Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

An investigation of joint tissue derived stem cells for articular cartilage tissue engineering

Tatiana Vinardell, D.V.M., I.P.S.A.V., M.Sc.

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

Doctor in Philosophy

Trinity College Dublin

October 2011

Supervisor

Dr. Daniel J. Kelly

External Examiner

Dr. Martin J. Stoddart

(AO Foundation, Switzerland)

Internal Examiner

Dr. Biqiong Chen

Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

I agree to deposit this thesis in the University's open access institutional repository or allow the Library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.

Tatiana Vinardell

October, 2011

Abstract

Tissue engineering has emerged as a promising approach to repair damaged articular cartilage. The associated drawbacks with the isolation and expansion of chondrocytes from autologous tissue have prompted significant research into alternative cell sources such as mesenchymal stem cells (MSCs). Such stem cells have been found in numerous tissues in the body, including bone marrow, periosteum, adipose tissue as well as joint tissues such as infrapatellar fat pad and synovial membrane. In this thesis it was hypothesized that stem cells isolated from within the synovial joint would possess a superior potential to generate phenotypically stable functional cartilaginous tissues compared to other stem cell sources.

This thesis began by comparing the ability of chondrocytes and bone marrow MSCs to integrate and form neo-cartilage in an *in vitro* cartilage explant defect model. This model demonstrated that chondrocytes accumulate greater amounts of cartilaginous matrix than bone marrow MSCs. Next the ability to engineer functional cartilaginous grafts using infrapatellar fat pad derived stem cells seeded in agarose hydrogels was demonstrated. Tissues engineered using fat pad derived stem cells were next compared to those derived from bone marrow MSCs and chondrocytes. Stem cells isolated from the fat pad appeared to possess a potential to generate functional cartilaginous tissues at least comparable to bone marrow MSCs, although chondrocytes possessed a stronger cartilaginous phenotype.

The functionality and phenotypic stability of cartilaginous tissues engineered using bone marrow MSCs, fat pad derived stem cells and synovial membrane derived stem cells were next compared *in vitro* and in an *in vivo* ectopic environment. Synovial membrane stem cells possessed the potential to generate functional cartilaginous tissues superior to the other stem cell sources *in vitro*, but when transplanted *in vivo* stem cell isolated for all the different tissue types embedded in agarose were phenotypically unstable and failed to form ectopic cartilage *in vivo*. Bone marrow MSCs underwent hypertrophy and terminal differentiation while joint tissue derived stem cells appeared to undergo fibrous dedifferentiation.

The final objective of the thesis was to investigate how mechanical load would influence both the initiation and maintenance of a chondrogenic phenotypic in joint tissue derived stem cells. This study revealed that HP acted synergistically with TGF-β3 to increase the expression of chondrogenic markers and inhibited the expression of markers associated

with pre-hypertrophy and terminal differentiation. In the context of stem cell based therapies for cartilage repair, this thesis demonstrates the importance of considering how joint specific environmental factors interact to regulate not only the initiation of chondrogenesis, but also the development of a stable hyaline-like repair tissue.

Acknowledgements

I would like to take this opportunity to thank my supervisor Dr Daniel Kelly for his support, guidance and availability over the last 3 years. Thanks for providing me with the opportunity to undertake this challenging, but inspiring experience. The guidance and knowledge that you imparted over the course of this project is greatly appreciated and your shared enthusiasm for research inspired me to see this research through to completion.

Thanks to my colleagues from the office and Kelly's group: Steve, Alanna, Eoghan, Thomas, Darren, Eamon, Tariq, Iratxe, Andy, Kevin, Pavel, Conor, Oana, Lu, Yurong, Grainne, Eric, Rebecca, Hanifeh, Katey, Colin and Matt. As a vet being surrounded by engineers, it was really interesting and motivating to share 3 years with you.

Thank you to all my family and friends, who have supported and encouraged me during my PhD. I would like to thank my husband Florent, for all the encouragement and support, he has given me since the day I met him and finally to the baby I am carrying: you gave me strength to accomplish the end of this PhD with enthusiasm, you are the best deadline I have ever had!

Table of contents

List of Figures

List of Tables

Nomenclature

ACI	Autologous chondrocyte implantation
ADSCs	Adipose derived stem cells
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
BM	Bone marrow
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CC	Chondrocytes
CFU-F	Colony forming unit-fibroblast
cm	Centimetres
CO_2	Carbondioxide
DAB	Diaminobenzide
Dex	Dexamethasone
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediamine-tetraacetic acid
EGF	Endothelial growth factor
f	Force
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FP	Infrapatellar fat pad
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H_2O	Water
H_2O_2	Hydrogen peroxide
IGF	Insulin growth factor
ITS	Insulin transferrin selenium
HP	Hydrostatic pressure
Hz	Hertz
MACI	Matrix-assisted autologous chondrocyte implantation
mg	Milligram

min	Minute
mm	Millimetre
mM	Millimolar
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MSCs	Mesenchymal stem cells
N	Newton
ng	Nanogram
nm	Nanomolar
OA	Osteoarthritis
OCD	Osteochondritis dissecans
PBS	Phosphate buffered saline
PEG	Poly (ethylene glycol)
PGA	Polyglycolic acid
PLGA	Poly (lactic-co-glycolic acid)
PDGF	Platelet derived growth factor
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Real-time polymerase chain reaction
s	Seconds
SM	Synovial membrane
SEM	Standard error of the means
t	Time
TGF-β	Transforming growth factor beta
w/w	Wet/weight
Ø	Diameter
µm	Micrometres
µl	Mircrolitres
µg	Micrograms
Pa	Pascals
kPa	Kilopascals
MPa	Megapascals
2D	2 dimensional
3D	3 dimensional

1 Introduction

1.1 Background

Articular cartilage is the tissue that lines the ends of bones within synovial joints and makes low friction and painless movement possible throughout life (Mankin et al. 2007). The tissue consists of chondrocytes embedded within an extracellular matrix consisting of collagens, proteoglycans, water and non-collagenous proteins. Over time and/or with recurrent trauma, the cartilage can fail leading to the clinical syndrome recognized as osteoarthritis (OA) (Mankin et al. 2007). The clinical finding that articular cartilage "once destroyed, is not repaired," has not changed since it was first reported by Hunter in 1743 (Chiang and Jiang 2009). Due to its limited ability to regenerate and self repair, as well as a paucity of therapeutic options, degeneration of articular cartilage has severe consequences (Pelttari et al. 2008).

Clinical procedures for cartilage defect repair include shaving, subchondral abrasion arthroplasty, microfracture and autologous chondrocyte implantation (ACI) among others. Those techniques generally achieve a temporary partial repair, with a biomechanically inferior repair tissue that may ultimately degenerate leading to OA (Schumann et al. 2006). Of all of these approaches, ACI is perhaps the only that aims to regenerate hyaline like cartilage. However there are several problems associated with these techniques including difficulties in obtaining a sufficient number of chondrocytes for transplantation, the necessity of creating donor-site defects within the articular cartilage and variability in the quality of repair among others (Horas et al. 2003). Taken together, the current state of the art necessitates improved therapeutic options for treating articular defects. The aim is to develop new cell- and tissue-engineering-based methods that may also overcome the limitations of current technologies and potentially extend the utility of such approaches to the treatment of OA (Pelttari et al. 2008).

Over the last two decades, the emerging discipline of tissue engineering has generated a tremendous interest in different fields but especially in cartilage repair (MacArthur and Oreffo 2005). Engineering functional cartilage tissue using mesenchymal stem cells (MSCs) seeded into scaffolds or hydrogels represent a promising alternative treatment option for articular cartilage defects. This thesis will focus on four important aspects of stem cell based therapies for articular cartilage repair: the integration of engineered

cartilaginous constructs in an *in vitro* cartilage defect model, the potential of different MSCs sources for engineering functional cartilaginous grafts, the phenotypic stability of these cells *in vivo* and their response to mechanical stimuli.

1.2 Challenges associated with stem cell based cartilage tissue engineering

The evaluation of the efficacy and safety of cell based therapies using animal experimental models is essential to realize clinical applications. However, the development of *in vitro* evaluation systems that enable preliminary screening before animal experiments are needed, in order to minimize problems related to the use of a large number of animals, including interspecies variability, ethical concerns and practical issues such as the increased cost of large animals (Iwai et al. 2011). The poor integration of repair tissue with the surrounding cartilage constitutes one of the primary causes of long term failure in cell based therapies for cartilage repair (Ahsan and Sah 1999; Gratz et al. 2006; Djouad et al. 2009). *In vitro* cartilage explant models are widely used to investigate cartilage integration and produce a model system to study the mechanics of cartilage repair (Obradovic et al. 2001; Hunter and Levenston 2004; Gilbert et al. 2009). Chondrogenesis and integration of tissue engineered cartilage depends on the choice of scaffold, the developmental stage, the adjacent tissue architecture and composition of the construct (Obradovic et al. 2001; Hunter and Levenston 2004; Tognana et al. 2005).

As previously stated, MSCs are an appealing cell source for cartilage repair. Bone marrow (BM) derived stem cells have been widely studied because they overcome the problem of creating a new defect in the articular surface of the joint associated with ACI, they have demonstrated a high rate of proliferation and a capacity to differentiate and synthesize markers associated with adipocytes, chondrocytes and osteoblasts (Maniatopoulos et al. 1988; Caplan 1991; Johnstone et al. 1998; Pittenger et al. 1999). However there are drawbacks associated with their use; while they have been used to generate cartilaginous tissues, the mechanical properties achieved were inferior to those obtained using chondrocytes (Mauck et al. 2006; Mauck et al. 2007; Thorpe et al. 2008; Erickson et al. 2009; Huang et al. 2010). Traditional procurement procedures for obtaining BM MSCs may be painful, time consuming, expensive and risk cell contamination and loss (Pelttari et al. 2008; Khan et al. 2009). An ideal source of autologous cells would be easy to obtain and result in minimal patient discomfort during harvesting (Zuk et al. 2001). Other sources of stem cells have recently been identified in the placenta (Dzierzak and Robin 2010),

umbilical cord (Forraz and McGuckin 2011), and other adult skeletal tissues such as subcutaneous fat, synovial membrane (SM) and infrapatellar fat pad (FP) (De Bari et al. 2001; Zuk et al. 2001; Zuk et al. 2002; Dragoo et al. 2003; Wickham et al. 2003; Huang et al. 2005; English et al. 2007; Khan et al. 2007; Pei et al. 2008). These cells have a good proliferative capacity *in vitro* and recent studies have found that stem cells isolated from the synovial joint would appear to possess a potential to generate cartilaginous tissues at comparable levels to chondrocytes in terms of matrix production (De Bari et al. 2001; Mochizuki et al. 2006; English et al. 2007; Pei et al. 2008).

In order to implant a scaffold seeded with stem cells into the human body the phenotypic stability of the engineered tissues need to be evaluated (Richter 2009). The back of genetic modified mice is commonly used as an *in vivo* bioreactor to test the phenotypic stability of cell seeded scaffolds (Dobratz et al. 2009). When BM MSCs are inserted in this environment, the chondrogenic phenotype adopted *in vitro* prior to implantation is lost, with cells proceeding to terminal differentiation as evident by markers of hypertrophy and mineralization (Pelttari et al. 2006; Karlsson et al. 2007). Similar results were obtained for SM derived stem cells, which fail to form ectopic stable transplants, loosing their *in vitro* acquired deposition of collagen type II and dedifferentiating, or even degenerating at ectopic sites (Dell'Accio et al. 2001; De Bari et al. 2004; Dickhut et al. 2009). On the other hand promising results have recently been observed in a study comparing different types of hydrogels. In the ectopic environment when adipose derived stem cells were seeded in Matrigel™, a gelatinous protein mixture, chondrogenesis and suppression of the calcification was observed, making these types of hydrogel attractive for MSC-based tissue engineering approaches in cartilage repair (Dickhut et al. 2008).

Chondrogenesis is the earliest phase of skeletal development and involves the differentiation of MSCs into chondrocytes. This is a multistep process involving the recruitment of mesenchymal chondroprogenitor cells, their condensation and differentiation into chondrocytes, and the formation of an extracellular matrix that is characteristic of cartilage (de Crombrugghe et al. 2000). The subsequent development of the musculoskeletal system is regulated by the coupling of biochemical and biomechanical factors *in vivo*. The mechanical environment is a key component of the *in vivo* environment and regulates the differentiation of skeletal tissues, such as the rate of bone formation, the calcification of the growth plate and the regulation of fracture repair (Kelly and Jacobs 2010). Tissue engineering approaches have incorporated bioreactors to apply

loading regimes to constructs to maximise cell synthesis, but it needs to be demonstrated how mechano-regulatory mechanisms will translate into the chondrogenic differentiation of MSCs *in vitro* (Huang et al. 2004; Mouw et al. 2007; Stoddart et al. 2009). Mechanical loading such as dynamic compressive strain and hydrostatic pressure (HP) has been shown to promote MSCs chondrogenesis (Angele et al. 2003; Angele et al. 2004; Huang et al. 2004; Campbell et al. 2006; Miyanishi et al. 2006; Mauck et al. 2007; Kisiday et al. 2009; Li et al. 2010; Li et al. 2010) and positively modulate the functional development of cartilaginous constructs engineered using MSCs encapsulated in agarose hydrogels (Huang et al. 2010; Meyer et al. 2011). Finite element models have also been used to support the hypothesis that mechanical loading can regulate tissue differentiation and that HP can promote cartilage formation and potentially suppress endochondral ossification and hypertrophy (Carter et al. 1998; Loboa et al. 2001). HP has also been shown to enhance chondrogenesis of SM stem cells at the gene and protein level (Sakao et al. 2008; Wu et al. 2008), however the interaction between HP and transforming growth factor-β (TGF-β) (member of the family of growth factors that regulates the initiation and maintenance of a chondrogenic phenotype in joint tissue derived stem cells) remains poorly understood.

1.3 Aim of the thesis

The over-riding objective of this thesis is to compare the chondrogenic potential of stem cells derived from different tissue sources for cartilage tissue engineering purposes. The underlying hypothesis of the thesis is that MSCs isolated from within the synovial joint possess a superior potential to generate functional cartilaginous tissues compared to other MSC sources. The aims of the thesis are as follows:

Firstly, to compare the ability of BM derived MSCs to form a chondrogenic tissue which integrates with the surrounding articular cartilage, to that of chondrocytes. Specifically, the aim is to adapt a well-established cartilage explant model that has been used to assess chondrocyte-based therapies for cartilage repair to investigate MSC-based therapies.

Secondly, to explore the potential of FP derived stem cells seeded in agarose hydrogels to generate mechanically functional cartilaginous grafts. Having demonstrated this, the chondrogenic potential of this cell source will be compared to BM MSCs and chondrocytes.

Thirdly, the *in vitro* functionality and *in vivo* phenotypic stability of cartilaginous tissues engineered using SM, BM and FP derived stem cells will be compared.

Fourthly, the chondrogenic response of joint derived stem cells from FP and SM subjected to 10 MPa of HP and to different concentrations of TGF-β3 will be compared to chondrocytes in a pellet culture system.

Ultimately, it is hoped that by comparing the chondrogenic potential of stem cells isolated from different tissues, the behaviour of those cells for cartilage tissue engineering purposes can be better understood, and may be optimized for cell-based therapies for articular cartilage repair.

2 Literature Review

2.1 Introduction

Articular joint disease is one of the most frequent conditions affecting the elderly population all over the world. The outcomes of surgery and other treatments for articular joint repair are variable and in most instances additional questions have been raised about the clinical dilemma of damaged articular cartilage and its limited capacity for natural healing (O'Driscoll and Saris 2007). This chapter gives an overview of the structure and function of articular cartilage, its pathology and different treatment options. The results and limitations of clinical repair are stated as well as future cartilage repair developments such as tissue engineering strategies, involving the use of chondrocytes and more recently different sources of MSCs.

2.2 Structure and function of the articular cartilage

2.2.1 Articular cartilage

Articular cartilage is a thin layer of highly specialized tissue that functions to permit smooth, nearly frictionless movement and load-bearing force distribution throughout the joints; this tissue is aneural, alymphatic and avascular (Clair et al. 2009). Microscopically, articular cartilage is composed of water (approximately 75 %), type II collagen (approximately 15 %), proteoglycans (approximately 10 %) and chondrocytes (only 2 %). Its metabolism is anaerobic, oxygen content is low due to the lack of blood supply with levels ranging from 1 to 8 % (Dewire and Einhorn 2001). Glucose reaches the cells by diffusion both from the joint surface and the underlying bone (Mow and Ratcliffe 1997; Ge et al. 2006; Goodrich and Nixon 2006). Articular cartilage is composed of 4 layers each with distinct cellular phenotype and matrix composition (Fig. 2.1). The **superficial zone** (10-20 %) has the highest chondrocyte density and the lowest proteoglycan content, the collagen fibres run parallel to the articular surface as well as the chondrocytes which lie close to each other in a dense arrangement. In the **middle zone** (40-60 %) the chondrocytes are distributed in groups of 2 or 3, they are rounded and bigger than the chondrocytes of the superficial layer; the collagen fibres are randomly oriented and more loosely packed. The **deep zone** (30 %) is composed of spherical chondrocytes distributed in columns. This layer has a high proteoglycan content and thick collagen fibres orientated perpendicular to

the subchondral bone surface. The **calcified zone** is separated from the deep zone by the tidemark: an irregular calcified line. This last zone has a low proteoglycan content and hypertrophic chondrocytes; the collagen fibres are anchored to the epyphyseal bone allowing the attachment of cartilage to the bone (Mow et al. 1989; Dewire and Einhorn 2001; Poole et al. 2001; Frisbie 2006; Ge et al. 2006; Getgood et al. 2009).

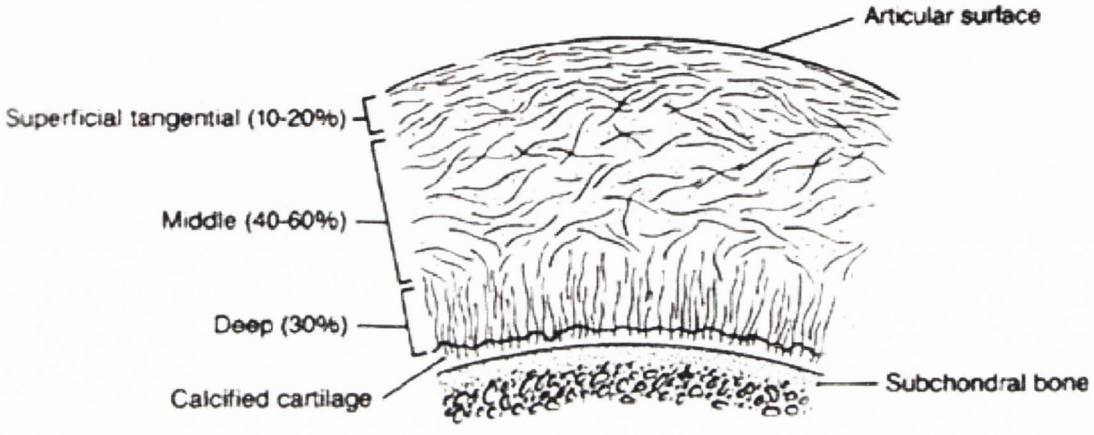


Figure 2.1: Zonal organization in articular cartilage (Mow et al. 1980).

2.2.2 Extracellular matrix

The extracellular matrix (ECM) consists of two components: the tissue fluid and the framework of structural macromolecules that give the tissue its form and stability. The interaction between both provide the tissue its unique mechanical properties (Mankin et al. 2007). Water is the most abundant component of articular cartilage (80 % of its volume), it is most concentrated near the articular surface of the articular cartilage and decreases linearly with increasing depth to an approximate concentration of 65 % in the deep zone (Fig. 2.2). A small percentage of the water is intracellular and 30 % is associated with collagen fibrils. The water that occupies the intermolecular space is free to move when a load or pressure gradient is applied to the cartilage. Thus movement is important in controlling cartilage mechanical behaviour and joint lubrication (Mow et al. 1989). The cartilage macromolecules collagens, proteoglycans, and non-collagenous proteins contribute 20 % to 40 % of the wet weight of the tissue. The three classes of macromolecules differ in their concentrations within the tissue and in their contributions to the tissue properties (Mankin et al. 2007).

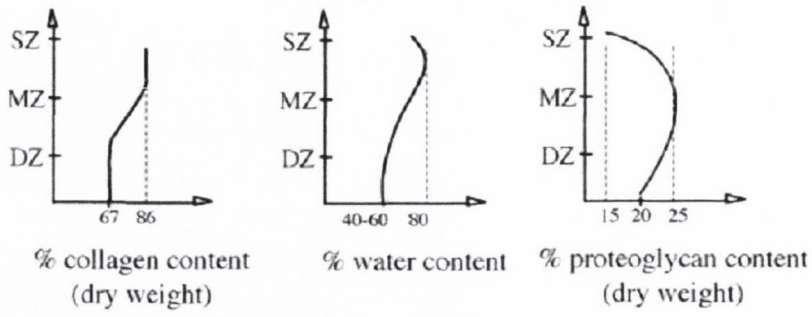


Figure 2.2: Distribution of collagen, water and proteoglycan content per dry weight as a function of depth from the articular surface. (SZ: superficial zone, MZ: middle zone and DZ: deep zone) (Mow and Guo 2002).

