus, but the full extent of the relationship between load and TGF- β 1 has not been characterised. The investigation presented in chapter 5 was therefore designed to observe if the effects of mechanical load on the secretome of stimulated cells was similar or different to cells stimulated by application of exogenous TGF- β 1 and vice versa. The second aim of the project was to identify soluble protein factors that might act as potentially novel markers for MSC chondrogenesis.

The results of secretome analysis showed that nineteen factors changed significantly between control, TGF- β 1 stimulated or loaded groups. Of the 19 factors, 11 factors changed between the two groups;

- Leptin, leptin receptor and MDC were upregulated in TGF- β 1 stimulated scaffolds alone compared to controls,
- MIP3 α , uPAR, LAP and angiogenin were significantly increased in the media analysed in loaded groups compared to controls,
- Angiopoietin 2, osteoprotegrin and DR6 were found at a higher concentration in loaded samples compared to TGF- β 1 stimulated samples.

The remaining eight factors changed in two of the three statistical comparisons made between control, TGF- β 1 stimulated and loaded groups;

- TGF- β 1 was significantly higher in the media of TGF- β 1 stimulated scaffolds than control and loaded scaffolds,
- ALCAM was significantly higher in loaded samples than control and TGF- β 1 stimulated samples,
- BLC, MCP3, MIF, VEGF, MMP13 and PDGF $\alpha\alpha$ were significantly higher in loaded and TGF- β 1 stimulated samples than controls.

These results clearly show that there is a cohort of proteins with similar expression patterns in response to both forms of chondrogenic stimulation (e.g. BLC, MMP13,

VEGF and GRO), whilst there are other factors which are differentially upregulated in response to TGF- β 1 (leptin and leptin receptor or load (e.g. uPAR and angiopoietin 2). Interestingly, these data demonstrate that there are clear similarities between the effect of TGF- β 1 and multiaxial mechanical load on MSCs, which is to be expected, given the role of TGF- β 1 in both systems, but also that these two forms of chondrogenic stimulation are not analogous. In fact, both forms of stimulation appear to have effects on factors and pathways that are not affected by the other (e.g. the effect of exogenous TGF- β 1 on the leptin pathway).

A wide search of the literature was performed for each factor identified by this secretome study with regards to MSCs, chondrogenesis, mechanical load etc. in order to gain an understanding for how the bioactive factors that were differentially expressed in this study respond to TGF- β 1 and various forms of mechanical load in previously published work, and how that matches the results presented here. Many of the factors discussed have not been broadly studied with regards to MSC chondrogenesis, therefore, the aim of this literature search was to assess whether the effects of these stimuli on other cells in other culture conditions broadly agreed or disagreed with the results presented in this study in the absence, in most cases, of a directly relevant body of literature.

Shear loading is a fundamental part of the multiaxial load applied in this model system; shear load in the form of fluid shear or surface interaction has been shown to have similar positive effects to those that were observed in this system on factors such as MCP3 in osteocytes (Kitase et al., 2014), angiopoietin-2 in endothelial cells (Goettsch et al., 2008, Tressel et al., 2007, Li et al., 2014), osteoprotegerin in a bone marrow derived stromal cell line (Kim et al., 2006) and PDGF in endothelial cells (Bao et al., 1999, Palumbo et al., 2002). However, there were also differences between the results presented in this thesis and the literature e.g. it has been shown that shear can increase GRO production in osteoblasts (Govey et al., 2014), although the choice of cell type may also affect GRO production. Shear loading has been shown to reduce the activity of MMP13 in chondrocyte cultures (Hamamura et al., 2013), however in my study no measurement was made of MMP13 activity but an increase in total MMP13 was detected in response to load (and TGF- β 1 stimulation). In support of the results presented here, MMP13 has been shown to be induced at an mRNA level in cartilage explants by shear and compression loading (Fitzgerald et al., 2008). High levels of shear have also been shown to reduce Angiopoietin 2 levels in HUVECs (Goettsch et al., 2008). Due to the importance of shear within the vascular system the majority of research into shear forces which overlap with these factors has been performed in endothelial cells and other vasculature derived cells. The results described in this chapter, however, reflect the effects of shear loading in these systems despite their

very different nature. This similarity in the effects of shear load on MSCs compared to other cell types in the secretome analysis was reflected in the increased presence of NO as determined indirectly via the Griess reaction which has previously been described in endothelial cells stimulated with shear forces (Goettsch et al., 2008, Bao et al., 1999).