Collagens contribute about 60 % of the dry weight of cartilage. The collagen fibrillar meshwork gives cartilage its form and tensile stiffness and strength, is also responsible for maintaining the physical location of the chondrocyte and contributes to the cohesiveness of the tissue by mechanically entrapping the large proteoglycans (Fig. 2.3). The collagen fibrils in the articular cartilage are predominantly type II (95 %), but also includes types VI, IX and XI with type X in the calcified zone (Poole et al. 2001).

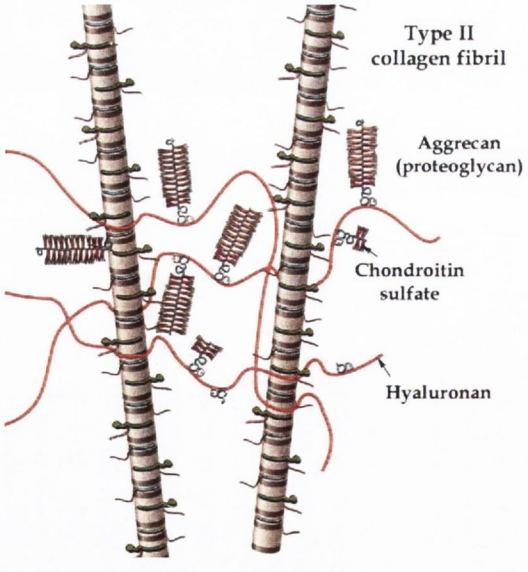


Figure 2.3: Schematic representation of collagen and aggrecan network in the articular cartilage (Poole et al. 2001).

Proteoglycans are negatively charged and are responsible for sequestering water molecules into the tissue (Mankin et al. 2007). Inorganic ions such as sodium, chloride and potassium are the common ions dissolved in the tissue water. These ions balance the fixed charges on

the proteoglycans and generate significant osmotic swelling pressure (Cowin and Doty 2007), contributing to the compressive properties of the tissue. Proteoglycans molecules consist of sulphated glycosaminoglycans (GAG) chains linked covalently to a core protein, fixed to the hyaluronan or hyaluronic acid molecule (Fig. 2.4) (Dewire and Einhorn 2001; Mow and Hung 2001; Poole et al. 2001).

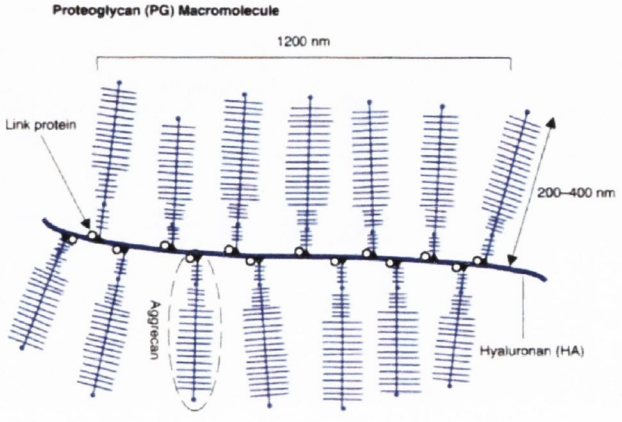


Figure 2.4: Schematic representation of a proteoglycan macromolecule (Mow and Hung 2001).

GAG consists of repeating disaccharides that contain an amino sugar. Each dissacharide unit has at least one negatively charged carboxylate or sulphate group, so the glycosaminoglycans form long strings of negative charges that repel one another and attract cations (Mankin et al. 2007). Such a conformation gives rise to a significant increase in the osmostic swelling effect and bring the fixed negative charges into closer proximity, thereby increasing the electrostatic repulsive forces. The magnitude of the osmotic pressure in articular cartilage has been measured to approach 0.35 MPa and is dependent upon the negative charge density on the proteoglycan (Fig. 2.5) (Cowin and Doty 2007).

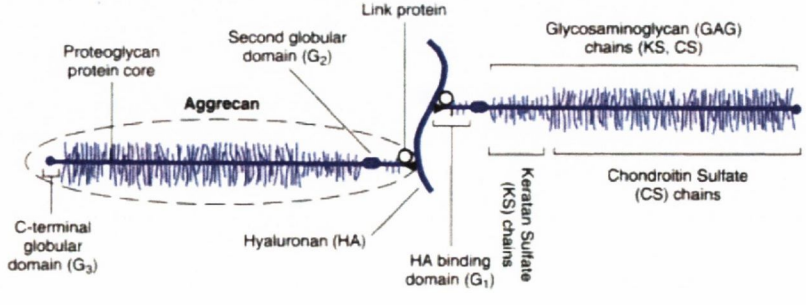


Figure 2.5: Diagram of aggrecan structure. The keratan sulphate and chondroitin sulphate chains bound covalently to a link protein (Mow and Hung 2001).

The remaining non-collagenous proteins within cartilage are mainly composed of small proteoglycans, such as decorin and biglycan which bind to collagen fibres and thus promote aggregation of the fibres into a collagen meshwork, whereas large proteoglycans with many branching side chains, such as aggrecan and versican, are entrapped in the tissue through frictional interactions with the collagen meshwork (Ge et al. 2006). This matrix provides structural support to the articular cartilage and provides inherent biomechanical properties such as elasticity (Meyer and Wiesmann 2006).

2.2.2.1 Chondrocytes

Adult articular cartilage has long been proposed to consist of a single cell type: chondrocytes. In recent years hypotheses have been put forward to counter act this idea, based on the presence of a progenitor cell population residing in the superficial and middle zone of the cartilage (Karlsson and Lindahl 2009; Pretzel et al. 2011). Chondrocytes are derived from MSCs; those cells are called chondroblasts and are located alone, in pairs or grouped in lacunae in the ECM. From the superficial to the deep zone, chondrocytes decrease in number and increase in size and metabolic activity (Ge et al. 2006). They secrete lubricin, a molecule which is responsible for reducing the coefficient of friction and thus providing cartilage with such favourable properties (Getgood et al. 2009). Chondrocytes are responsible for the production and maintenance of the ECM. They have both anabolic (synthesis) and catabolic (degradation) capabilities.

2.2.3 Biomechanical properties of articular cartilage

Articular cartilage is subjected to a wide range of static and dynamic mechanical loads during daily activity. The ability of cartilage to withstand physiologic compressive, tensile, and shear forces depends on the composition and structural integrity of its extracellular matrix (Mankin et al. 2007). The ECM with associated interstitial fluid, solutes and ions, can be imagined as a mechanical signal transducer that receives input in the form of joint loading and yields an output of various extracellular signals (e.g., deformation, pressure electrical), as well as fluid, solute (e.g., nutrient) and ion flow fields (Mow and Guo 2002). The water phase of the cartilage is an important constituent in controlling many physical properties of the tissue. It occupies up to 80 % of articular cartilage wet weight and it appears that most of this water is intra-fibrillar, therefore not available for transport under

mechanical loading (water is being absorbed by the collagen fibres). This exclusion raises the density of the fixed charges within the tissue, increasing the osmotic pressure.

The dominant load-carrying structural components of the solid matrix by composition are the collagen molecules and the negatively charged proteoglycans. As described previously, collagen fibres are distributed and orientated in a non homogeneous way by zonal variations in the cartilage. This layering inhomogeneity appears to serve an important biomechanical function by distributing the stress more uniformly across the loaded regions of the joint tissue (Cowin and Doty 2007). The most important mechanical properties of collagen fibres are tensile stiffness and strength (Mow et al. 1989). On the other hand proteoglycans contribute to the compressive properties of the tissue in two ways: a swelling pressure associated with the charged nature of proteoglycan aggregates and the bulk compressive stiffness of those immobilised within the collagen network (Mow and Guo 2002). The superficial zone of the cartilage has relatively low proteoglycan content and a low permeability, which is important in providing for a barrier of high resistance against fluid flow when cartilage is compressed. In the middle zone, the proteoglycan content will increase to 25 % of its total volume, giving rise to a high swelling pressure, particularly in tissues with a damaged surface zone (Mow and Guo 2002).

Due to the interaction of the solid and fluid phases during loading, cartilage tissue exhibits creep when subjected to a constant load and stress relaxation during an applied constant displacement. The viscoelasticity of articular cartilage due to interstitial fluid flow is known as biphasic viscoelastic behaviour and the component of viscoelasticity due to macromolecular motion is known as flow-independent or intrinsic viscoelastic behaviour of the collagen-proteoglygan solid matrix (Mow et al. 1980; Mow et al. 1989). Different tests can be used to determine the mechanical properties of the articular cartilage including confined compression, unconfined compression, indentation, contact analyses among others (Mow et al. 1989; Mow and Guo 2002). A common test carried out to determine the mechanical properties of articular cartilage is the unconfined compression test. Two impermeable steel platens are used to compress a sample of cartilage that is free to bulge in the transverse direction. This stress relaxation test enables us to determine the intrinsic equilibrium Young's modulus, which is a measure of the inherent compressive properties of the solid phase and is positively correlated with GAG content. In this test the parallel organisation of the collagen fibres in the superficial zone restrains lateral expansion which generally acts to enhance fluid pressurisation (Fig. 2.6).

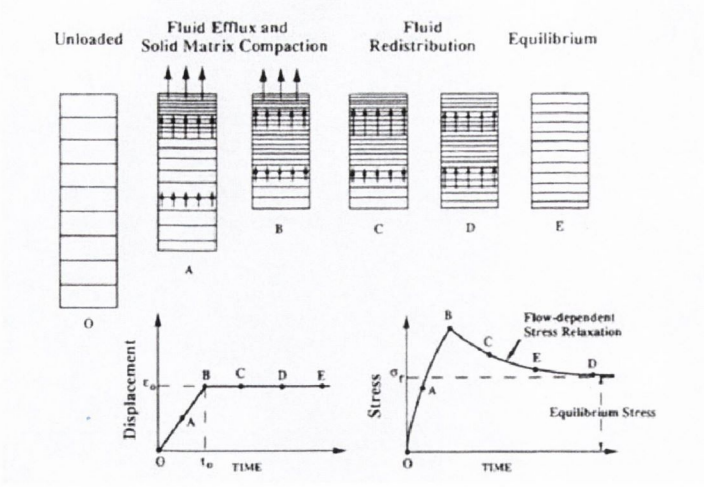


Figure 2.6: Schematic representation of fluid exudation and redistribution within cartilage during a rate-controlled compression stress-relaxation experiment. The horizontal bars in the upper figures indicate the distribution of strain in the tissue. The lower graph shows the stress response during the compression phase (O, A, B) and the relaxation phase (B, C, D, E) (Mow and Huiskes 2005).

By performing a cyclic loading test, the dynamic modulus can be calculated from the stress and strain peak to peak amplitudes, mimicking joint loading during daily activities. There are a variety of ways to study the biomechanics of cartilage depending on the objective of the observer, where the goal is to gain a better understanding of the tissue biomechanical properties and function of cartilage.

2.3 Articular cartilage pathology and treatment options

The chondrocyte plays a critical role in cartilage metabolism, organizing and regulating ECM composition. At different stages of development from growth to maturation, the relative levels of matrix degradation and synthesis are adjusted. The obtained result determines a growth, equilibrium or degradation status (Todhunter 1996). Chondrocytes, inflammatory cells and synoviocytes under the influence of biomechanical stimuli, matrix enzymes, cytokines, nitric oxide and prostaglandins regulate cartilage homeostasis. If a rupture of the homeostasis is produced by different etiologic agents (mechanical, biochemical or genetic factors) it can lead to a catabolic status and then to articular cartilage defects and pathology.

The paucity of chondrocytes within the ECM, their inability to migrate to the zone of injury, and their inability to regenerate large amounts of ECM, mean these defects will

usually progress. Full thickness defects, which penetrate the subchondral bone, do have the potential for intrinsic repair due to the communication gained with the marrow cavity and the MSCs population within (Getgood et al. 2009). A blood clot made of red and white cells, lipids, growth factors and MSCs from the BM facilitates the formation of a fibrous or fibrocartilaginous repair tissue. The mechanical properties of the newly synthesised fibrocartilage are inferior to normal cartilage and will generally degenerate over time (Bahuleyan et al. 2009). There are two main types of articular disorders in the joint that leads to articular defects: arthritis and internal derangements of the joint.

2.3.1 Arthritis

The term arthritis is used to include both inflammatory and degenerative lesions of a joint. It implies a diffuse lesion affecting the joint as a whole. Clinically, arthritis is generally characterised by pain and restriction of movement at a joint arising spontaneously. The four more common types of arthritis are: pyogenic arthritis, rheumatoid arthritis, tuberculous arthritis and OA. Of these, OA and then rheumatoid arthritis are the most frequent (Adams and Hamblen 1995). In this review we will detail the most relevant disease to the thesis: OA.

OA has long been considered as being the result of age or trauma. This concept has evolved, and it is now accepted that the aetiology of OA is multiple and includes various mechanical, biochemical, and genetic factors. Any alteration of a joint may be responsible, directly or indirectly, for the development of OA. The main predisposing factors are: congenital–ill development, irregularity of joint surfaces from previous fracture, internal derangements, such as loose body or a torn meniscus, previous disease such as rheumatoid arthritis or haemophilia, mal-alignment of a joint and obesity (Adams and Hamblen 1995). The progression of this disease is generally divided into three broad stages. Stage I is the proteolytic breakdown of cartilage matrix. In stage II, we have fibrillation and erosion of cartilage surface, which is accompanied by the release of breakdown products into the synovial fluid. During stage III, synovial inflammation begins when synovial cells ingest a breakdown product through phagocytosis and produce proteases and proinflammatory cytokines (Martel-Pelletier 2004). This is accompanied by hypertrophic bone changes with osteophyte formation and subchondral bone plate thickening. This is one of the two main hypothesis described in the literature regarding the etio-pathiogenesis of OA.

A controversy still persists in the literature, where some researchers believe that the phenomenon is initiated in the subchondral bone, as a result of different etiologic agents (as trauma, obesity, etc) leading to subchondral bone sclerosis and implicating cartilaginous lesions with a disorganisation of the ECM (Kawcak et al. 2001; Riggs 2006). OA involves crosstalk between tissues on the cellular and cytokine levels, at least in some subgroups or phenotypes, and not all pathways have been elucidated yet (Martel-Pelletier 2004; Ge et al. 2006; Krasnokutsky et al. 2008; Samuels et al. 2008; Getgood et al. 2009) (Fig. 2.7). Drugs have been developed to target these mediators which have shown promising results. However, it is likely that more than one causative factor will need to be addressed, especially as the severity of the disease progresses. Therapy for OA is aimed at relieving symptoms and at maximizing function. Therapies can be considered as either symptom modifying OA drugs or as disease modifying OA drugs.

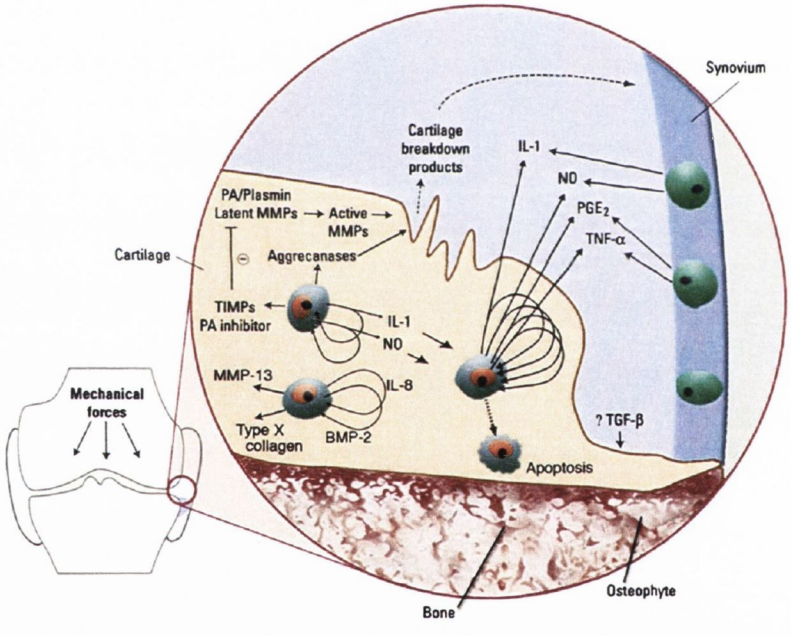


Figure 2.7: Molecular pathogenesis of OA. Potential biomarkers and targets for disease modification are released as a result of events in cartilage, bone, and synovium (Krasnokutsky et al. 2008).

To date only symptom modifying OA drugs therapies are available, if the condition worsens and little or no cartilage remains, the bones may rub against each other causing great pain. At this stage joint replacement is often the only therapeutic option (Brady et al. 1997; Pelletier et al. 2001).

2.3.2 Internal derangements

Internal derangements imply a localized mechanical fault which interferes with the smooth action of a joint. This type of mechanical damage can be considered in two groups: loose body formation and osteochondritis dissecans (OCD). Intra-articular loose bodies may be derived from bone, cartilage or SM. They may be entirely free within the joint or they may retain a pedicle of soft tissue resulting in free-floating islands which can travel throughout the joint. The commonest causes of loose bodies are OCD and fracture of the articular end of the bone. Loose bodies cause symptoms when they become caught between the joint surfaces. Osteochondritis itself signifies the disturbance of the usual growth process of cartilage and OCD is the term used when this affects joint cartilage causing a fragment to become loose. OCD is considered a developmental and metabolic disorder related to cartilage growth and endochondral ossification. Failure of the developmental process leads to a disturbance in the formation of joint cartilage and the bone supporting it. The resultant cartilage and subchondral bone is irregular in thickness and weaker than in normally developing areas. There may be a loss of blood supply and necrosis of the abnormal tissue. Forces placed on these abnormal areas when the patient ambulates may cause further damage to the abnormal tissue. This can lead to several problems, including local detachment and fracture of cartilage and bone (Adams and Hamblen 1995) (Fig. 2.8).

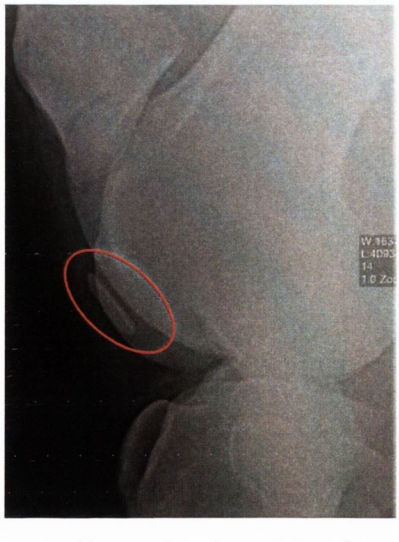


Figure 2.8: Equine knee radiograph of an OCD fragment. The OCD fragment is circled in red. Notice that the fragment seems to "float" within a defect in the main bone.

2.3.3 Cartilage repair

In 1743, Hunter stated that "From Hippocrates down to the present age, we shall find that an ulcerated cartilage is universally allowed to be a very trouble–some disease; that it admits of a cure with more difficulty than a carious bone; and that, when destroyed, it is

never recovered". Again in 1851, Paget said, "There are, I believe, no instances in which a lost portion of cartilage has been restored, or a wounded portion repaired, with new and well-formed permanent cartilage, in the human subject." Since then, numerous studies have confirmed that articular cartilage has limited capacity for spontaneous repair, except for formation of fibrous or fibro-cartilaginous tissue and its unique mechanical functions are never re-established spontaneously after a significant injury (Ahsan and Sah 1999; Messner 2001).

In terms of cartilage repair it is essential to differentiate between an injury involving cartilaginous tissue solely (partial-thickness injury) or one that also penetrates subchondral bone to the marrow cavity (full-thickness injury). Similar to cartilage fissures in OA, partial thickness defects above a certain size do not usually heal spontaneously, but some repair may be initiated by special means. Beside the extent of injury in depth, its width seems to be of importance; defects 3 mm in size and larger heal less satisfactorily than smaller ones (Messner 2001). The spectrum of alternative treatments for focal, chondral or osteochondral lesions is wide. Treatment options are divided into four categories:

1. Symptomatic treatment to reduce clinical symptoms, like lavage and debridement.
2. Recruitment of stem cells from the BM; this group includes multiple drilling, microfracturing, abrasion arthroplasty and spongialization.
3. Cellular induction of chondrogenesis; this group includes periosteal and perichondrial transplantation and ACI.
4. Transplantation of osteochondral allografts or autografts (Peterson 2001).

2.3.4 Symptomatic treatment

This is the most simple of procedures but has been shown to have the least predictable outcome. It consists of lavage to washout synovial fluid (with released enzymes and inflammatory mediators) and debridement, aimed at relieving mechanical symptoms associated with unstable flaps of cartilage, loose bodies, osteophytes which interfere with joint function. The symptomatic relief from debridement has positive feedback with up to 80 % improvement in the first year but these results gradually decline over time. Moseley et al performed a study which compared arthroscopic lavage to placebo and found that neither arthroscopic lavage nor debridement were better than placebo (Peterson 2001; Moseley et al. 2002; Getgood et al. 2009).

2.3.4.1 Marrow stimulation

The subchondral bone plate separates the rich vascular plexus of the BM from the avascular cartilage. This allows marrow derived cells including MSCs, platelets, fibroblasts and other chemotactic factors to invade the damaged area and produce repair tissue able to fill a chondral lesion. Repair tissue even if chondroid in appearance will undergo degeneration over time. Drilling and multiple microfracturing are 2 techniques that penetrate into the trabecular bone (Peterson 2001; Getgood et al. 2009) (Fig. 2.9).
Both techniques involve debridement of the peri-lesional cartilage; 3-4 mm deep holes are then made in the subchondral bone 2-3 mm apart, starting in the periphery, working into the centre of the defect. Multiple drilling technique changes the elastic characteristics of the subchondral bone, as are the shock-absorbing effects of the subchondral bone plate and the trabecular bone, which may contribute to the degeneration of the repair tissue after the treatment (Peterson 2001).

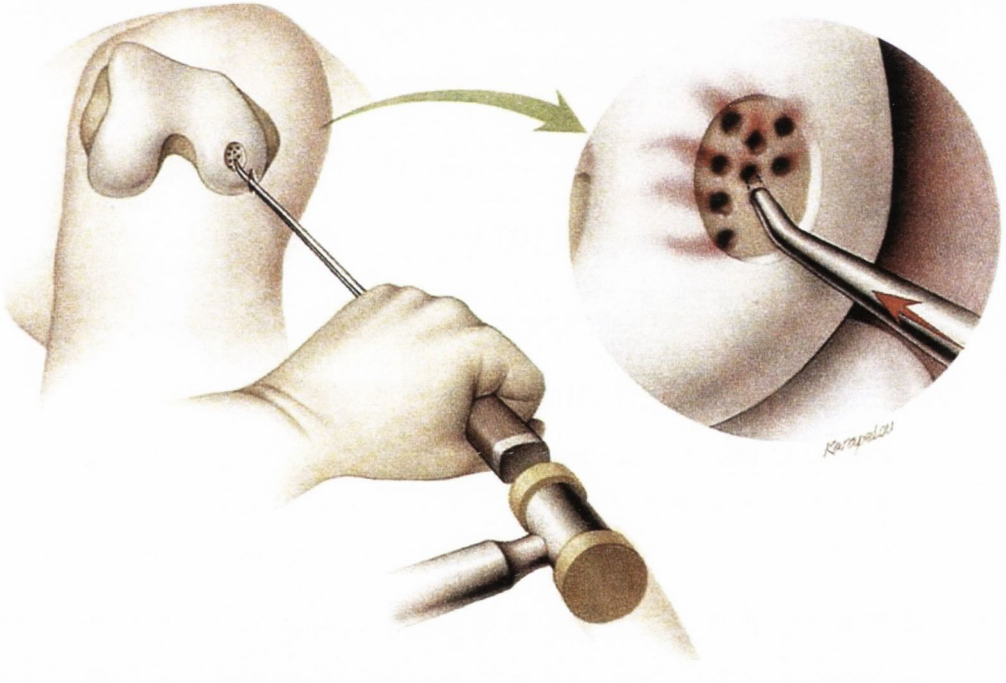


Figure 2.9: Microfracturing technique. Awls with various degrees of angulation are introduced arthroscopically and used to penetrate the subchondral bone and encourage stem cells production of cartilage-like tissue (Peterson 2001).

Multiple microfracturing is not as aggressive as the drilling technique because it preserves the subchondral bone plate; however it may increase the stiffness of the subchondral bone plate in the long term. Another advantage of the microfracturing technique is the non production of heat necrosis (Steadman et al. 1999; Steadman et al. 2003; Minas et al.

2009). Seven years follow up with this technique has shown a success rate of 75 %. Frisbie *et al.* showed in an equine microfracturing study the importance of removing the calcified cartilage layer to allow an optimal amount and attachment of the repair tissue (Frisbie et al. 2006). Abrasion arthroplasty, affects only the superficial layer of the subchondral bone, it involves superficial abrasion of the subchondral bone with a motorized burr. The goal is to reach the cortical vascularization and allow stem cells migration and production of fibrocartilage. Over time the success of this technique decrease to 50 % after 5 years follow up (Getgood et al. 2009) (Fig. 2.10).

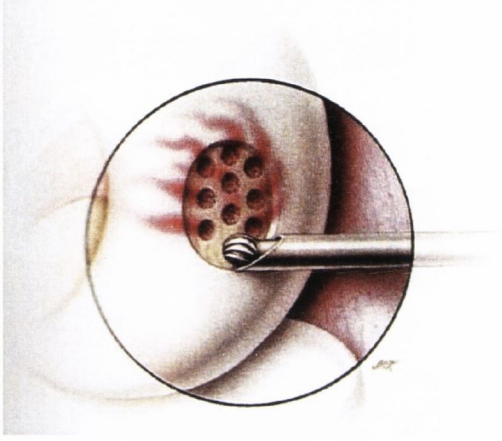


Figure 2.10: Abrasion chondroplasty technique (Peterson 2001).

Finally spongialization consists of removing the subchondral bone and leaving the trabecular spongious bone surface. This technique is not used for isolated lesions but for more widespread damage.

2.3.4.2 Autologous osteochondral transplantation

In this technique (also called mosaicplasty), osteochondral plugs are transferred from un-diseased and relatively non-weight bearing areas to the lesion site. It is the only procedure which produces hyaline cartilage within the defect. One advantage of the technique is that rapid bone healing provides good vertical fixation of osteochondral plugs into the implant site (Koga et al. 2009). Different studies have shown that this technique results in donor site morbidity and extensive cell death of chondrocytes in the superficial aspects and margins of the osteochondral plug leading to biomechanical failure at the interface regions and further cartilage degeneration (Khan et al. 2008). Attempts to counteract this problem have included microfracturing the gaps and inserting bone morphogenic protein 7 (BMP-7) in a collagen matrix. Mosaicplasty has shown mixed results, with a number of studies

showing superior outcomes to marrow stimulation techniques (Getgood et al. 2009) (Fig. 2.11) .

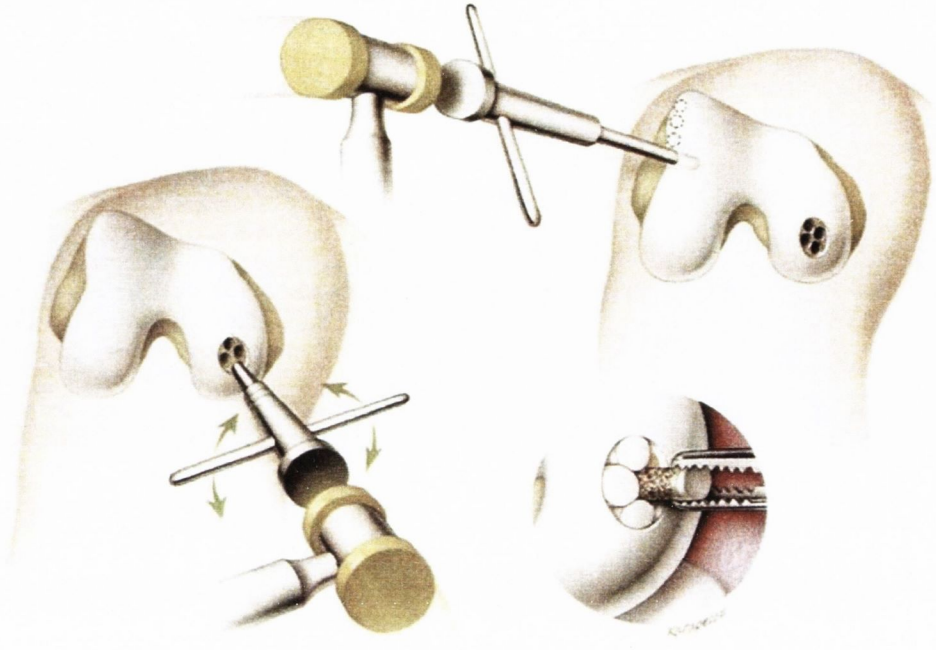


Figure 2.11: Drawing of autologous osteochondral transplantation technique (Peterson 2001).

2.3.4.3 Autologous chondrocyte implantation

The clinical use of ACI was first reported by Brittberg and collaborators (Brittberg et al. 1994). A cartilage biopsy from non-weight bearing areas is taken arthroscopically, chondrocytes are then enzymatically isolated and culture expanded *in vitro*. The expanded cells are harvested and transplanted with fibrin beneath a periosteal flap, harvested from the proximal tibia and sutured over the defect to keep the cells in place (Khan et al. 2008). Bentley *et al.* have since shown improved outcomes with ACI over mosaicplasty, with 88 % showing good to excellent results with ACI compared to 69 % with mosaicplasty at a mean time frame of 19 months, but at 24 months the histologic scores seems to show no difference between the two techniques (O'Driscoll and Saris 2007). Two operations are required for the ACI technique, it is very expensive to culture the cells and the repair tissue is still not like hyaline cartilage in terms of mechanical properties or phenotype (Getgood et al. 2009) (Fig. 2.12) .

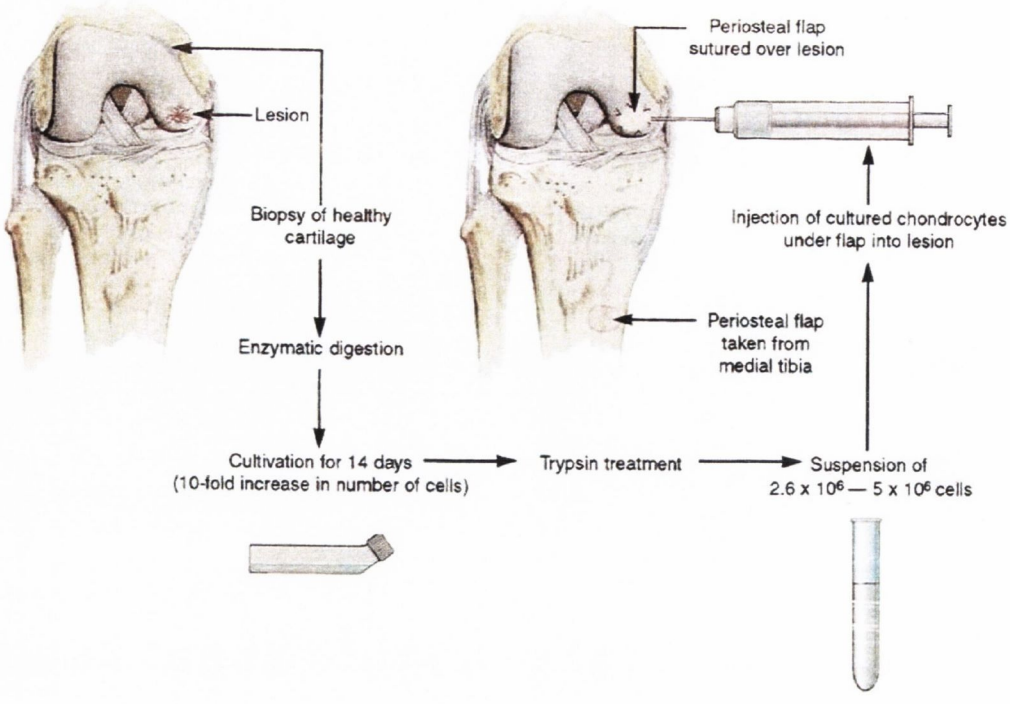


Figure 2.12: Diagram of ACI transplantation technique (Peterson 2001).

In recent years a new second generation of cartilage repair technique has emerged using a scaffold instead of the periosteal flap, called matrix-assisted chondrocyte implantation procedure (MACI). Its composed of a type I and III collagen porcine degradable membrane that covers temporarily the chondrocytes in a cell suspension and gives them support to grow until the cells can produce their own matrix (Clair et al. 2009). This technique reduces the risks of hypertrophy and ossification associated with periosteal tissue and increases the stability of the graft, however an open surgical technique with sutures is still required. When first and second generation techniques were compared, no significant differences in the clinical outcome was observed (Manfredini et al. 2007). The third generation technique consists of the same membrane but with the cells seeded on the top of the membrane. This technique overcame the shortcomings of the first- and second-generation ACI, is less invasive, requires less surgical time, has a low incidence of postoperative complications and can be used to access difficult-to-reach defect sites (Brittberg 2010). These new techniques and technologies hold much promise in the treatment of cartilage repair, as the field continues to advance. Further prospective, randomized and longer term outcomes are needed in order to validate the usage of these novel cartilage repair techniques.

2.4 Cartilage tissue engineering

Tissue engineering is a new technology, that applies the principles of biology and engineering to the development of functional substitute tissues for damaged tissue (Vunjak-Novakovic and Goldstein 2005). Cells, biomaterial scaffolds and regulatory factors can be utilized in a variety of ways to engineer functional tissue structures, either *in vitro* or *in vivo* (following surgical implantation); these three factors constitute the tissue engineering triad (Fig. 2.13).

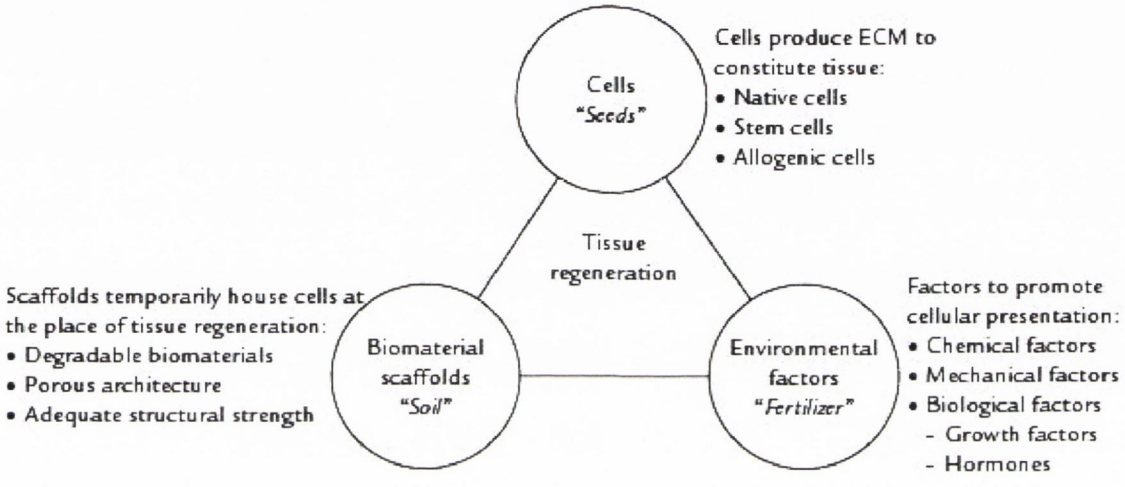


Figure 2.13: The tissue engineering triad (Chiang and Jiang 2009).

The primary function of orthopaedic tissues is biomechanical in nature, and the main goal of all orthopaedic tissue engineering is the restoration of normal biomechanical function (Vunjak-Novakovic and Goldstein 2005). The clinical utility of tissue engineering will likely depend on the ability to replicate the site specific properties of cartilage. In engineered constructs, the cells must conform to a specific differentiated phenotype and the composition and architectural organization of the ECM must provide the necessary biomechanical properties inherent to the tissue being replaced, as well as contiguity and strength between the replacement constructs with the neighbouring materials. Ideally an engineered cartilage graft should provide the regeneration of new functional tissue, rather than repair with weak fibrocartilage and undergo orderly remodelling in response to environmental factors (Vunjak-Novakovic and Goldstein 2005). The typical tissue engineering approach requires the use of a scaffold, but some researchers have been producing scaffold-free implants, such as aggregates of cells or using the scaffolds as moulds for creating a specific shape (Stoddart et al. 2009). The disadvantage of tissue engineering approaches is the costly and long *in vitro* culture period required to develop the graft prior to implantation. The ideal situation would be a one step procedure with

appropriate rehabilitation program, but a variety of parameters, including cell source, cell density, carrier, expansion, chemical supplements need to be tested before initiating *in vivo* animal and clinical studies (Grad et al. 2011). Bioreactors have been developed to test all those parameters; the purpose of those systems is to reproduce the *in vivo* environment, offering the possibility to evaluate and compare novel therapeutics effects. Bioreactors are particularly useful for understanding of regulation of chondrocytes and MSC fate (Grad et al. 2011)

2.4.1 3D environment and biomaterials used for cartilage regeneration

In articular cartilage, chondrocytes are embedded in a lacuna immersed in the ECM, if those cells are harvested and isolated in monolayer culture; they tend to lose their phenotype adopting a fibroblast-like morphology and secreting type I collagen (Darling and Athanasiou 2005; Vinatier et al. 2009). In order to retain their phenotype chondrocytes need to be condensed into a 3D environment. First described by Holtzer and collaborators in 1960 for use in studying embryonic chick vertebral chondrocytes, the pellet culture system is the most popular differentiating system used. After isolation and expansion, chondrocytes are centrifuged in a defined medium (approximately 250 000 cells); 24 hours later the cells form a free-floating aggregate, leading to chondrogenic differentiation (Holtzer et al. 1960; Manning and Bonner 1967; Zhang et al. 2004).

The 3D environment seems to be a critical step supporting the chondrocytic phenotype, for those purposes scaffolds are used to deliver cells and growth factors to the injury site and to guide the new regenerative tissue into a stable chondrogenic phenotype in order to support cartilage tissue assembly and growth (Gabay et al. 2010). Such biomaterials should meet certain fundamental requirements including being non immunogenic, biodegradable, non toxic, porous and permeable (Hunziker 2002; Pelttari et al. 2009; Stoddart et al. 2009; Vinatier et al. 2009). The materials used to produce these scaffolds have been classified as:

- **Hydrogels:** mimics the hydrated nature of cartilage, have viscoelastic properties and maintain the spherical shape of the cells as they become encapsulated within the hydrogel. Homogeneous cell distribution within the material and the capacity of adaptation and fixation in the defects are further advantages of hydrogels (Dickhut et al. 2008). Hydrogels used for cartilage tissue engineering include agarose (a polysaccharide derived from marina algae), alginate, hyaluronic acid, gelatin, fibrin

glue, collagen derivatives and acellular collagen matrix (Kon et al. 2008; Almqvist et al. 2009; Erickson et al. 2009; Kim et al. 2009; Gabay et al. 2010).

- **Meshes and sponges**: highly porous, have easy moulding characteristics, relatively easy production. Examples include Poly (lactic-co-glycolic acid) (PLGA) sponges, Polyglycolic acid (PGA) fibers, Poly (ethylene glycol) (PEG) and nanofibers (Getgood et al. 2009; Gabay et al. 2010).
- **Material composites**: provide features such as enhanced stiffness and surface lubricity. Most commonly used are polymer and ceramic biphasic inorganic materials formed through treatment with heat. These are often porous and brittle bioactive glass, hydroxyapatite and polymer used to tailor biologic response of cells and drug delivery (Gabay et al. 2010).
- **Structural composites:** hydrogel incorporation of polymer meshes and sponges used to enhance gel mechanical properties, for example polyactid sponge + alginate gel (Gabay et al. 2010).

2.4.2 Engineering cartilaginous tissues using chondrocytes

Among the various cell types that have been contemplated for cartilage tissue engineering, chondrocytes from hyaline cartilage have been considered the logical cells of choice. Chondrocytes grown in three-dimensional culture are capable of synthesizing a cartilaginous ECM which is similar to that of the native tissue. Although much progress has been made in refining this approach, it still has proven difficult to accumulate enough ECM to produce a tissue with properties that mimic native articular cartilage (Buschmann et al. 1992; Buschmann et al. 1995). These cells are subject to two major concerns: instability in monolayer culture, in fact chondrocytes tend to loose their ability to produce hyaline cartilage like matrix and produce mainly type I collagen and the lack of donor availability (Vinatier et al. 2009). As a consequence of dedifferentiation and loss of phenotypic traits during *in vitro* expansion, a progressive loss of cell ability to form stable ectopic cartilage *in vivo* has been observed (Stewart et al. 2000). It is conceivable that a regenerate composed of poorly differentiated fibrocartilage may arise from the application of such dedifferentiated cells (Pelttari et al. 2008).