Literature on other forms of mechanical load e.g. stretch and compression were also examined and corroborate the results of this study. Exposure to cyclic stretch increases the expression of MIF in gingival cells, MIP3 α in hTERT periodontal ligament cells, and angiopoietin-2 and PDGF in HUVECs (Wang et al., 1997, Lee et al., 2012, Hashimoto et al., 2002, Morimoto et al., 2003, Chang et al., 2003). Compression has been shown to induce the expression of MMP13 and uPAR correlating with the results of this study (Chen et al., 2013, Chu et al., 2006, Fitzgerald et al., 2008). As with the effects of shear load in other systems it is impossible to draw direct comparisons with our system as these investigations used different types of cells and different types of load in different culture systems, however, the bulk of evidence reported corroborates the effects of mechanical shear observed in our system.

Similarities and differences between the effects of TGF- β 1 stimulation on factors in this system and those reported in the literature can also be found. Previous work has shown that TGF- β stimulation induces MCP3 in murine fibroblasts, decreases GRO expression in human lung carcinoma epithelial cells (Lo et al., 2013), increases PDGF expression in an osteoclast cell line (Wang et al., 1997), induces VEGF and osteoprotegerin in vascular smooth muscle cells (Toffoli et al., 2011, Tanabe et al., 2006), upregulates uPAR expression in an epithelial cell line (Yue et al., 2004) and stimulates BLC expression at seven days of hBMSC chondrogenesis (Cristino et al., 2008). These results corroborate the observations in this system and in the absence of further experiments to provide greater, more specific information. There were also effects of TGF- β 1 reported in the literature that do not fit the results produced by the model system used in this study. Cell associated leptin receptor and its ligand leptin were decreased in response to TGF- β 1 in BMSCs (Zeddou et al., 2012), however this does not necessarily reflect the effect of TGF- β 1 on soluble Leptin receptors as observed in the work presented here, as the shedding of leptin receptor into the culture medium may be due to processes outside the influence of TGF- β . TGF- β 1 has been associated with the shedding of ALCAM from the cell's surface, however this was not observed in this system despite the clear increase in shedding in response to mechanical load. Recent work carried out by Rodriguez et al. investigated the secretome of adipose derived MSCs cultured in monolayer stimulated with 3ng/ml TGF- β 1 (Rodriguez et al., 2015). The results of this work bare some similarities to the data presented in this chapter e.g. a decrease in GRO α in response to TGF- β 1

stimulation. However, clear differences were also apparent to the work in this chapter e.g. no change in VEGF expression in response to TGF- β 1 and a decrease in leptin expression in response to TGF- β 1 stimulation. These differences are likely to result from a combination of the different tissue source used for the MSCs (adipose rather than bone marrow) and the different culture system (2D monolayer culture as opposed to 3D culture within a scaffold).

The results of this work not only bare similarities to work carried out in other systems but also to secretome studies previously carried out on MSCs. Factors such as GRO, VEGF, MIF, MIP3 α and OPG were detected in the media of MSCs cultured in high density monolayers by Liu et al. (Liu and Hwang, 2005). Furthermore, a number of factors found in the secretome of embryonic stem cell derived MSCs (the HuEs9.E1 MSC cell line) were also found in this investigation (ALCAM, Angiogenin, MCP3, GRO, BLC, MIF, MMP13 and VEGF) (Sze et al., 2007). With specific regard to studies focusing on MSCs undergoing chondrogenesis, expression of MMP13 and VEGF have been shown to increase in chondrogenic conditions (Grassel et al., 2009, Arufe et al., 2011), as observed in both TGF- β 1 and load induced chondrogenesis in this system.

The differential expression of specific factors identified in my study may act as potential markers to track the progression of chondrogenesis in MSCs, particularly those factors whose expression was affected by either TGF- β 1 stimulation or mechanical load compared to controls (angiogenin, BLC, GRO, MCP3, MIF, VEGF, MMP13 and PDGF $\alpha\alpha$). Among these factors are those whose role in, or relation to, MSC chondrogenesis still needs to be established; examples of this include BLC, which has previously been shown to be upregulated in response to MSC chondrogenesis induced over the first week of stimulation with TGF- β 1 (Cristino et al., 2008), MIF and GRO, as well as those that have been studied with regards to MSCs but not extensively within MSC chondrogenesis such as angiogenin, and VEGF. Others, such as MMP13 have been well studied with regards to MSC chondrogenesis (Mueller and Tuan, 2008). The factors presented here may in fact provide options for determining the progression of MSCs towards hypertrophy, rather than the induction of chondrogenesis. Currently the determination of chondrogenic induction in MSCs is performed by looking for a range of gene expression markers (e.g. Sox9, aggrecan, collagen type II) and accompanying matrix molecules, whereas hypertrophy is most commonly characterised using, just one, collagen type X. It has even been suggested that collagen type X may not be a good marker of hypertrophy (Mwale et al., 2006). A number of the factors detected in this system such as GRO (Merz et al., 2003, Olivotto et al., 2007), MMP13 (Mueller and Tuan, 2008) and VEGF (Bluteau et al., 2007) have all been linked to hypertrophy. With further characterisation these factors may be useful to provide further details about the progression of MSCs towards hypertrophy.

Although not a factor detected in the cytokine array used to investigate the secretome of chondrogenically stimulated cells, the presence of nitric oxide was also shown to be differentially affected in loaded scaffolds compared to both controls and TGF- β 1 stimulated scaffolds. Nitric oxide has previously been shown to be produced in response to shear loading in endothelial cells (Goettsch et al., 2008) and MSCs (Riddle et al., 2006, Tjabringa et al., 2006). However, to the best of my knowledge, this is the first time nitric oxide has been shown to be produced in MSCs being chondrogenically stimulated with multiaxial mechanical load. The absence of nitric oxide production by MSCs in scaffolds stimulated with exogenous TGF- β 1 demonstrates that NO production is directly linked to the mechanical loading and not the chondrogenesis of MSCs.

As a result of the experimental design used in this study there are certain weaknesses which would need to be addressed for continuation of this work.

The first is that the samples used for the secretome analysis were collected in a separate experiment from those used for the real-time PCR gene expression analysis. The gene expression corroboration was not included in the initial experiment as whilst designing this set-up it was not considered important. This however, was a gross oversight, and may well have caused increased variation between these two data sets that would not have been present had the RNA for gene expression analysis been isolated from the same scaffolds used to condition media for the secretome analysis. The collection of samples from different experimental repeats, even though they were performed with cells from the same bone marrow preparations and had undergone the same passage number, may have played a role in the low rate of subsequent corroborative confirmation of changes detected at a protein level with the gene expression data. However, for factors such as angiopoietin 2 and osteoprotegrin, whose media protein levels were matched by their gene expression, this suggests a certain degree of robustness in the changes detected in their production in response to TGF- β 1 stimulation and mechanical load.

The work presented in chapter 5 was also limited to a certain extent by the cytokine antibody array used to perform the secretome analysis. The first of these limitations was based around the method of analysis itself. As covered in the discussion of Chapter 5, this kind of analysis is suitable for the binary identification of factors in different media samples, but does not provide as sensitive a level of quantification that is comparable to other methods e.g. ELISA. As a result, comparison between TGF- β 1 stimulated samples and their respective controls and loaded samples and respective controls may provide clearer differences between groups than the direct comparison

between the two chondrogenically stimulated groups where the changes in expression are likely to be more subtle. Further work could use the ELISA method to more accurately determine the relative levels of target factors in media collected from different experimental groups. The choice of kit also presents certain limitations in terms of the proteins detected by the array; it would have been advantageous for the kit to detect e.g. extracellular matrix molecules such as aggrecan and collagen type II, and focus on more factors that are known to be associated with cartilage and chondrogenesis e.g. PTHrP. However, the inclusion of factors that are not widely studied with regard to MSC chondrogenesis has also provided interesting results (e.g. the down regulation of GRO in response to both TGF- β 1 stimulation and mechanical load) that would otherwise not have been observed. Further work could further enhance the analysis of the secretome of secreted cells e.g. the use of mass spectroscopy would allow for the detection of a much larger range of proteins including matrix molecules allowing for further comparison between the different groups (Sze et al., 2007, Wagner and Ho, 2007).

Efforts were made to ensure that both TGF- β 1 stimulated and loaded groups received similar chondrogenic stimuli through the use of 1 ng/ml in the TGF- β 1 stimulated group. This concentration was chosen, as a concentration of 1 ng/ml TGF- β 1 is regularly detected in the media collected from MSC containing fibrin-poly(ester-urethane) scaffolds in response to the multiaxial mechanical load described in this thesis. However, in order to ensure that the chondrogenic stimulation was as similar as possible it would also have been necessary to apply TGF- β 1 only from day 2, which is the point that loading began and therefore the point at which cells in the loaded scaffolds would start being exposed to TGF- β 1. This was not done, and as a result TGF- β 1 stimulated MSCs received an additional two days of chondrogenic stimulation than loaded scaffolds. This may have affected the results of the comparisons between the two groups, however, the extent to which it may have had an effect is not easily determined.