The chondrogenic differentiation media plays a key role in the phenotypic stability of chondrocytes. For the last ten years an effort seems to be appearing in the scientific world moving towards a serum free chondrogenic medium. Previously chondrocytes and articular

cartilage explants were cultured in the presence fetal bovine serum (FBS). The use of this serum has significant drawbacks as is not a well-characterized culture supplement and possesses batch-to-batch compositional variations (Honn et al. 1975; Lima et al. 2007).
To be specific, FBS presents unknown concentrations of binding proteins and growth factors making it difficult to determine the effects of experimentally added growth factors. Moreover, the capacity of FBS to support chondrogenesis is so variable that some investigators test or even pool the serum from different donors before use. Based on these concerns regarding the variable biological effects of FBS, a serum-free media is preferred approach for tissue engineering applications (Fitzsimmons et al. 2004). However, the common practice still continues to exist where cells are culture in basal medium such as Dulbecco's modified Eagle's medium (DMEM) in the presence of 10 % FBS during the expansion phase. Then once the cells are seeded in scaffolds and the goal is to engineer cartilage like tissue the media is changed to a serum free media (Lima et al. 2007; Thorpe et al. 2008; Erickson et al. 2009).

The main components of such serum free media are cytokines, defined as small cell-signaling protein molecules that are secreted by different cells of the body (Goldring 2000). Cytokines play an important role in the regulation of cartilage homeostasis and can be grouped into 3 categories: catabolic, anabolic and inhibitory cytokines (Martel-Pelletier et al. 1999; Oliviero et al. 2010). The main cytokines used to engineer cartilage-like tissue are the anabolic cytokines, including members of the TGF superfamily, fibroblast growth factor (FGF) and insulin growth factor (IGF). TGF-β and bone morphogenic proteins (BMP) are the two major branches of the TGF superfamily (Groppe et al. 2008). They are known to play a key role in chondrogenesis and to stimulate the proliferation and synthesis abilities of cartilaginous matrix *in vitro* and *in vivo* (Tang et al. 2009).

TGF-β is composed of three isoforms TGF-β1, TGF-β2 and TGF-β3; they differ in their effects on chondroprogenitor cells but the all three are used for cartilage tissue engineering purposes. TGF-β1 is responsible for the cell interaction between condensing cells, has anti-inflammatory properties and stimulates matrix synthesis by chondrocytes. TGF-β2 mediates hypertrophic differentiation of chondrocyte. Both TGF-β1 and 2 have been reported to possess osteoinductive potential while TGF-β3 has been observed to be upregulated in dedifferentiated human chondrocytes (Hao et al. 2008) (Fig. 2.14).

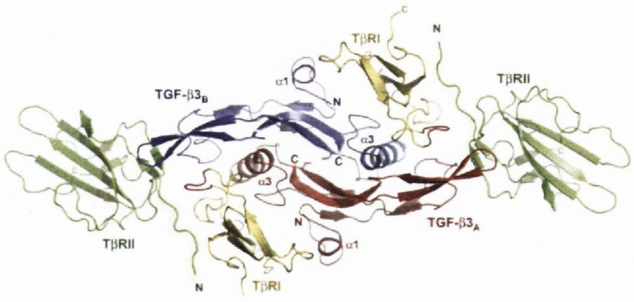


Figure 2.14: Structure of TGF-β3 (Groppe et al. 2008).

Dexamethasone is also used in the serum free media to enhance chondrogenesis of chondrocytes. The use of this synthetic glucocorticosteroid is controversial and different research groups have shown that glucocorticosteroids either promote or inhibit the elaboration of a cartilaginous matrix by chondrocytes *in vitro* and *in vivo* (Miyazaki et al. 2000; Celeste et al. 2005; Sekiya et al. 2005; Buxton et al. 2011).

2.4.3 Engineering cartilaginous tissues using MSCs

One of the major limitations of using chondrocytes for tissue engineering is the need to take biopsies from healthy cartilage for cell isolation, which implies additional donor site morbidity for the patient (Hildner et al. 2011). There is also evidence to suggest that age diminishes the potential of autologous chondrocytes to synthesize cartilaginous ECM (Adkisson et al. 2001). This has led to the search for alternative cell sources for cartilage repair therapies. MSCs are a highly available cell type that can be isolated from diverse tissue, without irreversible damage.

Stem cells are defined as undifferentiated cells capable of self renewal that can differentiate into more than one specialized cell type. The potential degree of cellular differentiation varies among stem cell populations:

a) Totipotent stem cells (e.g. early mammalian embryos) have unlimited differentiation capability and can form all cell types of the conceptus, that includes the embryo as well as the embryonic part of the placenta and its associated membranes.
b) Pluripotent cells (e.g. embryonic cells) can produce cells that derive from all three germ layers: endoderm (liver, pancreas, thymus, thyroid and lung), mesoderm (bone, cartilage, muscle, heart and kidneys) and ectoderm (skin, brain, eyes and neural tissue).
c) Multipotent cells give rise to lineage-restricted, tissue specific cell types (e.g. hepatic stem cells giving rise to hepatic or cholangiocytic cells).
d) Unipotent stem cells only generate one cell type.

There is much controversy surrounding the use of totipotent and pluripotent stem cells for research. They carry many social and ethical issues. Furthermore, legal constraints prevent the use of these cells in many countries. In contrast, multipotent stem cells like MSCs isolated from the BM are becoming increasingly recognized for their potential to generate different cell types (Fig. 2.15) and thereby function effectively *in vitro* or *in vivo* to either engineer grafts or regenerate tissues (Schumann et al. 2006).

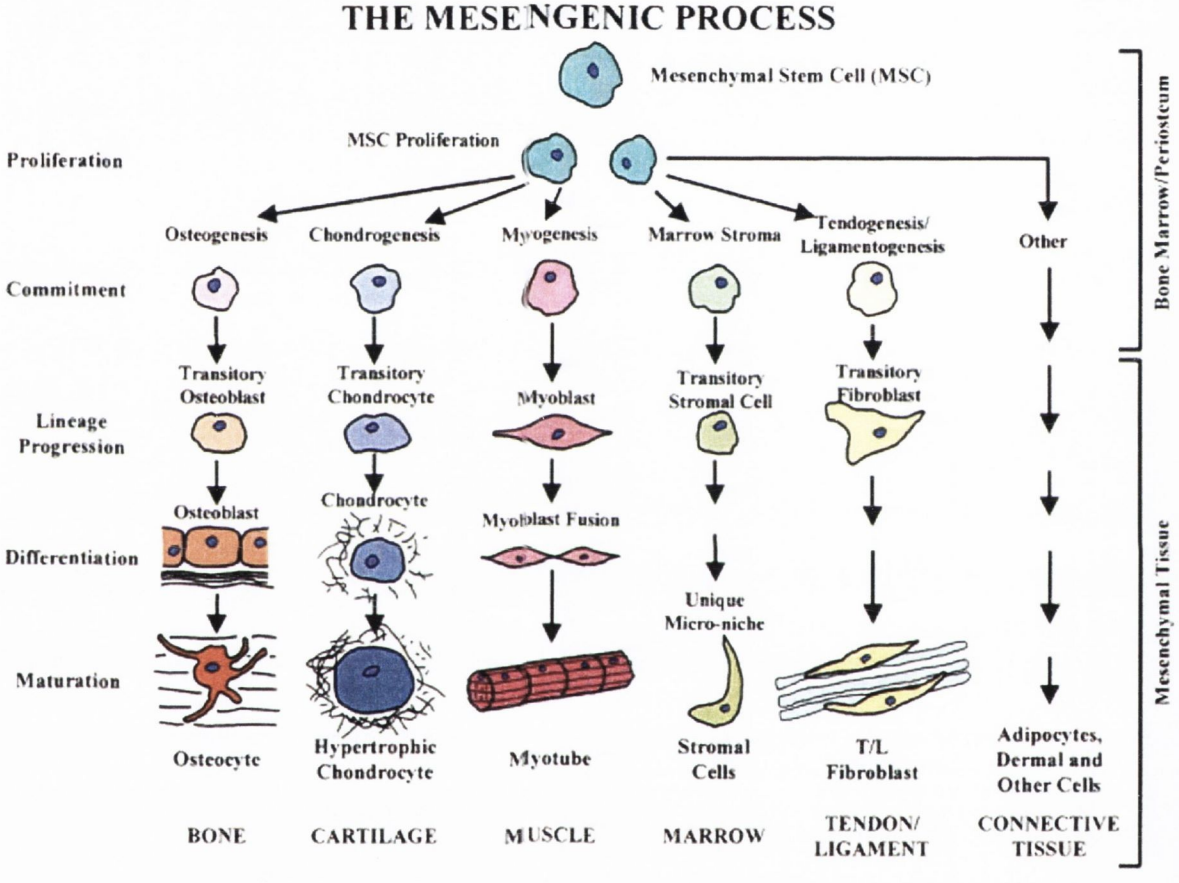


Figure 2.15: The mesengenic process diagram. MSCs have the capacity to differentiate into bone, cartilage, muscle, marrow stroma, tendon/ligament, fat and other connective tissues. The sequence of this differentiation involves multistep lineages controlled by growth factors and cytokines. This figure is structured in a manner comparable to hematopoietic lineage progression and involves well-described lineages for osteogenic differentiation with decreasing information available from left to right (Caplan 2005).

MSCs which reside within the stromal compartment of BM were first identified in the pioneering studies of Friedenstein and Petrakova in 1966, who isolated bone forming progenitor cells from rat marrow, developed the plastic adherence technique for the

isolation of such precursor cells and noted that a rare cell population developed into colony forming units that were fibroblastic (CFU-F) (Friedenstein 1976). Following *in vitro* culture expansion, clonal cultures derived from individual CFU-Fs could be introduced into diffusion chambers in experimental models where the formation of bone, cartilage and stromal elements was observed (Jones and McGonagle 2008). The impression is that these stem cells are associated with blood vessels (Tavian et al. 2005) and that every blood vessel in the body has a MSC on the tissue side of endothelial cells. These vascular associated cells are referred to as perycites or vascular support cells (Caplan 2007).

MSCs represent a phenotipically heterogeneous population of cells and a very small fraction 0.001-0.01 % of the total population in marrow (Pittenger et al. 1999). There is a considerable interest in establishing MSC-specific cell surface markers and several monoclonal antibodies have been raised in an effort to provide reagents for the characterization and isolation of cells found in the human marrow stromal compartment. Different techniques are used to detect the surface markers to isolate and sort the cells including flow cytometry and magnetic beads isolation. However many researchers continue to use the plastic adherence technique to isolate MSCs from the BM.

The chondrogenic differentiation of MSCs is based on the knowledge of chondrogenic development, cartilage homeostasis and function. The use of MSCs for articular cartilage repair is based on the awareness that *in vivo*, during embryogenesis, limb formation occurs through the condensation of MSCs which then differentiate to the chondral pre-skeleton and form the cartilage covering the articulating surface of the joint (Mobasheri et al. 2009). The phenomenon occurs *in vitro* under conditions that include (1) a three-dimensional culture format such as pellets or scaffolds (Johnstone et al. 1998; Awad et al. 2004; Erickson et al. 2009), (2) nutrient medium and (3) addition of a member of the TGF-β super-family (Zuk et al. 2001; Huang et al. 2004). When these conditions are met, the cells rapidly lose their fibroblastic morphology and begin to initiate expression of a number of cartilage-specific ECM components. This involves biosynthesis of GAG and is accompanied by an alteration in cell morphology (Barry and Murphy 2004).

Media used for tissue culture have an important impact on growth and differentiation of MSCs. As discussed previously in the chondrocytes section, MSCs are also cultured in basal medium such as DMEM medium in the presence of 10 % FBS (Pittenger et al. 1999). MSCs adhere to the tissue culture substrate and have a fibroblastic morphology. Primary

cultures are usually maintained for 12–16 days, during which time the nonadherent haematopoietic cell fraction is depleted. Once the MSCs are expanded the media that facilitates the differentiation of MSCs into chondrocyte like cells, includes TGF-β and dexamethasone. The *in vitro* chondrogenesis of BM-derived MSCs was first reported by Johnstone in 1998 utilizing scaffold-free pellet culture (Johnstone et al. 1998). Since these initial findings, other research groups have shown this relationship and/or studied the effect of these chondrogenic factors on MSCs derived from other sources such as adipose tissue, SM and periosteum (Miura et al. 2002; Awad et al. 2004; Pei et al. 2008; Buxton et al. 2011). As previously described the use of dexamethasone has shown controversial effects on the production of a chondrogenic matrix by chondrocytes but since Johnstone showed that dexamethasone combined with TGF stimulated chondrogenesis, these two factors have been added on a routine basis to chondrogenic media irrespective of the tissue origin of the MSC. Preliminary data shown by Shintani and collaborators demonstrate that the effects of dexamethasone on chondrogensis of MSCs is subjected to the culture environment, tissue source and nature of stimulating growth factors; more specifically they observed that dexamethasone was not necessary or could even inhibit chondrogenesis of SM stem cells (Shintani and Hunziker 2011). Taken together, these results illustrate the complexity of culturing MSCs, the need to evaluate isolation techniques with care, and the need to identify new cell-specific markers. As MSCs are expanded in large-scale culture for human applications it will be important to identify defined growth media, without or with reduced FBS, to ensure more reproducible culture techniques and enhanced safety (Barry and Murphy 2004).

2.5 Alternative sources of stem cells for articular cartilage repair

For many years BM derived MSCs were considered the major source of stem cells for tissue engineered applications (Tapp et al. 2008). However recent reports have provided new insights into stem cell populations within a variety of adult tissues including infrapatellar FP, periosteum, SM and subcutaneous fat. Such cells possess significant plasticity in their multi-lineage capabilities and produce proteins associated with the adipocyte, chondrocyte and osteoblast phenotype (Dragoo et al. 2003; Huang et al. 2005; English et al. 2007; Pei et al. 2008). These tissues represent attractive cell sources for tissue engineering because they are generally accessible with minimal donor site morbidity. The differentiation characteristics, the purity and the yields are quite different for all of the stem cell preparations from different tissues. It has recently been

demonstrated that stem cells isolated from joint derived tissues and chondrocytes share similar gene expression profiles distinct from other stem cells such as BM and adipose derived stem cells (ADSCs) (Segawa et al. 2009).

2.5.1 Synovial membrane derived stem cells

The SM is a vascular connective tissue that lines the inner surface of the joint capsule and articulates with itself or with the margins of the articular cartilage (Radin 2001) (Fig. 2.16). The SM is responsible for the homeostasis in the joint and the synthesis of synovial fluid, a mixture of a protein-rich filtrate of blood and synoviocyte-derived hyaluronic acid and is also the key element of the immune system in the joint (Goodrich and Nixon 2006). In 2001, human derived SM stem cells were identified and shown to have the ability to proliferate extensively in culture and maintain their multilineage differentiation potential *in vitro*, establishing their progenitor cell nature (De Bari et al. 2001).

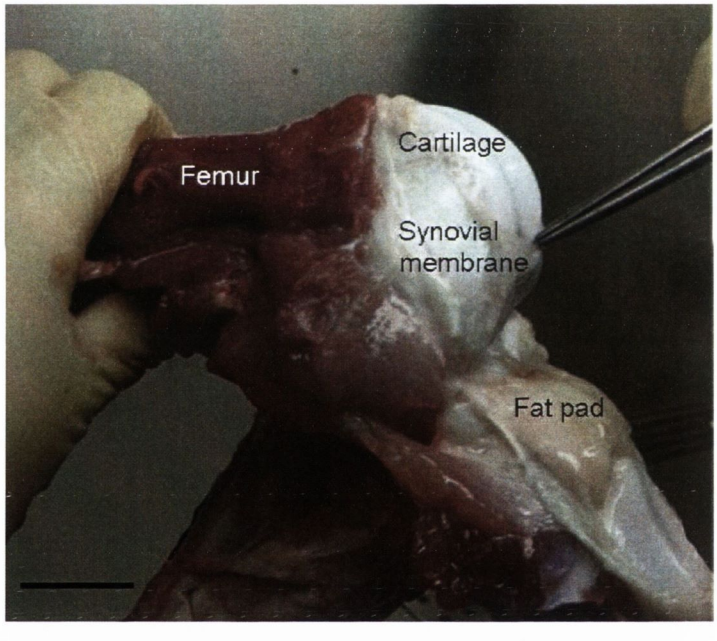


Figure 2.16: Picture of a porcine knee showing different structures of the joint. Scale bar 5 cm.

SM tissue can be easily harvested from the knee joint by arthroscopy, with a low degree of invasiveness and causing minimal complications at the donor site. This tissue has high self-regenerative capability, the potential to fully heal after surgical synovectomy (Theoret et al. 1996) and it has been observed that the removal of SM has no deleterious clinical consequences (Fowler et al. 2002). A minimal amount of synovial tissue is required to extract the SM stem cells successfully, based on the CFU-F data obtained in our laboratory from 9 different porcine donors, 1 gram of synovial tissue contains 4 % of SM stem cells.

These cells have been successfully extracted from pathological synovial derived tissue from rheumatoid arthritis and OA patients (Zimmermann et al. 2001; Nagase et al. 2008). In addition, SM stem cells multipotent capacity is not altered by cell passages and donor age (De Bari et al. 2001; Yoshimura et al. 2007).

Since De Bari and collaborators have shown that SM stem cells may be an optimal alternative source of chondrogenic cells for joint surface defect repair, numerous authors have further characterized their chondrogenic potential and surface profile obtaining promising results (De Bari et al. 2001; Jo et al. 2007; Pei et al. 2008; Pei et al. 2008). Furthermore some authors have compared their chondrogenic potential with other cell sources. In a previous study, rat MSCs derived from BM, SM, periosteum, adipose tissue and muscle were compared for their chondrogenic potential in an *in vitro* pellet culture system, with SM stem cells demonstrating the greater chondrogenic response (Yoshimura et al. 2007). Similarly a patient-matched quantitative comparison of MSCs derived from diverse human tissues revealed that SM stem cells had greater chondrogenic differentiation ability and proliferative capability than MSCs derived from adipose and skeletal muscle (Sakaguchi et al. 2005). It has also been demonstrated that cartilage pellets from human SM stem cells were significantly larger than those from BM MSCs (Shirasawa et al. 2006). Finally a recent study by Koga et al. demonstrated that both SM stem cells and BM MSCs had greater *in vivo* chondrogenic potential than adipose- and muscle-derived MSCs (Koga et al. 2008).

Many challenges still remain concerning the use of SM stem cells, including determining the optimal concentration and combination of growth factors. Increase in their chondrogenic potential has been observed when supplemented with a combination of TGF and IGF (Sakimura et al. 2006; Pei et al. 2008) and others with BMPs (Park et al. 2005; Sakaguchi et al. 2005; Mochizuki et al. 2006; Miyamoto et al. 2007; Shintani and Hunziker 2007). SM stem cells have been used successfully in different types of scaffolds to engineer chondrocytes tissues *in vitro* (Pei et al. 2008; Sampat et al. 2011) although SM stem cells fail to obtain ectopic formation of stable cartilage *in vivo* (De Bari et al. 2004). When implanted in the joint of small animal models *in vivo* statisfactory chondrogenesis was obtained (Koga et al. 2008). Finally, experiments using animals of large sizes are still rare and need to be further studied as are the detailed differentiation mechanisms of SM stem cells (Fan et al. 2009).

2.5.2 Infrapatellar fat pad derived stem cells

Intra-articular fat pads are a feature of some joints. They are composed of adipocytes and adipose connective tissue containing collagen and glycosaminoglycans. Its surface is covered with SM and its role is to facilitate the distribution of synovial fluid within the joint and act as a cushion protecting exposed articular surfaces (Vahlensieck et al. 2002; Nakano et al. 2004) (Fig. 2.16). In comparison with the SM, more tissue is required in order to obtain cells (Khan et al. 2008). Based on the literature there is no clinical evidence to suggest that taking biopsies from the FP could be harmful for the patient. Biopsies of FP could be taken during a diagnostic arthroscopy as suggested for the SM and it has also been shown that FP stem cells in a pellet system produced from human patients suffering from OA were more chondrogenic than control BM MSCs and presented similar chondrogenic capabilities to cartilage derived cultures (English et al. 2007).

To date the *in vitro* results using FP stem cells have been really promising. They can be isolated within a short time frame, harvested in clinically relevant quantities for treatment of focal cartilage defects, have a multipotent capacity and when seeded in 3D scaffold they are able to differentiate into the chondrogenic lineage, as demonstrated by upregulation of chondrospecific genes, formation of chondrogenic ECM and production of GAG (Dragoo et al. 2003; Wickham et al. 2003; English et al. 2007; Khan et al. 2007; Khan et al. 2008; Lee et al. 2008; Jurgens et al. 2009). When compared to other tissue sources they seem similar to SM stem cells and present a higher proliferative and chondrogenic potential than MSCs derived from subcutaneous fat (Mochizuki et al. 2006). Recently promising results have been observed in rabbit models of OA where the injection of FP stem cells reduced the development of osteoarthritic lesions (Toghraie et al. 2011). Taken together these results suggest that FP stem cells could be suitable for a one-step surgical procedure to regenerate cartilage tissue.

2.5.3 Adipose derived stem cells

The subcutaneous adipose tissue represents an accessible source of stem cells often termed ADSCs. Subcutaneous adipose tissue is abundant and readily available. Approximately 400,000 liposuction surgeries are performed in the United States each year. These procedures yield anywhere from 100 mL to >3 L of lipoaspirate tissue (Gimble et al. 2007). Those cells can be obtained by a simple surgical procedure, repeatedly and in large quantities (Schaffler and Buchler 2007). ADSCs are multipotent when cultivated under

appropriate conditions (Zuk et al. 2001; Zuk et al. 2002) and a number of studies report engineering cartilaginous tissue with these cells (Awad et al. 2004; Cheng et al. 2009; Cui et al. 2009; Diekman et al. 2010). Under certain conditions these cells would appear to possess a more limited chondrogenic capability in comparison with BM MSCs (Awad et al. 2004; Im et al. 2005; Kisiday et al. 2008; Kim and Im 2009). In terms of engineering cartilage tissue from ADSCs, limited mechanical properties have been achieved in agarose gels, despite positive evidence of chondrogenesis (Awad et al. 2004) . To overcome this problem it seems that a number of growth factors such as BMP-6 and BMP-7 are needed in order to promote robust chondrogenesis of ADSCs (Estes et al. 2006; Kim and Im 2009). TGF-β and FGF-2 have also been found to enhance growth and proliferation of these cells, as it has also been observed with a commercial growth medium when compared with DMEM; thus it is possible that synergism among the other supplement factors may exist (Tapp et al. 2008).

Overall, these results suggest that optimisation of the biochemical environment during both the expansion and differentiation phase may be necessary to make true comparisons of the chondrogenic capacity of the different cell sources. Even if previous studies have used different isolation methods and culture conditions, there appears to be a consensus emerging that the cells derived from the joint present a higher chondrogenic intrinsic potential than the other stem cell sources.

2.6 Role of biophysical stimuli in cartilage tissue engineering

As previously described in this chapter, mechanical factors play an important role in the development and maintenance of healthy cartilage *in vivo*. In the context of cartilage engineering it has been shown that loading applied to chondrocytes in 3D culture enhances their biosynthetic activity and stimulate the synthesis of cartilaginous ECM macromolecules (Buschmann et al. 1992; Buschmann et al. 1995; Mauck et al. 2003). The process by which cells sense and respond to mechanical signals is called mechanotransduction (Wang et al. 1993). Among the different mechanical forces studied, HP and direct compression are perhaps the most representative of the knee joint and their effects on chondrocytes *in vitro* have been studied in depth (Toyoda et al. 2003; Toyoda et al. 2003; Kelly et al. 2004; Ng et al. 2006). To date, moderate levels of mechanical loading has shown positive stimulus on chondrogenesis (Angele et al. 2004; Huang et al. 2004; Schumann et al. 2006; Stoddart et al. 2009). Table 1 shows a selection of some of the most

interesting studies that demonstrates the positive effects of biomechanical stimulation on articular cartilage constructs.

First Author	Proteoglycan	Collagen	Notes
Davisson 2002 (perfusion, shear, 1-3 week ovine knee, $25x10^6$ cell/cm^3 PGA, 9 days)	40% increase in GAG synthesis	Not evaluated	Heavily dependent on the level and duration of perfusion velocities
Pei 2002 (shear, 2-4 week bovine knee, $25x10^6$ cell/cm^3 PGA, 4 weeks)	600% increase in GAG composition	300% increase in collagen composition	Compressive modulus of 400 ± 80 kPa
Mauck 2000(compression, 3-5 month bovine ankle, $22x10^6$ cell/cm^3 agarose, 4 weeks)	45% increase in GAG composition	37% increase in collagen composition	Aggregate modulus of 100 kPa
Mizuno 2002 (hydrostatic pressure, 2-3 week bovine shoulder, $52x10^6$ cell/cm^3 collagen sponge, 15 days)	310% increase in ^{35}S-sulfate incorporation	Intracellular, pericellular and extracellular immunoreactivity	2.8 MPa stimulated chondrogenesis

Table 1: Mechanical stimulation of articular cartilage constructs (modified from (Darling and Athanasiou 2003).

Over the past decade researches have concentrated on replicating the native environment *in vitro* by combining mechanical forces and growth factors with synergistic effects reported (Bonassar et al. 2001; Demarteau et al. 2003; Miyanishi et al. 2006; Miyanishi et al. 2006; Lima et al. 2007). Research is now focusing on the specific variables involved in these experiments, such as loading regimens, growth factor concentrations and stimulation protocols (Darling and Athanasiou 2003).

As previously stated, stem cells represent an attractive cell source for cartilage regeneration but the exploration of the mechanical factors affecting chondrogenesis need to be studied in greater detail. Different *in vivo* studies have confirmed the beneficial effect of motion on cartilage healing with periosteal grafts or BM MSCs (O'Driscoll et al. 1988; Wakitani et al. 1994; Salisbury Palomares et al. 2010). Various types of loading have been hypothesized to influence the chondrogenic differentiation of MSCs *in vitro* (Kelly and Jacobs 2010). The most common being confined compression; it has been suggested that compression induces chondrogenesis of stem cells, but this effect was diminished at lower frequencies (Elder et al. 2001). In addition cyclic compressive loading at a frequency of 1Hz and an amplitude of 10 % strain has been shown to promote chondrogenic gene expression and TGF-β1 protein production in rabbit BM MSCs in agarose culture (Huang et al. 2004). A similar response was observed in human BM MSCs seeded in hyaluronan

gelatin composite scaffolds after 7 days of loading (Angele et al. 2004). The addition of growth factors, specifically TGF-β1 and dexamethasone in combination with the dynamic compression was found to promote chondrogenic gene expression and protein expression (Li et al. 2010) and increase cell viability (Mouw et al. 2007). In contrast an inhibition of MSCs chondrogenesis has been described if the loading was initiated after immediately after MSC encapsulation in agarose (Thorpe et al. 2008; Huang et al. 2010; Thorpe et al. 2010).

2.6.1 Hydrostatic pressure

HP is the other mechanical force well studied in the literature that is believed to induce or enhance chondrogenesis of MSCs (Kelly and Jacobs 2010). Researchers have been mimicking the physiological walking regime and cadence applied to human joints which is typically exposed to stresses between 3 and 10 MPa and frequencies of 1Hz (Elder and Athanasiou 2009). In the majority of the studies HP is applied by compressing the fluid phase within a pressure chamber attached directly to a hydraulic system controlled by a computer (Smith et al. 1996; Hu and Athanasiou 2006). HP has been shown to induce chondrogenesis at a gene level where an upregulation of collagen type II and aggrecan mRNA expression was observed in human BM MSC (Miyanishi et al. 2006; Wagner et al. 2008) and at a protein level as evidenced by increased proteoglycan and collagen content for human BM MSCs (Angele et al. 2003). Studies assessing the effects of HP on chondrogenic gene expression and cartilage matrix production have also shown that the effects of HP seems to decrease with diminishing amplitude of dynamic HP and an optimum regime of 10 MPa at a frequency of 1Hz has been proposed (Miyanishi et al. 2006; Miyanishi et al. 2006; Finger et al. 2007; Bahuleyan et al. 2009).

The effects of growth factors, such as TGF-β or BMP-2, have also been studied in combination with HP. Some authors have observed positive effects of these combination (Miyanishi et al. 2006; Miyanishi et al. 2006; Wagner et al. 2008), with others reporting little or no effect on chondrogenic gene expression or matrix accumulation in MSC aggregates (Zeiter et al. 2009).

2.7 In vivo studies

Different techniques have been studied regarding the healing potential of stem cells in articular cartilage defects. In this section the main approaches are summarized:

a) **Cartilage repair with cells alone:** this supports the idea that MSCs can migrate to damaged tissues i.e. MSCs could reside in remote tissues and be mobilized in response to regenerative clues or a systemic route could be potentially used to deliver MSCs with the support of regenerative/homing molecules to implement MSC-based therapies (Granero-Molto et al. 2008). MSCs migration to injured tissues has been reported in brain, myocardium, kidneys and radiation-induced multi-organ failure (Shake et al. 2002; Chapel et al. 2003; Hou et al. 2003; Morigi et al. 2004). The mechanisms that regulate the MSCs migration to the injured tissues are still unknown but Murphy and collaborators have demonstrated the *in vivo* regenerative properties of MSCs via this technique. Cells retarded cartilage destruction and OA progression when injected in a caprine model of OA (Murphy et al. 2003). In a model of rat articular cartilage lesion, it has been shown that MSCs, that were genetically modified to express IGF-I or BMP-2, repaired the damaged cartilage whereas uninfected MSCs either failed to fill up the defects or formed fibrous tissue mainly composed of type I collagen. These findings suggest that MSCs engineered to express anabolic growth factors become more effective for chondrogenic regeneration but these systems need to be refined and other chondro-inductive growth factors need to be explored (Gelse et al. 2003). Promising results have also been observed after 14 weeks post surgery in a rabbit model but in terms of histological results the new cartilage repair tissue did not seem completely integrated and was in actuality more compliant than normal cartilage tissue (Im et al. 2001). Recently, some promising human case reports have shown MRI evidence of increased meniscus volume and/or cartilage volume after the injection of BM MSCs into degenerated joints (Centeno et al. 2006; Centeno et al. 2008) but unfortunately no cartilage biopsy was taken during the follow up, so it is hard to determine if a change towards fibrocartilage or true hyaline cartilage had been made.

b) **Cartilage repair with cell seeded scaffolds:** Since the healing process is extremely demanding MSCs alone seem insufficient and more beneficial effects need to be achieved through the engineering of MSCs with scaffolds, most probably combined with growth factors. In an animal model of OA, BM MSCs were seeded in type I collagen gels; after 24 weeks post surgery newly cartilage

repair tissue was observed but a progressive thinning of the repair tissue was discerned (Wakitani et al. 1994). In the human field, an improvement of the clinical symptoms was observed when an athlete was treated for an articular cartilage defect with autologous BM MSCs seeded in a collagen gel (Kuroda et al. 2007). However, Wakitani and collaborators observed that autologous BM MSCs seeded in collagen scaffolds in patients with OA, presented no significant clinical improvement compared with the control cell free group (Wakitani et al. 2002).

All the above scaffold based-cartilage defect treatments involve abrasion of the subchondral bone and the question remaining is: could the repair tissue be just simply generated by the bleeding involved in the procedure by the MSCs released from the subchondral bone? Today, there still exists a lack of controlled designed animal models with quantitatively reported outcome measures and as previously stated researchers have to be careful when comparing results as there is an important variability in terms of animal species used, types of MSCs or variety of scaffolds.

2.8 Summary

In conclusion, the management of articular cartilage lesions is a challenging problem. The nonvascular nature of cartilage makes it very difficult for lesions therein to selfheal. In clinic, apart from surgical approaches, cell-based therapy utilizing chondrocytes to repair cartilage injury has been well established for a period of time now. However, it can only improve cartilage regeneration to some extent and is not very effective in the long term, due to the limited availability of autologous chondrocytes and the loss of cartilage-related phenotype and functionality in the chondrocytes after several passages of *in vitro* culture. While a number of surgical approaches have been described, it remains difficult to compare the efficacy of these techniques because of a paucity of well-designed randomized controlled trials in the literature. The current evidence suggests that BM stimulation procedures and whole-tissue transplantation of allografts or autografts can achieve favorable outcomes when used for the management of focal chondral defects of the knee, although they typically only result in temporary repair.

As a result, cell-based therapies, including tissue engineering strategies for growing clinically relevant grafts, are being intensively researched; to summarize cartilage tissue engineering approaches involves seeding harvested cells onto a scaffold (agarose, fibrin) with stimulatory growth factors such as TGF-β3. Mechanical loading can be applied then to

replicate the native environment and promote chondrogenesis. Once an *in vitro* cartilage engineered construct is engineered and the biomechanical properties are sufficient to withstand physiological loading without collapsing, it can potentially be implanted into an *in vivo* articular cartilage defect (Fig. 2.17).

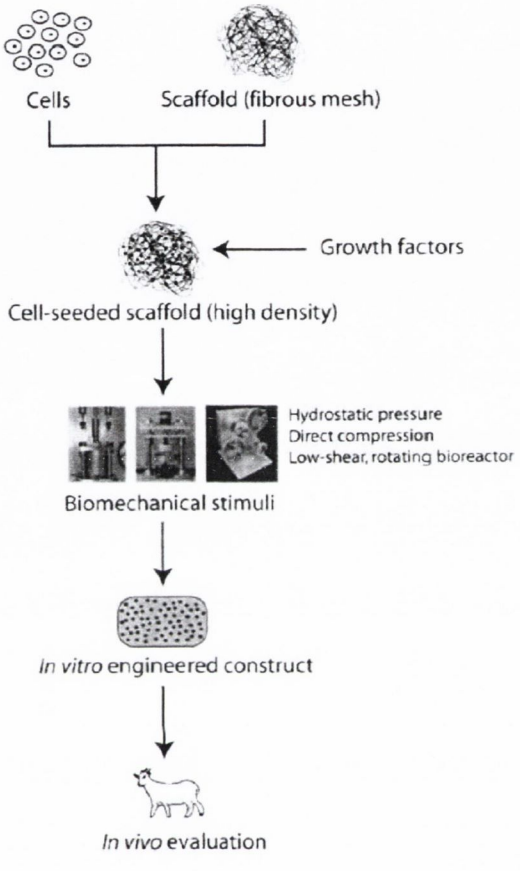


Figure 2.17: Tissue regeneration approach (Darling and Athanasiou 2003).

Cell based techniques performed with or without a scaffold have demonstrated early promise in animal and basic-science models, but several questions need to be addressed. Do we use well characterized populations? Are mixtures of stem cells with differentiated CC the way ahead? Do we need to apply them to the defect directly or do they work through trophic means? What is the best cell source? Do we need scaffolds? To date, we have not determine a suitable MSC-based construct with mechanical properties matching that of native cartilage, or even that produced by chondrocyte based constructs (Kisiday et al. 2008; Huang et al. 2010). The future is exciting but additional studies need to be completed in order to validate the efficacy of stem cell based therapy in achieving successful human clinical outcomes

3 Chondrogenesis and integration of mesenchymal stem cells within an in vitro cartilage defect repair model

3.1 Introduction

ACI (Brittberg et al. 1994; Peterson et al. 2000) and scaffold-based variants whereby cells are incorporated within supporting three dimensional (3D) scaffolds or (Marcacci et al. 2007; Selmi et al. 2007; Selmi et al. 2008) are promising alternative strategies for articular cartilage repair. However, there are several problems associated with the ACI procedure, including difficulties in obtaining a sufficient number of chondrocytes for transplantation, the necessity of creating donor-site defects within the articular cartilage and variability in the quality of repair (Horas et al. 2003). MSCs possess the ability to proliferate extensively *ex vivo* while maintaining their multipotent differentiation capabilities (Bruder et al. 1997; Kadiyala et al. 1997), making them an attractive cell type for cell based cartilage repair strategies. These cells can be isolated from the BM using minimally invasive techniques from noncritical locations such as the iliac crest and have the capacity to differentiate along a number of different mesenchymal lineages including bone, cartilage and fat (Maniatopoulos et al. 1988; Caplan 1991; Johnstone et al. 1998; Pittenger et al. 1999). The chondrogenic differentiation potential of MSCs can be demonstrated *in vitro* using well-established procedures (Johnstone et al. 1998; Barry et al. 2001). A major challenge with MSC-based cartilage repair therapies is to generate cells with features of stable chondrocytes which are resistant to hypertrophy and terminal differentiation, as found in hyaline articular cartilage (Pelttari et al. 2008).

Transplantation of isolated autologous BM-derived MSCs suspended in hydrogels have been shown to promote the repair of articular cartilage defects in young and/or active patients (Kuroda et al. 2007; Wakitani et al. 2007). Successful long-term regeneration of articular cartilage defects using chondrocytes, MSCs or otherwise, requires integration of the repair tissue with the surrounding host cartilage (Ahsan and Sah 1999). It has been demonstrated that 8 months following implantation of chondrocytes in an equine model, the integration strength of the repair tissue, as measured by a uniaxial tensile test, is approximately half that compared to intact control samples (Gratz et al. 2006). Poor

integration could lead to an altered stress state within the regenerating tissue and ultimately its degeneration. *In vitro* explant models of cartilage defect repair have contributed significantly to our understanding of tissue integration and the ability of cell-based therapies to fill defects with articular cartilage-like repair tissue (Obradovic et al. 2001; Hunter and Levenston 2004). These models allow for a systematic analysis of various factors (e.g., cells, biophysical and biochemical cues, inflammatory components, etc.) governing successful repair, without the natural variability found in animal models, such as the host immune response and levels of physical activity (Hunter and Levenston 2004). Using such explant models it has been possible to demonstrate that chondrogenesis and subsequent integration of tissue-engineered cartilage depends on factors such as the choice of scaffold (Hunter and Levenston 2004), the developmental stage of the construct (Obradovic et al. 2001) and the adjacent tissue architecture and composition (Tognana et al. 2005). Integration of such tissues has been further investigated following subcutaneous implantation in nude mice (Peretti et al. 1999; Silverman et al. 2000; Johnson et al. 2004). These models have revealed that the tensile strengths of bonds formed between articular cartilage and engineered cartilage increase with time, with little observed differences between the quality of integrative repair using articular or non-articular chondrocytes (Johnson et al. 2004). Multiple *in vitro* studies have demonstrated chondrogenesis of MSCs in pellet culture or on scaffolds in the presence of TGF-β family members (Johnstone et al. 1998; Silverman et al. 2000; Worster et al. 2001; Williams et al. 2003; Awad et al. 2004; Huang et al. 2004; Mauck et al. 2006; Thorpe et al. 2008).

These studies have typically characterized chondrogenesis through the expression and synthesis of cartilage-specific matrix molecules. A number of studies have also investigated the functional mechanical properties of cartilaginous tissues engineered using MSCs, suggesting that the mechanical properties (e.g., equilibrium Young's modulus, dynamic modulus) of such constructs are lower than that produced by chondrocytes under identical conditions (Mauck et al. 2006; Erickson et al. 2009). What remains unclear is what effect the complex milieu of factors and stimuli that such constructs will experience *in vivo* will have on chondrogenesis of MSCs. For example, what role the surrounding articular cartilage and the associated factors released (e.g., matrix metalloproteinases, cathepsins, nitric oxide, etc.) will have on chondrogenesis of MSCs is poorly understood. The aim of the present study is to adapt a well-established cartilage explant model that has been used to assess chondrocyte-based therapies for cartilage repair to investigate MSC-based therapies. Such models, while obviously not incorporating many of the stimuli

present *in vivo*, do recapitulate certain aspects of the environment of a cartilage defect absent in traditional pellet cultures and other *in vitro* systems. The specific objectives of the present study were to (i) compare the ability of chondrocytes and MSCs to form neocartilage in an *in vitro* cartilage explant defect model and (ii) to evaluate the mechanical integrity of the bond formed between the engineered and normal tissue using these two cell types.

3.2 Materials and methods

3.2.1 Cell, cartilage isolation and construct assembly

Articular cartilage was aseptically harvested from the femoropatellar joints of two immature/young pigs (four month old). Full depth articular cartilage explants were obtained using a 6 mm biopsy punch (Kai Medical Europe, Germany) and the height standardised to 2 mm through removing both the superficial and deep zones using a custom-built rig. Full depth concentric circular holes (3 mm diameter) were cut using a biopsy punch (Kai Medical Europe, Germany) to form annuli of tissue.

CC and MSCs were harvested from the same donor pigs from which the cartilage explants were harvested. CC were isolated from articular cartilage harvested from the femoropatellar joints. Briefly, cartilage slices were rinsed with phosphate buffered saline containing penicillin/streptomycin (200 U/ml). The cartilage chunks were then combined and digested via serial digestion with pronase (1 mg/ml) for 1 hour, then rinsed in phosphate buffered saline (PBS) followed by incubation with DMEM/F12 containing collagenase type II for 16-18 hours under constant rotation at 37°C. The resulting cell suspension was then filtered through a 40 μm pore-size cell sieve (Falcon Ltd, Sarstedt, Ireland) and the filtrate centrifuged and rinsed with PBS twice. Cells were seeded at a density of 50,000 cells/cm^2 in 175 cm^2 T flasks and expanded to passage one (P1). Viable cells were counted using a hemacytometer and 0.4 % trypan blue staining. Isolated CC from all joints were pooled and maintained in DMEM/F-12 (Sigma–Aldrich, Dublin, Ireland) supplemented with 10 % v/v FBS and 100 U/ml penicillin/streptomycin me during the expansion phase. Porcine MSCs were isolated from marrow obtained from the femoral shaft and expanded according to a modified method developed for human MSCs (Lennon and Caplan 2006). MSCs were sub-cultured at a ratio of 1:2 following colony formation and expanded to passage three. CC and MSCs were suspended in 2 % agarose at a density of 15 million cells/ml. The solution was aspirated with a 1 ml warm syringe (BD, Belgium)

and 18 gauge needle (BD, Microlance, Ireland) and injected into the cores created in the cartilage explants, see Figure 3.1.