Both of the stimuli used in this system have been shown to be chondrogenic in previously published work (Li et al., 2009, Li et al., 2010a). However, due to the time frame chosen in this experiment it was not possible to tie the effect on different factors to a chondrogenic outcome. Performing the same analysis (as well as including histological analysis) at a later time point e.g. day 21 or day 28 would allow for such a comparison. The reason an earlier time point was chosen was to investigate potential factors involved in the induction of MSC chondrogenesis.

6.4 Future work

The results of the work carried out in this thesis lay the platform for a large amount of potentially interesting follow on work. Some of the most interesting avenues of investigation are detailed below.

The activation of TGF- β 1 in response to multiaxial mechanical load was demonstrated in Chapter 4. Work conducted by another group has shown that latent TGF- β 1 within samples of synovial fluid could be activated by the application of fluid shear (Albro et al., 2012), but that latent TGF- β 1 within articular cartilage explants could not be activated by the application of uniaxial compression (Albro et al., 2013). These findings pose an interesting question with regards to the activation of latent TGF- β 1 in the system described in Chapter 4. As a result it would be informative to apply shear and compression alone and in combination to MSC loaded fibrin-poly(ester-urethane) scaffolds to determine the effect of the individual loading components on TGF- β 1 production and activation.

A mechanism could not be elucidated to explain the differences in matrix deposition detected histologically and immunohistochemically between groups 1 and 2 in Chapter 4. The investigations performed to date focused on the use of markers of chondrogenesis and articular cartilage to detect differences between the groups. A change in approach may, however, prove to be more informative with regards to the differences between the groups. An important next step would be to investigate the response of the three different scaffold configurations to exogenous TGF- β 1 rather than mechanical load. This would demonstrate if the response is due to the specific effect of load on the surface population, or if the effect is independent of mechanical load. It would also be interesting to investigate the presence of gap junctions in group 1 and group 2 scaffolds as the increase in cell density may affect cell-cell signalling which in turn may affect chondrogenesis. Proliferation was identified by Mesallati et al., (2015) who investigated the effect of a similarly structured co-culture approach on chondrogenesis; this has not so far been investigated in this system and labelling of proliferation cells with BrdU may be informative as to the differences observed between groups 1 and 2.

The nature of the secretome analysis performed in Chapter 5 leads to a large number of potential follow on studies that could be performed. Of particular interest are factors such as GRO, leptin and nitric oxide.

GRO has been shown to be associated with hypertrophy in studies by Merz et al. (2003) and Olivotto et al. (2007) and is also the only factor to be down regulated in response to either factor. The role of GRO in MSC chondrogenesis has not been characterised to date. Characterisation of the expression of GRO by chondrogenically

differentiating MSCs and investigation of the effect of the addition of exogenous GRO in culture media, or knock down of GRO expression on MSC chondrogenesis would also be extremely interesting and may provide a novel mechanism for the chondrogenic differentiation of MSCs.

Media from TGF- β 1 stimulated cells showed an increase in the presence of leptin and leptin receptor that was not detected in media from loaded scaffolds. Leptin signalling has been associated with osteoarthritis (Dumond et al., 2003), MMP expression and activation (Hui et al., 2012, Iliopoulos et al., 2007) and has also been localised to a population of hypertrophic chondrocytes in the growth plate (Kume et al., 2002). Therefore, the upregulation of two members of this pathway in response to TGF- β 1 but not mechanical load is interesting. Characterisation of the expression of leptin in chondrogenically differentiating MSCs as well as its exogenous application and knock down (in a similar manner to that suggested for GRO) may provide an insight in to the progression of MSCs towards hypertrophy in response to TGF- β 1 stimulation.

Nitric oxide has been shown in a number of cell types (including MSCs) to respond to various forms of shear stress (Goettsch et al., 2008, Riddle et al., 2006, Tjabringa et al., 2006). Nitric oxide was shown to be specifically produced in response to multiaxial mechanical load in Chapter 5. The nitric oxide produced in response to load may be involved upstream in the production of TGF- β 1 in response to load, or may be separate from this process. This could be determined through the application of a nitric oxide synthase inhibitor to the MSCs subjected to load and observing the effect of such an inhibitor on chondrogenesis. Should nitric oxide be involved in the chondrogenic response to load then this would provide another step in the mechanism involved in load induced chondrogenic stimulation of MSCs. If nitric oxide is not involved in this response then blocking nitric oxide synthase may prove to be a way of reducing the presence of this anti-chondrogenic stimulus without affecting the chondrogenesis of cells within a loaded scaffold. This may also have potential clinical benefits for those undergoing cartilage repair e.g. microfracture.

6.5 Summary

The novel results presented in this thesis allow for the drawing of certain conclusions about the chondrogenesis of MSCs in fibrin-poly(ester-urethane) scaffolds in response to, and in the absence of mechanical load.

The first is that separate populations of cells within the same construct can interact and effect the induction of chondrogenesis in the other cell population. This was first, partially, demonstrated by the potentially chondrogenic effects of predifferentiated bottom scaffolds on top scaffolds containing naïve MSCs in Chapter 3. This was then subsequently demonstrated in Chapter 4 where a substantial improvement in matrix deposition was noted when a population of MSCs was seeded onto the loaded surface of an MSC containing fibrin-poly(ester-urethane) scaffold.

The work presented in Chapter 4 described the activation of endogenously produced TGF- β 1 by the application of multiaxial mechanical load. This novel observation improves our understanding of the response of MSCs to multiaxial mechanical load by demonstrating that mechanical load not only induces the production of TGF- β 1 but is also required for its activation, and therefore the chondrogenic effects of TGF- β 1.

Secretome analysis performed in Chapter 5 demonstrated that exogenous TGF- β 1 stimulation and multiaxial mechanical load have notable differential effects on the soluble proteins secreted by MSCs over the first week in culture. The results showed that although both forms of stimulation had similar effects on a number of factors, which is likely to be linked to the role of TGF- β 1 in both systems, both forms of stimulation also led to changes in factor expression that were not noted in response to the other stimulus.

Subsequent to the secretome analysis in Chapter 5, nitric oxide was also identified as being upregulated in response to the application of mechanical load. To the best of my knowledge, the upregulation of nitric oxide production in MSCs chondrogenically stimulated with multiaxial load is also a novel finding.

These data provide useful information with regards to the spatial design of potential constructs for cartilage tissue engineering/regenerative medicine as well as providing a further insight into the effect of joint-like mechanical load on the induction of chondrogenesis in MSCs.

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Appendix 1

The 174 proteins detected by the RayBio Human Cytokine Array G-series 2000 described in Chapter 5.

Adiponectin (ACRP30)

Activin A

AgRP

ALCAM (CD166)

Amphiregulin

Angiogenin

Angiopoietin-2

Axl

CD80 (B7-1)

BDNF

bFGF

BLC (CXCL13)

BMP-4

BMP-5

BMP-6

BMP-7

beta-NGF

Betacellulin (BTC)

Cardiotrophin-1 (CT-1)

CCL28 (MEC)

CD14

Ck beta 8-1 (CCL23)

CNTF

CTACK (CCL27)

CXCL16

DR6 (TNFRSF21)
Dtk
EGF
EGFR
ENA-78 (CXCL5)
Endoglin (CD105)
Eotaxin-1 (CCL11)
Eotaxin-2 (MPIF-2/CCL24)
Eotaxin-3 (CCL26)
ErbB3
E-Selectin
Fas (TNFRSF6/Apo-1)
Fas Ligand (TNFSF6)
FGF-4
FGF-6
FGF-7 (KGF)
FGF-9
Flt-3 Ligand
Fractalkine (CX3CL1)
GCP-2 (CXCL6)
GCSF
GDNF
GITR (TNFRSF18)
GITR Ligand (TNFSF18)
GM-CSF
GRO alpha/beta/gamma
GRO alpha (CXCL1)
HCC-4 (CCL16)
HGF

I-309 (TCA-3/CCL1)
ICAM-1 (CD54)
ICAM-2 (CD102)
ICAM-3 (CD50)
IFN-gamma
IGFBP-1
IGFBP-2
IGFBP-3
IGFBP-4
IGFBP-6
IGF-1
IGF-1 R
IGF-2
IL-1 R2
IL-1 R4 (ST2)
IL-1 R1
IL-10
IL-10 R beta
IL-11
IL-12 p40
IL-12 p70
IL-13
IL-13 R alpha 2
IL-15
IL-16
IL-17A
IL-18 BP alpha
IL-18 R beta (AcPL)
IL-1 alpha (IL-1 F1)