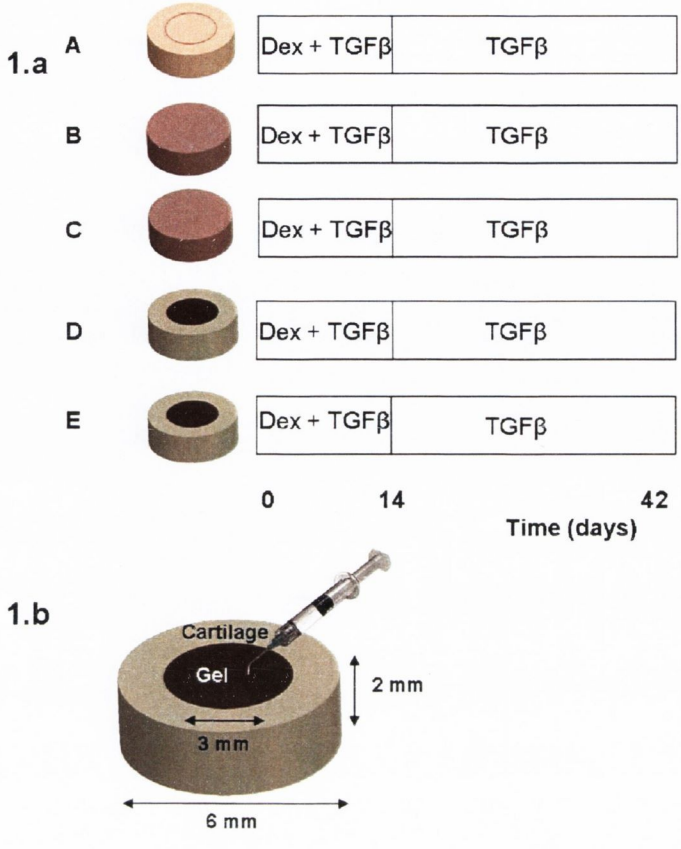


Figure 3.1: 1.a. Diagram illustrating research design. Samples were supplemented with dexamethasone (Dex) and TGF-β3 for 14 days and then with just TGF-β3 until day 42. A) Cartilage in cartilage. B) Agarose gels seeded with chondrocytes. C) Agarose gels seeded with MSCs. D) Cartilage annular rings filled with MSCs seeded hydrogels. E) Cartilage annular rings filled with chondrocyte seeded hydrogels. 1.b. Diagram illustrating the injection method: Chondrocytes and MSCs were suspended in 2 % agarose at a density of 15 million cells/ml (n = 5 per group). The solution was aspirated with a 1 ml warm syringe and 18 gauge needle and injected into the cores created in the cartilage explants.

The constructs were set for 3 minutes in petri dishes to allow the agarose to cool and then they were transferred to 6 well plate dishes with culture medium (2 samples per well with 2.5 ml of medium per construct, see below). Free swelling (FS) controls (no surrounding cartilage) (n=5) and cartilage constructs (cartilage core in cartilage explant - histological analysis only) were also kept in similar conditions (Fig. 3.1). All constructs were maintained for 6 weeks in a chemically defined chondrogenic medium consisting of DMEM GlutaMAX supplemented with penicillin (100 U/mL)-streptomycin (100 µg/mL) (both GIBCO, Biosciences, Ireland), 100 µg/ml sodium pyruvate, 40 µg/ml L-proline, 50

μg/ml L-ascorbic acid-2-phosphate, 1 mg/ml bovine serum albumin (BSA), 1× insulin–transferrin–selenium (all from Sigma-Aldrich, Ireland) and 10 ng/ml recombinant human TGF-β3 (R&D Systems, UK). Medium was changed every 2-3 days. For the first 2 weeks of the experiment medium was supplemented with 100 nM dexamethasone.

3.2.2 MSCs Tripotentiality

Adipogenesis and Osteogenesis

MSCs were plated on 9.5 cm^2 six well plates at a density of 10^3 cells/cm^2 and cultured for 7 days in complete medium (DMEM GlutaMAX supplemented with 10 % v/v FBS and 100 U/ml penicillin/streptomycin) which was then changed to osteogenic or adipogenic medium for 21 days. Osteogenic medium consisted of complete medium supplemented with 100 nM dexamethasone, 10 mM β-glycerolphosphate and 0.05 mM ascorbic acid (Sigma). Adipogenic medium consisted of complete medium supplemented with 100nM dexamethasone, 0.5 mM isobutylmethylxanthine and 50 μM indomethacin (Sigma). Adipogenesis was assessed by ethanol fixing followed by staining with 1 % Oil Red solution, while for osteogenic differentiation the plates were fixed with ethanol and stained with 1 % Alizarin Red solution.

Chondrogenesis

A pellet culture was used to assess chondrogenesis. 250,000 cells were placed in a 1.5 ml conical microtube and centrifuged at 650 G for 5 minutes. The pellets were cultured in chondrogenic medium. For histological evaluation the pellets were embedded in paraffin, cut into 5 μm thick sections and stained with 1% alcian blue 8 GX (Sigma–Aldrich, Ireland) in 0.1 M HCl to assess GAG content and picrosirius red to detect collagen.

3.2.3 Mechanical testing

The integration strength of the MSCs and CC seeded agarose hydrogels to the cartilage explant was evaluated at week 6 using a push out test. The engineered tissue was pushed out with a 2.5 mm diameter plunger, while the cartilage explant was supported on a rigid annulus (6 mm outer diameter, 3.5 mm inner diameter), similar to other tests reported in the literature (Obradovic et al. 2001; Hunter and Levenston 2004). The maximum force achieved before separation of the tissues (Fig. 3.2.) was normalized by the lateral area of

the core, with the resulting value considered as the failure stress, as described elsewhere (Hunter and Levenston 2004).

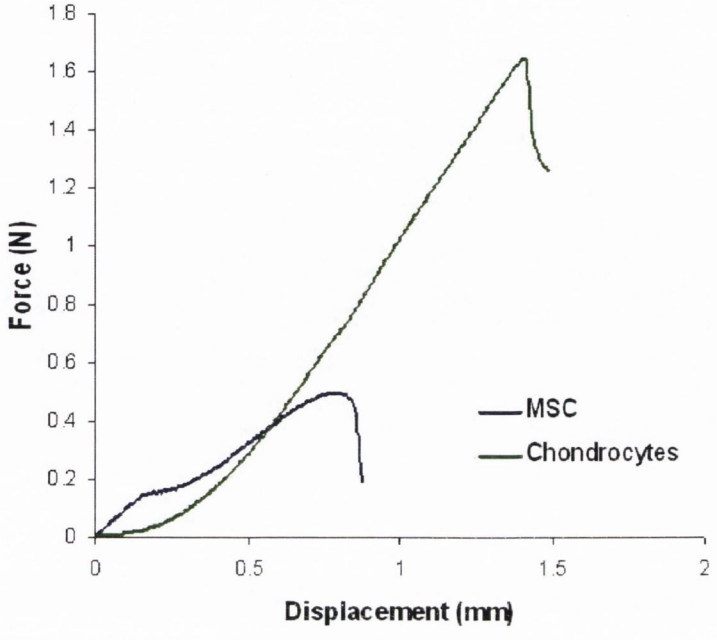


Figure 3.2: Force-displacement curves for chondrocytes and MSCs seeded constructs. The peak force to push out the gel seeded with chondrocytes is higher than the force needed to push out the MSCs seeded gel from the construct.

3.2.4 Cell viability, histology and immunohistochemistry

Viability of agarose encapsulated cells within explants was assessed 48 hours after encapsulation using fluorescent membrane integrity assay, LIVE/DEAD® Assay (Invitrogen, Biosciences, Ireland). Explants were incubated with 4 μM calcein-AM and 2 μM ethidium homodimer for 1 hour and observed using a confocal microscope (Zeiss, LSM-510-META) with a laser excitation wavelength of 490 nm and fluorescent emissions collected at wavelengths above 520 nm.

Following the 6 week culture period, constructs were fixed in 4 % paraformaldehyde overnight, rinsed in PBS, processed on an automated tissue processor (ASP300 Leica, Germany), embedded in wax and sectioned to 10 μm thickness. The histological sections were stained with 1 % alcian blue 8 GX (Sigma–Aldrich, Ireland) in 0.1 M HCl to assess glycosaminoglycan content and picrosirius red to detect collagen. Type I and type II collagen content were evaluated with a standard immunohistochemical technique. Briefly sections were treated with chondroitinase ABC (Sigma) in a humidified environment to enhance permeability of the extracellular matrix by removal of chondroitin sulphate. Slides

were rinsed with PBS, quenched of peroxidase activity and blocked with goat serum for 2 hours. Sections were then incubated overnight at 4°C with mouse monoclonal collagen type I diluted 1:400 (Abcam, UK) (concentration 5.4 mg/ml) or mouse monoclonal anti-collagen type II diluted 1:100 (Abcam, UK) (concentration 1 mg/ml). After washing in PBS, the secondary antibody for type I and type II collagen (Anti-Mouse IgG Biotin antibody produced in goat) (concentration 1 g/L) binding was applied for 1 hour. Color was developed using the Vectastain ABC reagent (Vectastain ABC kit, Vector Laboratories, UK) for 45 min and 5 min exposure to Peroxydase DAB substrate kit (Vector laboratories, UK). Negative and positive controls were included in the immunohistochemistry staining protocol for each batch. The cartilage sections were examined with an Olympus IX51 microscope and mounted with an Olympus video camera.

3.2.5 Biochemical analysis

Constructs were assessed after 6 weeks of culture. Free swelling controls were cored using a 3 mm biopsy punch, the wet mass of both annulus and core recorded and then frozen for subsequent analyses. These cores were compared to engineered tissue formed within the cell seeded explants. For the cell seeded explants, the engineered cartilage (after push-out tests) and surrounding cartilage tissue were also weighed and frozen for separate biochemical analyses. All samples were digested in papain (125 μg/ml) in 0.1 M sodium acetate, 5 mM cysteine HCl, 0.05 M EDTA, pH 6.0 (all from Sigma–Aldrich, Dublin, Ireland) at 60°C under constant rotation for 18 hours. Aliquots of the digest samples were assayed separately for DNA and sulfated GAG content. DNA content was quantified using the Hoechst Bisbenzimide 33258 dye assay as described previously (Kim et al. 1988). A standard curve was generated with calf thymus DNA (Sigma–Aldrich, Dublin, Ireland). The proteoglycan content was estimated by quantifying the amount of sulfated GAG in constructs using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Northern Ireland), with a chondroitin sulfate standard. Total collagen content was determined by measuring the hydroxyproline content, using a hydroxyproline-to-collagen ratio of 1:7.69. (Kafienah and Sims 2004; Ignat'eva et al. 2007) Each biochemical constituent (DNA, hydroxyproline and GAG) was normalised to the tissue wet weight.

3.2.6 Statistical analysis

Mechanical and biochemical properties of engineered constructs are expressed in the form of mean ± standard deviation (SD). Five samples were made per group; 3 samples were used for mechanical and biochemical analyses and 2 samples were used for histology. Differences in mechanical and biochemical properties with cell type and culture condition were determined by using either a student t-test or two way ANOVA with Bonferroni post-tests. All calculations were performed using commercially available software (GraphPadPrism 4, San Diego, USA). A level of $p<0.05$ was considered significant.

3.3 Results

Stromal cells isolated from the BM of young porcine femora demonstrated the ability to differentiate down the osteogenic, adipogenic and chondrogenic lineages (Fig. 3.3). Oil droplets were observed around cells cultured with adipogenic medium after 21 days. Calcified nodules were observed in plates supplemented with osteogenic medium, while pellets stained positive for both collagen (picrosirius red) and GAG (alcian blue).

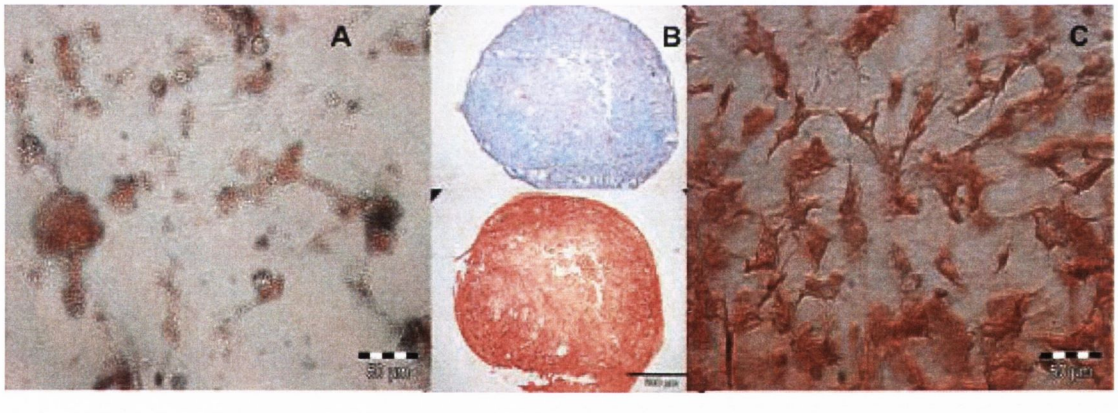


Figure 3.3: A) Adipogenic potential of mesenchymal stem cells: colonies positive for oil red staining. B) Chondrogenic potential of MSCs: colonies staining positive for glycosaminoglycan with alcian blue (top image) and for collagen with picrosirius red (bottom image). C) Osteogenic potential of MSCs: colonies positive for alizarin red staining.

The LIVE/DEAD® Assay demonstrated dual staining (red for dead cells and green for live cells) in the cartilage and in the seeded hydrogels 48 hours after cell encapsulation, with greater cellularity observed in the cartilage explant (Fig. 3.4). Dead cells were concentrated within the interface between the cartilage and agarose seeded gel. The interface was

characterized by a thin acellular region between the cartilage and hydrogel (data not shown). No obvious difference in the initial viability between MSCs and chondrocyte seeded constructs was observed.

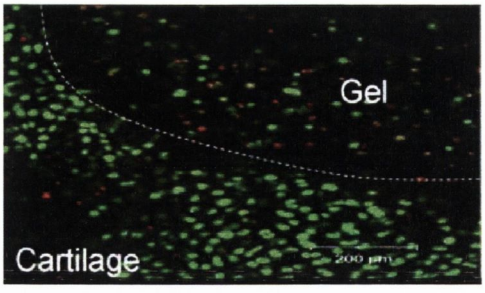


Figure 3.4: Representative image of dead (ethidium bromide labelled; red) and live (calcein labelled; green) cells in MSCs constructs after 48 hours.

Alcian blue and picrosirius red staining revealed that CC seeded in agarose hydrogels demonstrated enhanced GAG and collagen accumulation compared to MSCs in this cartilage explant model (Fig. 3.5).

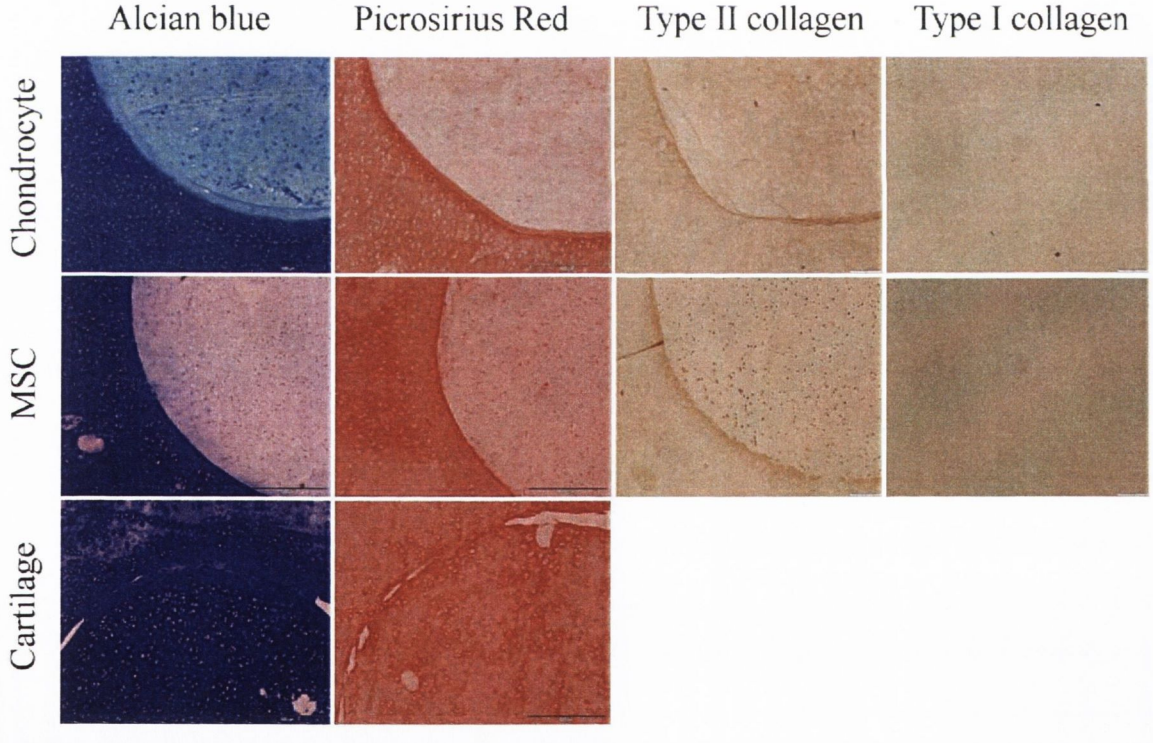


Figure 3.5: Microscopic appearance of cartilage explant and gel seeded with chondrocytes (first row), MSCs (second row) and cartilage (third row) at week 6. Sections taken from half-way through the depth of the construct were stained for Alcian Blue (stains glycosaminoglycan), picrosirius Red (stains collagen) and type II and I collagen by immunohistochemistry; scale bar 500 µm.

Little or no gaps were observed between the cell seeded hydrogels and the surrounding cartilage with minimal evidence of interdigitation between the two tissues for both MSCs and chondrocyte groups. In comparison small gaps were observed at the interface between explants filled with cartilage plugs that had not filled with neocartilage after 6 weeks in culture (Fig. 3.5). Immunohistochemistry demonstrated positive staining for collagen type II in both the chondrocyte and MSCs groups, with weak type I staining. Staining for type II collagen was more uniform in the chondrocyte seeded group, but more localised to the cells in the MSCs group. A similar trend was observed with the picrosirius red staining. The cartilage surrounding the hydrogel always stained positive for type II collagen.

Greater GAG accumulation was measured in chondrocyte seeded constructs (1.27 ± 0.3 %w/w) compared to MSCs seeded constructs (0.19 ± 0.03 %w/w) ($p<0.0001$) (Fig. 3.6b). A similar trend was observed for collagen content, with greater accumulation in the chondrocyte group (0.31 ± 0.08 %w/w) compared to the MSCs seeded group (0.09 ± 0.01 %w/w) ($p<0.05$) (Fig. 3.6c).