IL-1 beta (IL-1 F2)
IL-1 ra (IL-1 F3)
IL-2
IL-2 R beta (CD122)
IL-2 R gamma (Common gamma Chain)
IL-2 R alpha
IL-21 R
IL-3
IL-4
IL-5
IL-5 R alpha
IL-6
IL-6 R
IL-7
IL-8 (CXCL8)
IL-9
IP-10 (CXCL10)
I-TAC (CXCL11)
LAP
Leptin
Leptin R
LIF
Light (TNFSF14)
L-Selectin (CD62L)
Lymphotactin (XCL1)
MCP-1 (CCL2)
MCP-2 (CCL8)
MCP-3 (MARC/CCL7)
MCP-4 (CCL13)

M-CSF
M-CSF R
MDC (CCL22)
MIF
MIG (CXCL9)
MIP-1 alpha (CCL3)
MIP-1 beta (CCL4)
MIP-1 delta (CCL15)
MIP-3 alpha (CCL20)
MIP-3 beta (CCL19)
MMP-1
MMP-13
MMP-3
MMP-9
MPIF-1 (CCL23)
MSP alpha/beta
NAP-2 (PPBP/CXCL7)
NGFR (TNFRSF16)
NT-3
NT-4
Oncostatin M
Osteoprotegerin (TNFRSF11B)
PARC (CCL18)
PDGF-AA
PDGF R alpha
PDGF R beta
PDGF-AB
PDGF-BB
PECAM-1 (CD31)

PLGF
Prolactin
RANTES (CCL5)
SCF
SCF R (CD117/c-kit)
SDF-1 alpha (CXCL12 alpha)
SDF-1 beta (CXCL12 beta)
gp130
Siglec-5 (CD170)
TNF RII (TNFRSF1B)
TNF RI (TNFRSF1A)
TARC (CCL17)
TECK (CCL25)
TGF alpha
TGF beta 1
TGF beta 2
TGF beta 3
Thrombopoietin (TPO)
Tie-1
Tie-2
TIMP-1
TIMP-2
TIMP-4
TNF alpha
TNF beta (TNFSF1B)
TRAIL R3 (TNFRSF10C)
TRAIL R4 (TNFRSF10D)
uPAR
VE-Cadherin (CDH5)

VEGF-A

VEGFR2

VEGFR3

VEGF-D

Appendix 2

Tables grouping the 174 factors analysed using the RayBio Cytokine Antibody Array described in Chapter 5 by level of expression.

<100 (55)	Average:	SD:
BMP-6	20.50	18.21
EGF	75.33	10.40
Eotaxin-2	39.48	18.96
FGF-6	89.09	26.08
Fractalkine	56.08	13.77
GCP-2	59.52	13.74
I-309	76.70	36.40
IGFBP-4	65.48	18.32
IGF-I	38.10	23.48
IL-16	40.97	9.05
IL-4	59.48	9.60
Leptin	93.79	81.84
MDC	54.81	9.98
MIP-1-delta	39.08	9.73
MIP-3-alpha	75.85	26.79
NAP-2	60.29	9.54
SCF	91.19	48.69
TARC	62.49	15.76
TGF-beta 3	47.79	10.99
Acrp30	92.64	28.07
Amphiregulin	78.92	26.50
Axl	74.55	15.60
bFGF	50.84	15.77
b-NGF	31.23	6.16

CCL-28	96.26	19.50
Dtk	92.56	36.24
FGF-4	48.68	11.16
FGF-9	57.97	12.64
GITR-Ligand	94.24	19.10
GITR	91.73	19.21
ICAM-1	70.90	16.08
ICAM-3	23.25	8.45
IGFBP-3	62.24	17.13
IGF-I SR	95.10	16.48
IL-1 RI	95.14	20.68
IL-11	94.45	20.56
IL-12 p40	47.83	12.96
IL-12 p70	67.27	11.85
IL-17	81.16	22.51
IL-6 R	81.78	18.31
I-TAC	32.52	12.19
Lymphotactin	44.30	12.07
MIP-1alpha	66.21	14.88
MIP-1beta	56.87	14.37
MIP-3beta	78.51	14.50
MSP-alpha	60.32	12.03
NT-4	85.51	31.98
Oncostatin M	97.90	17.10
sTNF RII	26.36	7.70
Thrombopoietin	92.97	17.15
TRAIL R4	94.62	17.76
VEGF-D	59.82	14.37