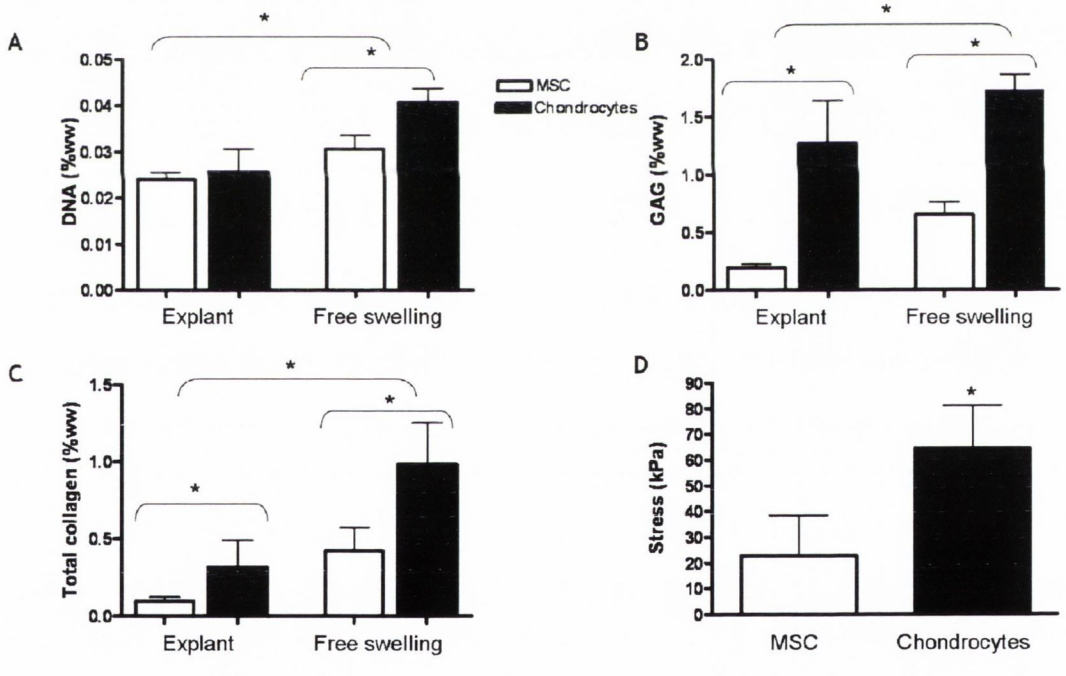


Figure 3.6: A: DNA content, B: GAG content and C: Total collagen content of MSCs and chondrocyte explant core and free-swelling control gels at week 6. * $p< 0.05$. Typical DNA content in cartilage disks was 0.036 %ww, GAG content was 5.3 %ww and total collagen content was 8 %ww. D: Failure stress during push-out testing for MSCs and CC seeded constructs at week 6. * $p=0.0026$.

DNA content was not significantly different for MSCs and chondrocyte seeded constructs (Fig. 3.6a). Control constructs cultured in free swelling conditions (i.e. not in cartilage explants) demonstrated higher GAG, DNA and collagen ($p<0.05$) content than those cultured in cartilage explants. For chondrocyte-seeded controls not surrounded by cartilage, GAG content was 1.7 ± 0.1 %w/w, DNA content was 0.04 ± 0.003 %w/w and collagen content was 0.98 ± 0.13 %w/w. For MSCs-seeded controls not surrounded by cartilage, GAG content was 0.65 ± 0.01 %w/w, DNA was 0.03 ± 0.002 %w/w and collagen content was 0.42 ± 0.08 %w/w. After 6 weeks in culture chondrocyte seeded constructs demonstrated a significantly higher failure stress (64.4 ± 8.3 kPa) during push-out testing from the surrounding cartilage explant compared to MSCs seeded constructs (22.7 ± 5.9 kPa) ($p=0.0026$) (Fig. 3.6d). Earlier assessment (week 3) of the integrative mechanical properties of cell seeded hydrogels could not be accurately determined because of the relatively weak integration of the gels to the cartilage explants at these time points. Biochemical analysis of engineered tissues at week 3 revealed a similar trend to that observed at week 6 (data not shown).

3.4 Discussion

Integration of native tissue and repair tissue is a key indicator of the long-term success of tissue-engineered approaches to cartilage repair. In this *in vitro* model of cartilage defect repair, it has been demonstrated that chondrocytes accumulate greater amounts of cartilaginous matrix than MSCs in the agarose gels, which is in agreement with the findings of previous studies in FS culture (Mauck et al. 2006). Based on the results of the push-out test, cartilaginous tissue secreted by chondrocytes also integrates better with the surrounding tissue. This result may simply be a function of the higher GAG content associated with the chondrocyte-seeded constructs which results in greater swelling of the engineered tissue, thereby increasing the peak forces obtained from the push-out test utilized in this study.

Previous studies have suggested that the failure stress of such an interface is not purely a function of the total biochemical content of the engineered tissue (Hunter and Levenston 2004) and may depend more on the formation of cross-links between the adjacent tissues which were not measured in this study. The higher failure stress observed in chondrocyte-seeded hydrogels may also be due to factors other than the strength of the bond between the adjacent tissues. It has been demonstrated that the outgrowing fibrous tissue formed

during *in vitro* culture of cartilaginous specimens significantly increases the failure stress obtained from push-out tests (Moretti et al. 2005). While pilot studies revealed that the use of a chemically defined medium not supplemented with FBS generally reduced such tissue outgrowth, it was not completely absent in our explant model. Given that such tissue was not explicitly removed in this study, it may be that higher push-out forces observed in the chondrocyte seeded group are a result of greater tissue outgrowth associated with the higher levels of matrix accumulation by chondrocytes compared to MSCs. It is also possible that failure of the gel/tissue material itself, as opposed to the interface with the explant, contributes to the measured failure properties. The magnitudes of interface strength reported in this article are of a similar magnitude to other reported studies. For example, the strength of the chondrocyte-seeded group (64 kPa) is higher than that reported by Hunter et al. (~10 kPa), but lower than that reported by Obradovic et al. (~80–384 kPa) following bioreactor culture.

Differences in species, culture conditions, testing regimes, etc. may explain much of the reported differences. For example, Dhert et al. have demonstrated using finite element modelling that factors associated with the experimental setup of push-out tests will influence the resulting interfaces stresses (Dhert et al. 1992). Higher resolution imaging to assess neo-tissue organization at the interface should also be considered in future cartilage explant studies. Matrix accumulation in both the MSCs- and chondrocyte- seeded groups was inhibited by the presence of a surrounding cartilaginous ring, as evidenced by a significant decrease in GAG content in these groups compared to FS controls. Given that the diffusion coefficient of articular cartilage to key chondrogenic molecules is lower than that in free solution (Maroudas 1970; Torzilli et al. 1987; Torzilli et al. 1997; Leddy and Guilak 2003; Tognana et al. 2005), it would seem reasonable to assume that diffusional limitations associated with the surrounding cartilage may be partially responsible for this result. Related to this is the possibility that the surrounding articular cartilage may be acting as a sink for such regulatory molecules, as various growth factors have been observed to bind to cell receptors and cartilage matrix components (Yamaguchi et al. 1990; Ruoslahti and Yamaguchi 1991; Schneiderman et al. 1995); most likely proteoglycans and/or some other noncollagenous matrix proteins. Matrix components may also neutralize the activity of growth factors (Ruoslahti and Yamaguchi 1991). It is also unclear what role the physical confinement of the cartilage explant has on chondrogenesis. Confining self-assembled tissue-engineered cartilage for 2 weeks in agarose wells has been shown to increase the compressive stiffness of the construct without a change in the GAG or

collagen content (Elder and Athanasiou 2008). However, the growth dynamics of self-assembled cartilaginous tissues and those engineered in hydrogels are fundamentally different, leading to altered levels of physical stimuli acting on the developing tissues. This complicates comparisons between these two culture systems.

It has also been suggested that soluble factors released from surrounding cartilage can inhibit cell proliferation and matrix accumulation in chondrocyte seeded hydrogels within such explant models (Hunter and Levenston 2004). This inhibition was observed whether or not the cell-seeded hydrogel was cultured inside a cartilage annuli, or in close proximity to the explant (Hunter and Levenston 2004). Therefore, the inhibition of MSCs chondrogenesis observed in this study may also be due to the presence of such factors released by the surrounding cartilage tissue as nitric oxide, cathepsins, or MMPs that are released and activated when the cartilage is damaged leading to cell death and tissue degradation. However, there is also evidence to suggest that chondrogenesis of MSCs might be enhanced in the presence of viable cartilaginous tissue. For example, it has been demonstrated that chondrocytes can store latent pro-chondrogenic cytokines such as TGF-β and can regulate both the temporal and spatial activation of such molecules (Pedrozo et al. 1998). Co-culture of MSCs with chondrocyte-like cells has been proposed as a novel strategy to induce chondrogenic differentiation of MSCs (Boon et al. 2004; Richardson et al. 2006; Lu et al. 2007; Ni et al. 2008). Co-culture of xenogenic MSCs and chondrocytes has revealed that while the presence of MSCs can enhance chondrogenesis of chondrocytes, a chondroinductive effect by chondrocytes on MSCs was not observed (Tsuchiya et al. 2004). Similarly, co-culture of MSCs with nucleus pulposus cells has been demonstrated to enhance chondrogenesis (Richardson et al. 2006), but only if cell to cell contact is allowed, which is generally absent in the explant model employed in this study. Intimate contact between different cell types may lead to a more efficient transduction of molecular signals that induce chondrogenesis. Surface receptors of adjacent cells come into direct physical contact and the autocrine and paracrine factors secreted by one cell type readily interact with the other (Boon et al. 2004).

To completely de-couple these different possible effects, future studies will include controls where MSCs-seeded hydrogels are cultured in the proximity of cartilage explants, in media supplemented with and without known anabolic and catabolic cytokines. The results of this explant study suggest that alterations to the biochemical or biophysical environment may be required before MSCs produce similar results to primary

chondrocytes. In the future such models could also be improved by incorporating many additional factors known to be present in the *in vivo* environment, but missing in this *in vitro* model, such as bone morphogenic proteins or fibroblast growth factor. Cartilage explant models can also be extended to include the subchondral bone (Tam et al. 2007), which among other benefits, may be a source of soluble factors (e.g., bone morphogenetic proteins) that regulate chondrogenesis *in vivo*. Another critical factor to include in future *in vitro* models of MSCs-based cartilage repair is physiological levels of mechanical loading to the explant (Hunter and Levenston 2002), which has previously been demonstrated to regulate chondrogenesis of MSCs in various bioreactor systems (Huang et al. 2004; Campbell et al. 2006; Mouw et al. 2007; Thorpe et al. 2008). Dynamic loading will also influence the transport of large molecules in such constructs (Mauck et al. 2003; Albro et al. 2008). Cartilage treatments should also be investigated, as other authors have already shown in cartilage repair studies that treatment with highly purified collagenase and/or hyaluronidase improves integrative cartilage repair (Bos et al. 2002). Finally, the use of alternative scaffold materials to agarose should be investigated (e.g., fibrin, collagen), as agarose does not allow for significant cell movement, limiting their ability to migrate to the cartilage interface. Inclusion of these and other factors will significantly improve *in vitro* models of cartilage repair, potentially reducing the need for animal model trials and providing controlled experiments prior to clinical investigations.

4 Functional properties of cartilaginous tissues engineered from infrapatellar fat pad derived mesenchymal stem cells

4.1 Introduction

ACI is an established technique for the treatment of articular cartilage defects and is now in clinical use (Brittberg et al. 1994; Peterson et al. 2000; Horas et al. 2003; Ruano-Ravina and Jato Diaz 2006). However, limitations still exist with both first- and second-generation ACI techniques in relation to the cell source procured. Issues such as dedifferentiation during monolayer expansion of isolated CC (Benya et al. 1978; Diaz-Romero et al. 2005), age-related capacity (Barbero et al. 2004) and poor chondrogenic potential of osteoarthritic-derived cells (Tallheden et al. 2005) have prompted investigations into the therapeutic potential of utilising MSCs as an alternative cell source for cartilage repair (Wakitani et al. 1994; Bosnakovski et al. 2004; Bosnakovski et al. 2006; Mauck et al. 2006; Miyanishi et al. 2006; Coleman et al. 2007; Hannouche et al. 2007; Mauck et al. 2007; Huang et al. 2008; Kisiday et al. 2008).

MSCs possess the ability to proliferate extensively *ex vivo* while maintaining their multipotent differentiation capabilities (Bruder et al. 1997; Kadiyala et al. 1997; Barry et al. 2001), making them an attractive cell type for cell-based cartilage repair strategies. These cells can, for example, be isolated from BM aspirates taken from the iliac crest and have the capacity to differentiate along a number of mesenchymal lineages including bone, cartilage and fat (Maniatopoulos et al. 1988; Caplan 1991; Johnstone et al. 1998; Pittenger et al. 1999; Jones and McGonagle 2008). To date, MSCs derived from the BM have been the prime site of cell harvesting for cartilage therapy development (Yoo et al. 1998; Kuroda et al. 2007; Wakitani et al. 2007). More recently, interest has also extended to utilising MSCs from other locations, and in the case of knee joint cartilage repair, from non-cartilaginous knee joint tissues. It has been demonstrated that tissues from this region including the joint FP (Dragoo et al. 2003; Wickham et al. 2003; English et al. 2007; Khan et al. 2007; Lee et al. 2008), SM (Nishimura et al. 1999; Sakaguchi et al. 2005; Mochizuki et al. 2006; Shirasawa et al. 2006; Ando et al. 2007; Marsano et al. 2007; Pei et al. 2008; Pei et al. 2008) and synovial fluid (Jones and McGonagle 2008) possess significant

chondrogenic potential and perhaps provide a more readily available and clinically feasible source of chondroprogenitor cells. Through *in vitro* experiments it has been demonstrated that 80 % of OA-derived FP pellet cultures were more chondrogenic than control BM MSCs and in addition exhibited similar chondrogenic capacity to cartilage-derived cultures (English et al. 2007).

One of the key challenges for any cartilage tissue engineering strategy is ensuring sufficient functional properties so that the engineered construct can be implanted in a load bearing environment. BM MSCs undergoing chondrogenesis have been shown to synthesise less extracellular matrix ECM than chondrocytes (Worster et al. 2001). Furthermore, BM MSCs generate an ECM that is mechanically inferior to that produced by fully differentiated articular chondrocytes maintained in identical hydrogel culture conditions (Mauck et al. 2006; Erickson et al. 2009; Huang et al. 2009). It has been suggested that this may in part be due to some critical structural ECM components not being expressed and deposited by BM MSCs (Erickson et al. 2009). There is therefore a need to assess the functional properties of cartilaginous tissues engineered using MSCs isolated from other tissue sources. The objective of this study is to determine the functional properties of cartilaginous tissues engineered from stem cells isolated from the FP.

4.2 Materials and methods

4.2.1 Cell isolation and expansion

Porcine FPs were harvested from the knee joint capsule of two 4-month-old porcine donors (50 kg) within 3h of sacrifice. FPs were washed thoroughly in PBS and diced followed by overnight incubation under constant rotation at 37°C with high-glucose DMEM GlutaMAX™ (GIBCO, Biosciences, Ireland) containing collagenase type II (0.5 mg/ml,Sigma–Aldrich, Ireland) and 1 % penicillin (100 U/mL)-streptomycin (100 µg/mL). After tissue digestion, cells were washed in PBS, filtered through a 40 µm nylon cell strainer and centrifuged at 650 g for 5 min. Mononucleated cells were counted using a haemacytometer and plated in T-75 cm^2 flasks (Sarstedt, Wexford, Ireland) at a density of 5×10^3 cells/cm^2 and cultured in expansion medium consisting of hgDMEM containing 10 % FBS and 1 % penicillin (100 U/mL)–streptomycin (100 mg/mL) (all from GIBCO, Biosciences, Dublin, Ireland). Non-adherent cells were removed during the first medium change after 72h. After colony formation (8 days), cells were detached with 0.05 %

trypsin–EDTA (Sigma-Aldrich, Arklow, Ireland) for 5 min at 37°C and re-plated at 5 x10^3 cells/cm^2 (P1). Cultures were expanded to passage three (P3, 21 days from initial isolation) at a seeding density of 5 x10^3 cells/cm^2 at each passage in expansion medium. Complete medium exchanges were performed twice weekly. Cells were pooled from two donors prior to agarose encapsulation.

4.2.2 Hydrogel construct fabrication and culturing

FP MSCs (P3) were encapsulated in agarose (Type VII) at ~40°C, to yield a final gel concentration of 2 % and a cell density of 15×10^6 cells/mL. The agarose-cell suspension was cast in a stainless steel mould to produce cylindrical discs (Ø 5 mm×3 mm thickness). Agarose hydrogel constructs were maintained in a chemically defined chondrogenic medium (CDM) consisting of DMEM GlutaMAX™ supplemented with penicillin (100 U/mL)-streptomycin (100 µg/mL) (both GIBCO, Biosciences, Ireland), 100 µg/ml sodium pyruvate, 40 µg/ml L-proline, 50 µg/ml L-ascorbic acid-2-phosphate, 1 mg/ml BSA, 1× insulin–transferrin–selenium, 100 nM dexamethasone (all from Sigma-Aldrich, Ireland) and 10 ng/ml recombinant human TGF-β3 (R&D Systems, UK). Hydrogel constructs were cultured in standard 6 well plates (Sarstedt, Ireland) with two-three constructs per well. Each construct was maintained in 2.5 mL of complete medium with complete medium exchanges performed every 3-4 days for the total culture duration of 42 days. The surfaces of wells were monitored and changed when outgrowth occurred (~weekly) to avoid excessive nutrient demands from monolayer cells. The effect of removal of TGF-β3 from culture after 21 days was also examined in a parallel study. Agarose hydrogels were maintained in fully supplemented CDM with TGF-β3 for either the total culture period of 42 days (TGF+) or for the first 21 days, with subsequent removal of TGF-β3 (TGF-) from days 21 to 42.

4.2.3 Assessment of functional properties

Constructs were mechanically tested in unconfined compression at days 0, 21 and 42 using a standard materials testing machine with a 5N load cell (Zwick Z005, Roell, Germany). A preload of 0.01 N was applied to ensure that the surfaces of the gel constructs were in direct contact with the impermeable loading platens. Stress relaxation tests were performed, consisting of a ramp and hold cycle with a ramp displacement of 0.001 mm/s until 10 % strain was obtained and maintained until equilibrium was reached. Dynamic

tests were performed immediately after the stress relaxation cycle. A cyclic strain of 1 % was applied at 1 Hz. After mechanical testing, constructs were weighed wet and the total mass recorded. Constructs were cored using a 3 mm biopsy punch and separated from the annulus; the wet mass of both the core and annulus was recorded and frozen for subsequent biochemical analyses.

4.2.4 Quantitative biochemical analysis

Samples were digested at days 0, 21 and 42 in papain (125 μg/ml) in 0.1 M sodium acetate, 5 mM cysteine HCl, 0.05 M EDTA, pH 6.0 (all from Sigma–Aldrich, Ireland) at 60°C under constant rotation for 18 hours. Total DNA content was measured using a Quant-iT™ PicoGreen® dsDNA kit (Molecular Probes, Biosciences) with a lambda DNA standard. The proteoglycan content was estimated by quantifying the amount of sulfated GAG in constructs using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Northern Ireland), with a chondroitin sulfate standard. Total collagen content was determined by measuring the hydroxyproline content. Samples were hydrolysed at 110°C for 18 hrs in concentrated HCL (38 %) and assayed using a chloramine-T assay (Kafienah and Sims 2004) with a hydroxyproline-to-collagen ratio of 1:7.69 (Ignat'eva et al. 2007).

4.2.5 Histology and immunohistochemistry

Constructs were fixed in 4 % paraformaldehyde, wax embedded and sectioned at 5 μm to produce a cross section perpendicular to the disc face. Sections were stained with 1 % alcian blue 8 GX (Sigma–Aldrich, Ireland) in 0.1 M HCl which stains GAG, and picro-sirius red to stain collagen. The deposition of collagen types I and II were identified through immunohistochemistry. Briefly, sections were quenched of peroxidase activity, rinsed with PBS before treatment with chondroitinase ABC (Sigma-Aldrich, Ireland) in a humidified environment at 37°C to enhance permeability of the extracellular matrix by removal of chondroitin sulphate. Slides were rinsed with PBS and non-specific sites were blocked with goat serum. Sections were then incubated overnight at 4°C with the primary antibody; either mouse monoclonal collagen type I anti-body (1:400; 5.4 mg/mL; Abcam, Cambridge, UK) or mouse monoclonal anti-collagen type II (1:100; 1 mg/mL; Abcam, Cambridge, UK). After washing in PBS, sections were incubated for 1 hour in the secondary antibody for type I and type II collagen, anti-mouse IgG biotin antibody produced in goat (1:400; 1 mg/mL; Sigma-Aldrich, Arklow, Ireland). Colour was developed using the Vectastain ABC reagent (Vectastain ABC kit, Vector Laboratories,

Peterborough, UK) followed by exposure to peroxidase DAB substrate kit (Vector Laboratories Ltd., Peterborough, UK). Negative and positive controls of porcine ligament and cartilage were included for each batch.

4.2.6 Statistical analysis

Statistical analysis were preformed using either one way ANOVA with Tukey's post-hoc test for multiple comparisons, or two way ANOVA with Bonferroni posttests. Tests were performed using SigmaStat® (Version 3.1) software with 3 samples analysed for each group at each time point. Numerical and graphical results are displayed as mean ± standard deviation. Significance was accepted at a level of $p < 0.05$. The experiment was also replicated with cells pooled from different animals (n=2 donors). Comparable results were observed in both studies, and the results of the first replicate are presented in the thesis.