Activin A	69.51	14.01
B7-1(CD80)	68.33	16.72
CD14	54.92	11.76

100-500 (94)	Average:	SD:
BDNF	130.47	41.75
BLC	389.19	557.10
BMP-4	229.76	79.62
CK beta 8-1	113.93	19.88
CNTF	258.56	59.82
Eotaxin	132.06	54.81
Eotaxin-3	198.10	28.94
FGF-7	156.22	65.72
Flt-3 Ligand	189.15	17.52
GDNF	114.19	24.11
GM-CSF	325.87	36.63
GRO-alpha	172.27	34.27
IGFBP-1	152.43	72.68
IL-10	343.34	39.00
IL-13	389.92	43.08
IL-15	384.77	29.87
IL-1alpha	369.92	39.26
IL-1beta	205.15	40.82
IL-1ra	181.88	34.77
IL-2	324.53	35.47
IL-3	198.45	31.83
IL-5	464.88	47.99
IL-7	335.49	36.04

LIGHT	106.33	21.95
MCP-2	137.79	17.39
MCP-3	201.30	115.94
MCP-4	100.62	19.51
M-CSF	213.08	27.74
MIG	268.03	35.67
NT-3	119.82	24.16
PARC	176.32	30.46
PDGF-BB	177.73	23.14
RANTES	194.34	92.11
SDF-1	369.86	51.40
TGF-beta 1	356.93	52.22
TNF-alpha	394.93	34.84
TNF-beta	331.52	32.76
AgRP	138.77	27.18
BTC	110.81	19.19
CTACK	141.73	29.40
EGF-R	226.63	69.53
ENA-78	111.72	21.62
Fas/TNFRSF6	228.27	44.93
HCC-4	113.30	17.39
IGFBP-6	253.67	100.85
IL-1 R4/ST2	140.43	35.96
IL-2 Rapha	127.43	18.74
IL-8	254.23	46.30
PIGF	179.83	81.59
sgp130	179.39	55.54
sTNF-RI	261.63	52.62

TECK	103.40	19.38
TRAIL R3	149.90	46.16
uPAR	286.63	109.39
ALCAM	141.57	37.54
BMP-7	391.91	30.34
Cardiotrophin-1	283.75	22.50
CXCL- 16	310.47	246.81
DR6 (TNFRSF21)	475.82	43.25
Endoglin	216.47	24.47
ErbB3	250.70	23.87
E-Selectin	280.31	29.89
IGF-II	141.18	13.38
IL-1 R II	161.46	16.37
IL-10 Rbeta	167.60	15.98
IL-13 Ralpha2	281.21	26.02
IL-18 BPalpha	256.06	22.80
IL-2 Ralpha	264.41	36.49
IL-2 Rgamma	281.51	37.37
IL-21R	182.63	20.02
IL-5 Ralpha	117.80	26.67
IL-9	396.57	36.02
Leptin R	110.07	29.35
L-Selectin	271.88	24.01
M-CSF R	201.78	12.37
MMP-13	422.77	512.66
MPIF-1	275.30	29.12
PDGF AA	171.75	76.35

PDGF-AB	112.36	15.06
PDGF Ralpha	110.84	17.79
PDGF Rbeta	218.53	21.39
Prolactin	196.60	14.22
SCF R	354.31	27.75
SDF-1beta	253.54	24.27
Siglec-5	183.85	14.85
TGF-alpha	108.03	20.05
Tie-1	217.61	27.44
Tie-2	198.07	22.48
VE-Cadherin	199.29	19.49
VEGF R3	453.38	41.64
MMP-1	82.60	11.42
MMP-9	50.13	14.07
PECAM-1	66.09	10.20
VEGF R2	95.96	26.73

500-1000 (11)	Average:	SD:
IFN-gamma	642.52	73.33
GCSF	599.35	183.60
Osteoprotegerin	986.16	560.48
VEGF	938.11	397.31
BMP-5	599.05	62.87
Fas Ligand	659.85	75.51
ICAM-2	565.95	63.26
IL-18 Rbeta	757.35	106.17
IL-2 Rbeta	518.06	54.93
NGF R	549.36	67.07

TGF beta2	986.62	186.00
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1000-4000 (12)	Average:	SD:
IGFBP-2	1894.01	937.81
IL-6	1310.90	136.14
MCP-1	2371.32	1079.86
Angiopoietin-2	1341.87	1446.09
GRO	1326.36	1011.64
HGF	2217.95	1406.70
MIF	2290.84	1109.49
TIMP-1	1214.70	258.73
IP-10	1052.02	98.75
LAP	1302.15	752.86
LIF	1227.15	114.97
TIMP-4	1310.38	779.88

>4000 (2)	Average:	SD:
Angiogenin	40273.68	7037.95
TIMP-2	6778.68	2231.98

Appendix 3

List of Publications Oral Presentations and Poster Presentations

Publications

Gardner OFW, Archer CW, Alini M, Stoddart MJ. Chondrogenesis of mesenchymal stem cells for cartilage tissue engineering. *Histology and histopathology*. 2013;2823-42.

Glück M, Gardner OFW, Czekanska E, Alini M, Stoddart MJ, Salzmänn GM, Schmal H. Induction of osteogenic differentiation in human mesenchymal stem cells by crosstalk with osteoblasts. *Biores Open Access*. 2015 Jan 1;4(1)121-30.

Neumann AJ, Gardner OFW, Williams R, Alini M, Archer CW, Stoddart MJ. Human articular cartilage progenitor cells are responsive to mechanical stimulation and adenoviral-mediated overexpression of bone-morphogenetic protein 2. *PLoS One*. 2015 Aug 20;10(8)e0136229. doi 10.1371/journal.pone.0136229.

Articles Under Review

Gardner OFW, Musumeci G, Eglin D, Archer CW, Alini M, Stoddart MJ. Asymmetrical seeding of MSCs into fibrin-poly(ester-urethane) scaffolds and its effect on mechanically induced chondrogenesis.

Gardner OFW, Alini M, Stoddart MJ. Joint Mimicking Mechanical Load Activates TGF β 1 in Fibrin-Poly(ester-urethane) Scaffolds Seeded with Mesenchymal Stem Cells.

Gardner OFW, Fahy N, Alini M, Stoddart MJ. A Secretomic Comparison of the Differences in human Mesenchymal stem cell secretomes during chondrogenic induction.

Book Chapter

Gardner OF, Alini M, Stoddart MJ. Mesenchymal stem cells derived from human bone marrow. *Methods in Molecular Biology Cartilage Tissue Engineering – Methods and Protocols*. Humana Press. 2015.

List of Oral and Poster Presentations

Academica Rhaetia 2012

Oral presentation 'Cartilage Tissue Engineering'. Gardner OFW, Neumann AJ, Archer CW, Alini M, Stoddart MJ.

Prize: Science award for best presentation.

Belgium Symposium on Tissue Engineering 2012

Oral and poster Presentation 'Co-culture of mesenchymal stem cells at different stages of chondrogenic differentiation within a tissue engineering construct effects on chondrogenesis and hypertrophy'. Gardner OFW, Neumann AJ, Archer CW, Alini M, Stoddart MJ.

Gordon Research Conference - Cartilage Biology and Pathology 2013

Poster presentation 'The role of asymmetrical cell distribution during mechanically induced chondrogenesis of human bone marrow derived stem cells'. Gardner OFW, Musumeci G, Archer CW, Alini M, Stoddart MJ.

AO Foundation Exploratory Research Board "Where science meets clinics" 2013

Oral presentation 'Mechanically induced chondrogenesis of mesenchymal stem cells can be improved by manipulating the location of cells within a tissue engineering scaffold'. Gardner OFW, Musumeci G, Archer CW, Alini M, Stoddart MJ.

Orthopaedic Research Society Annual meeting 2014

Poster presentation 'Asymmetric Cell Seeding Enhances the Mechano-Induction of Chondrogenesis in Human MSCs in the Absence of Exogenous Growth Factors'. Gardner OFW, Musumeci G, Archer CW, Alini M, Stoddart MJ.

TERMIS EU 2014

Oral presentation 'Improving the deposition of cartilage-like matrix by mechanically stimulated MSCs in the absence of growth factors through the asymmetrical seeding of fibrin-polyurethane scaffolds'. Gardner OFW, Musumeci G, Archer CW, Alini M, Stoddart MJ.

European Cells and Materials 2014

Poster presentation 'Comparing the secretomes of unstimulated and mechanically loaded MSCs'. Gardner OFW, Archer CW, Alini M, Stoddart MJ.

Academica Rhaetia 2014

Poster presentation 'Improving the deposition of cartilage-like matrix by mechanically stimulated MSCs in the absence of growth factors through the asymmetrical seeding of fibrin-polyurethane scaffolds'. Gardner OFW, Musumeci G, Archer CW, Alini M, Stoddart MJ.

Stem cells in development and disease 2014

Poster presentation 'Tribological Tissue Engineering of Cartilage'. Gardner OF, Musumeci G, Archer CW, Alini M, Stoddart MJ.

Orthopaedic Research Society Annual meeting 2016

Oral presentation ' A Secretomic Comparison of the Induction of Chondrogenesis in Human Mesenchymal Stem Cells via TGF- β 1 and Mechanical Load'. Gardner OFW, Fahy N, Alini M, Martin Stoddart MJ