4.3 Results

Constructs increased in mechanical properties from an equilibrium modulus of 7.0 kPa (± 0.6) at day 0 reaching 26.2 kPa (± 1.1) by day 42 and a final 1Hz dynamic modulus, of 272.8 kPa (± 46.8) (Fig. 4.1A). DNA content increased with time in culture from 88.15 ng/mg w/w (± 6.64) at day 0, to 112.78 ng/mg w/w (± 15.57) at day 21 reaching 171.65 ng/mg w/w (± 3.17) by day 42 (1.9 fold increase), indicating significant cellular proliferation had occurred (Fig. 4.1B).

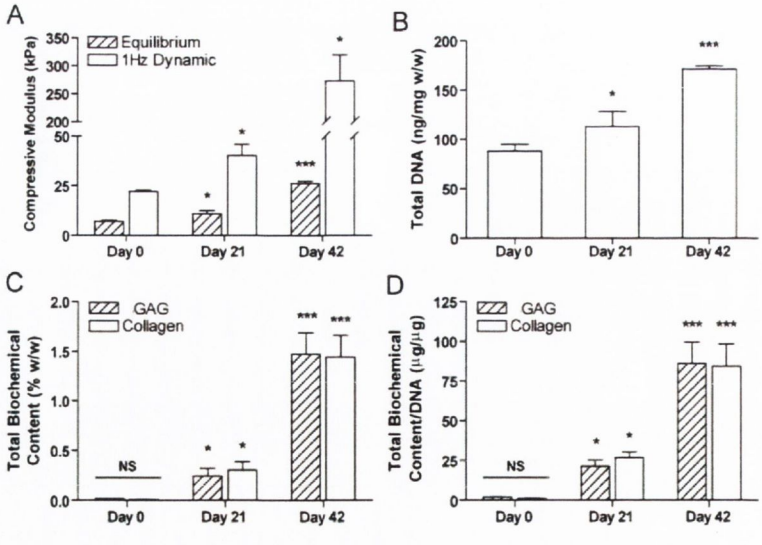


Figure 4.1: Mechanical and total biochemical properties of FP constructs at days 0, 21 and 42: (A) equilibrium modulus and 1Hz dynamic modulus; (B) total DNA content (ng/mg w/w); (C) total GAG and collagen content (%w/w) and (D) total GAG and collagen

content normalised to DNA (μg/μg). (NS) represents no significance, (*) $p<0.05$, (***) $p<0.001$ represents significance compared to earlier time point (s), n=3 for each group.

Increases in total biochemical content (Fig. 4.1C-D) were also observed with increasing time in culture ($p<0.001$). In terms of percentage wet weight (%w/w), GAG content reached a maximum of 1.47 % w/w (± 0.22) with collagen contents of 1.44 % w/w (± 0.22) attained by day 42 (Fig. 4.1C). Similarly, normalisation of the biochemical constituents by DNA content resulted in GAG accumulation of 85.93±13.57 (μg/μg) and 84.28 ±14.08 (μg/μg) for collagen (Fig. 4.1D). To gain an appreciation of the spatial distribution of biochemical constituents, the annuli and cores of samples were assessed separately (Fig. 4.2). By day 42, the core regions of constructs contained greater GAG accumulation compared to the corresponding annular region when expressed in terms of percentage of wet weight (annulus=1.37 ± 0.20 %w/w and core=1.64 ± 0.28 %w/w) (Fig. 4.2A), although this was not found to be statistically significant ($p>0.05$).

Similar differences were also observed in core-annulus GAG accumulation when normalised by DNA content (Fig. 4.2B), and were found to be statistically significant ($p<0.01$). Interestingly, collagen content was higher in the annular region of FP constructs compared to the core region when normalised by wet weight (annulus= 1.61 ± 0.24 %w/w, core=1.17 ± 0.18 %w/w, $p<0.05$), although no differences were observed when normalised by DNA content (annulus=82.88 ± 14.32 μg/μg, core=87.73 ± 15.00 μg/μg, $p>0.05$), (Fig. 4.2C and D). The DNA content increased with time in the annular regions from 94.36 ng/mg w/w (± 7.63) at day 0, reaching 195.31 ng/mg w/w (± 5.40) at day 42 (2.1-fold increase) indicating extensive proliferation. The FP core region DNA content also increased from 77.92 ng/mg w/w (77.42) at day 0, reaching 133.59 ng/mg w/w (± 5.68) at day 42 (1.7-fold increase), (Fig. 4.2E).

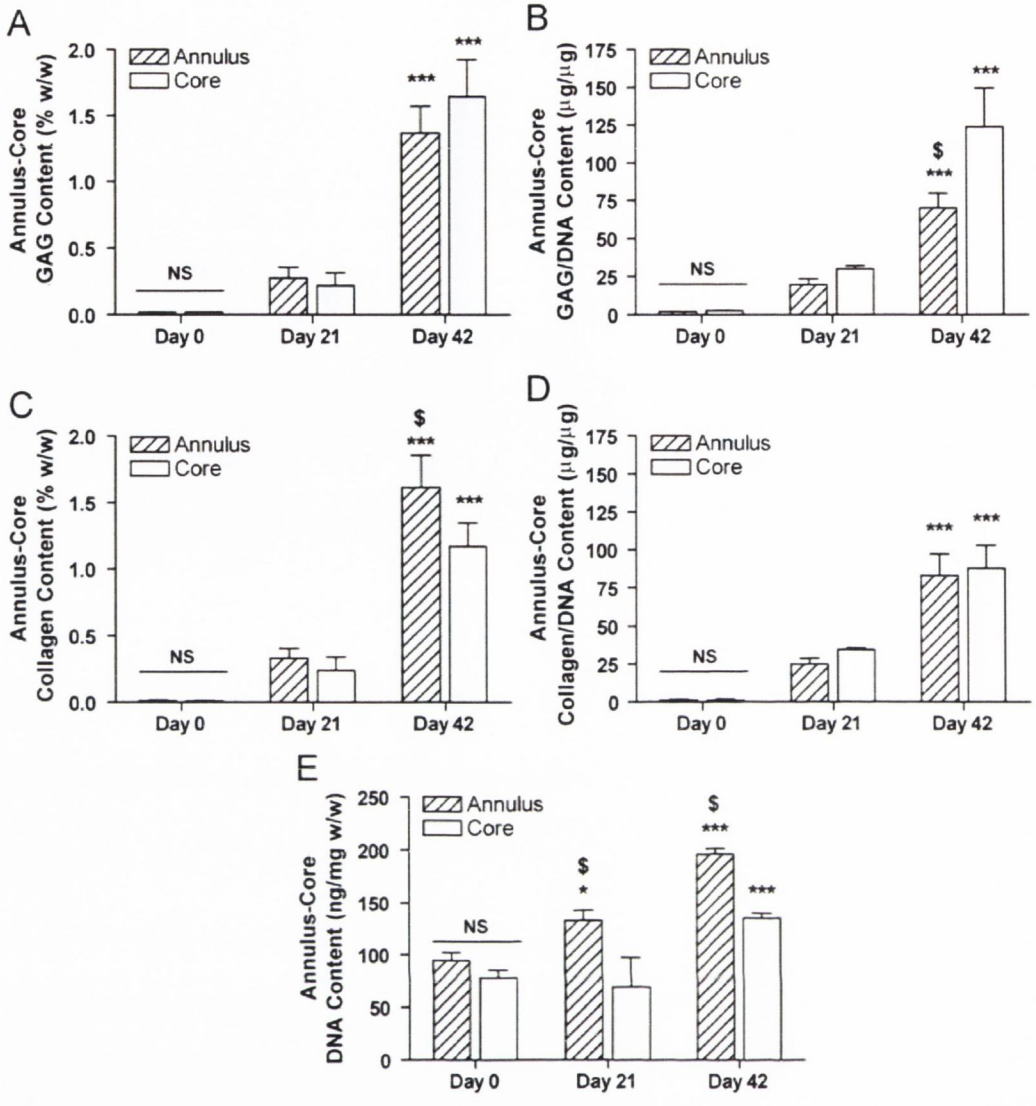


Figure 4.2: Annulus-core biochemical analysis of FP constructs at days 0, 21and 42: (A) GAG content (%w/w); (B) GAG/DNA content (µg/µg); (C) collagen content (%w/w); (D) collagen/DNA content (µg/µg) and (E) DNA content (ng/mg w/w).(NS) represents no significance,(*) $p<0.05$, (***) $p<0.001$ represents significance compared to earlier time point(s), ($) $p<0.01$ compared to corresponding core at the same time point, n=3 for each group.

Histological evaluation revealed that constructs stained positively for sulphated proteoglycan and collagen accumulation (Fig. 4.3A and B). Immunohistochemical results revealed that constructs stained weakly for collagen I, with more pronounced staining observed for collagen type II after 42 days of culture (Fig. 4.3C and D).

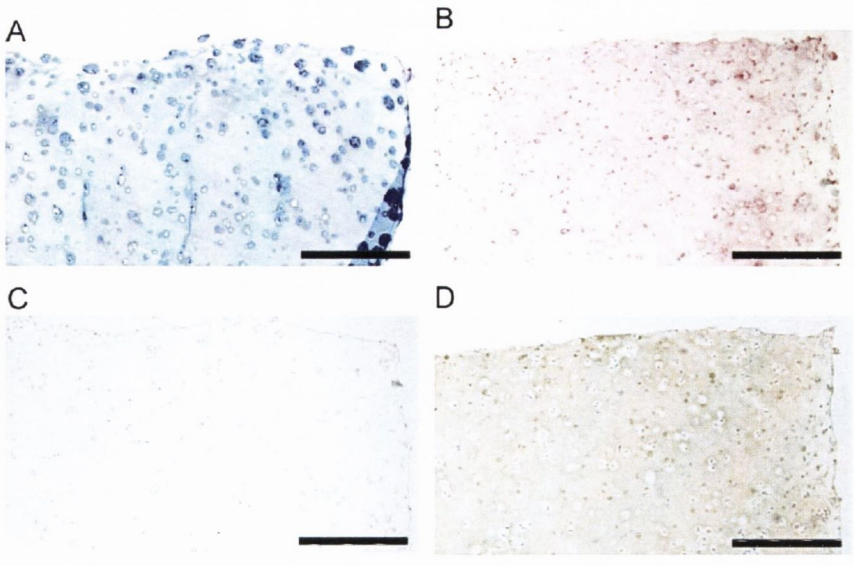


Figure 4.3: Histological evaluation of constructs after 42 days of culture: (A) Alcian blue staining for GAG; (B) Picro-sirius Red staining for collagen; (C) immunohistochemical staining for type I collagen and (D) immunohistochemical staining for type II collagen. Scale bar is 1 mm.

The removal of TGF-β3 from culture after 21 days was shown to have a significant effect on both the mechanical properties and biochemical content of FP constructs after 42 days (Fig. 4.4). No statistically significant increases occurred in any mechanical or biochemical measurement from day 21 to day 42 without the continued supplementation of TGF-β3 ($p>0.05$). Both the equilibrium (TGF^{+} =26.26 ± 1.10 kPa, TGF^{-} =12.30 ± 0.74 kPa, $p<0.001$) and 1Hz dynamic moduli (TGF^{+} =272.85 ± 46.81 kPa, TGF^{-} =74.35 ± 10.14 kPa, $p<0.001$) were higher for continued supplementation with TGF-β3 from day 21 to 42 compared to withdrawal of TGF-β3 (Fig. 4.4A). Significantly higher levels of GAG accumulation were observed after 42 days for constructs with continued supplementation of TGF-β3 (TGF^{+} =1.47 ± 0.22 %w/w; TGF^{-} =0.42 ± 0.08 %w/w, $p<0.01$), with similar differences for collagen accumulation (TGF^{+} =1.44 ± 0.22 %w/w, TGF^{-} =0.58 ± 0.06 %w/w, $p<0.01$) (Fig. 4.4B). These results were also reflected when normalised by DNA content (Fig. 4.4C). For DNA content (Fig. 4.4D), continued supplementation of TGF-β3 also enhanced cellular proliferation (TGF^{+} =171.65 ± 3.17 ng/mg w/w; TGF^{-} = 129.93 ± 11.82 ng/mg w/w, $p<0.01$).

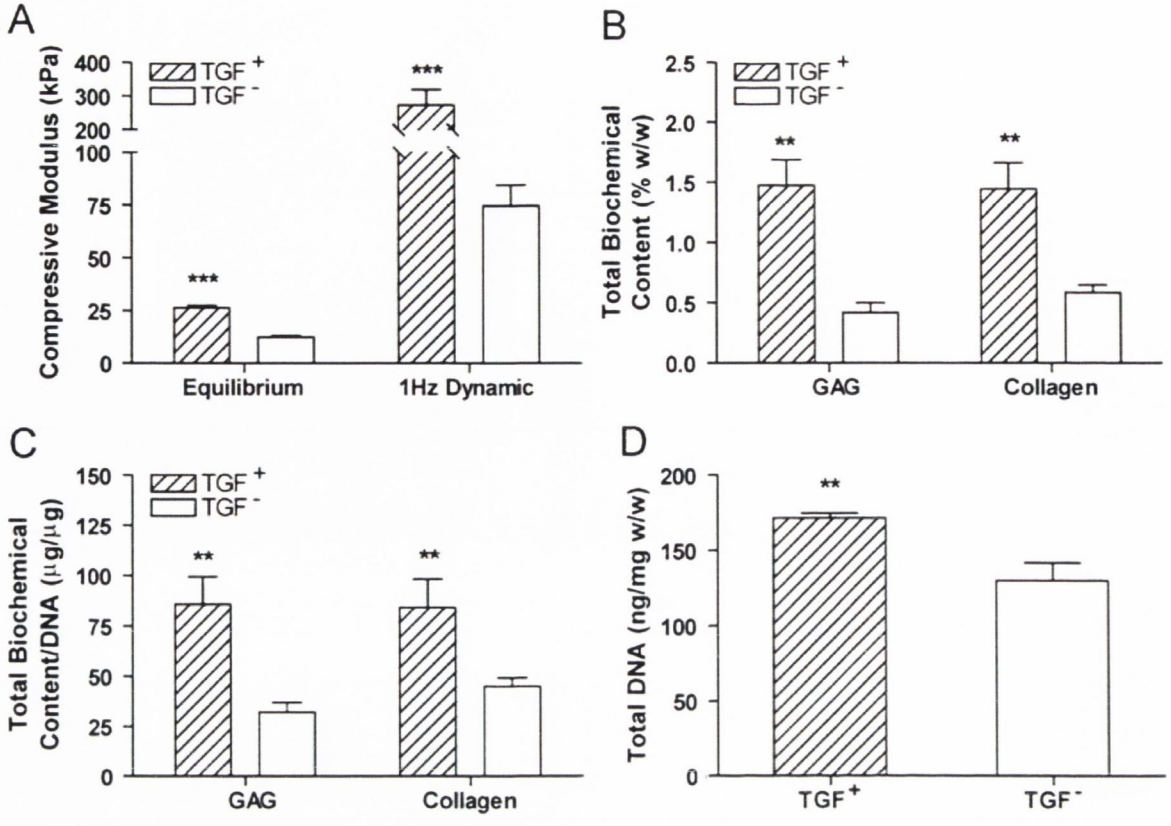


Figure 4.4: The effect of removal of TGF-β3 from culture after 21 days. Data represents mechanical properties and total biochemical analysis of constructs at day 42: (A) equilibrium modulus and 1 Hz dynamic modulus; (B) total GAG and collagen content (%w/w); (C) total GAG and collagen content normalised to DNA (µg/µg) and (D) DNA content (ng/mg w/w), n=3 for each group. (**) $p<0.01$, (***) $p<0.001$ represents significance of continued supplementation with TGF-β3 (TGF^{+}) compared to removal after 21 days (TGF^{-}), n=3 for each group.

4.4 Discussion

Alternative cell sources for articular cartilage repair strategies have received significant attention with the identification of chondro-progenitor populations within BM and in joint tissues such as synovium, FP and within the synovial fluid itself. In this study we have demonstrated that FP stem cells encapsulated in agarose hydrogels undergo chondrogenesis when exposed to TGF-β3. The FP of the knee is a heterogeneous tissue and is composed of a thin layer of synovium that covers a relatively large subsynovium adipose-like tissue. It has previously been demonstrated that the phenotype and chondrogenic potential of FP stem cells is more similar to fibrous SM stem cells than to subcutaneous fat-derived cells (Mochizuki et al. 2006). SM stem cells have already been

shown to possess significant promise as a cell source for cartilage tissue engineering (Sakaguchi et al. 2005; Yokoyama et al. 2005; Ando et al. 2007; Ando et al. 2008; Pei et al. 2008; Pei et al. 2008). The findings, in the present study, therefore extend observations in relationship to MSCs isolated from within the joint space (Mochizuki et al. 2006; Yoshimura et al. 2007) and suggest that FP stem cells proliferate and undergo robust chondrogenesis within agarose hydrogels.

The spatial distribution of GAG within the agarose hydrogels was generally different regionally, with greater amounts of GAG accumulation in the core of constructs compared to the annulus. Higher levels of core GAG content have previously been reported by our laboratory for agarose encapsulated porcine BM MSCs (Thorpe et al. 2008). This may simply be due to diffusion of GAG from the periphery of the hydrogel into the media, or alternatively spatial gradients in the concentration of nutrients, growth factors, oxygen, etc. could be leading to the development of a more chondrogenic environment within the core of the constructs. Interestingly, greater proliferation and collagen accumulation was observed in the annulus, suggesting an environment in this region that promotes a more proliferative phenotype compared to the construct core.

The mechanical properties of constructs increased significantly between day 21 and day 42 of culture. The reported mechanical properties of cartilaginous tissues engineered using chondrocytes from skeletal immature bovines embedded in agarose are generally higher than that reported here for porcine FP stem cells (Mauck et al. 2006; Byers et al. 2008). However differences due to age, species and culture conditions mean that future studies comparing donor matched chondrocytes and FP MSCs will be critical. In addition, longer term studies will be required to assess if the mechanical properties of cartilaginous tissue generated using FP MSCs plateau like that observed with BM MSCs after extended culture periods (Mauck et al. 2006).

The motivation behind the removal of TGF-β3 from culture was based upon the published findings of Byers and collaborators (Byers et al. 2008) for chondrocyte seeded agarose hydrogels. The authors demonstrated that 2 week transient exposure to TGF-β3 in a chemically defined medium elicited a superior response to that observed after continuous growth factor supplementation, with certain mechanical and biochemical levels achieved comparable to those of native cartilage. In the present study, the removal of TGF-β3 from culture after 21 days did not significantly enhance either the mechanical properties or

biochemical content of constructs at 42 days compared to constructs exposed to continued TGF-β3 supplementation. Removal of TGF-β has also been shown to be sub-optimal for BM MSCs in agarose hydrogels seeded at a similar density (Huang et al. 2009), although a pronounced positive effect on the equilibrium modulus and GAG accumulation was observed in that study for higher seeding densities (60 million/ml). Further studies to determine the optimal cell seeding density in order to engineer more functional cartilaginous tissues from FP stem cells are required.

An obvious limitation of the study is the use of MSCs isolated from skeletally immature pigs. Characteristics of MSCs such as the ability to rapidly proliferate is known to change with age (Tsuji et al. 1990; Egrise et al. 1992). Pigs share an underlying genetic and physiological similarity to humans (Vacanti et al. 2005) and due to this it has been suggested that they may provide a useful animal model system to evaluate tissue engineering strategies (Ringe et al. 2002). Regardless it is unclear if the results presented here would be comparable when using FP stem cells derived from aged or diseased human tissue, although studies investigating the chondrogenic potential of such cells are promising (Khan et al. 2007; Khan et al. 2008).

Given its anatomical location and ease of accessibility for harvesting biopsies of tissue, the FP may provide an attractive source of cells for articular cartilage repair. In the present work we add to this emerging concept by showing good *in vitro* functionality of cartilaginous tissues engineered using FP stem cells. A critical next step is to assess *in vivo* the ability of cartilaginous tissues engineered using FP stem cells-agarose constructs to repair large chondral or osteochondral defects. Clinical studies have already been undertaken by other groups investigating agarose-alginate hydrogels seeded with chondrocytes for cartilage defect repair (Selmi et al. 2007; Selmi et al. 2008). In this clinical context, the FP may also possess other benefits as a source of cells for cartilage repair. Although this work utilised expanded cells, future work will also investigate the feasibility and practicalities of utilising unexpanded cells from freshly digested FP tissue, since the yield of putative MSCs may be higher in such tissues. If successful, such advantages would significantly accelerate translation into a clinical setting by avoiding the time and cost hindrances associated with large-scale cell expansion. The present porcine studies therefore provide a rationale for the exploration of FP in man as a source of cells for cartilage regeneration.

5 Composition-function relations of cartilaginous tissues engineered from chondrocytes and mesenchymal stem cells isolated from bone marrow and infrapatellar fat pad

5.1 Introduction

Due to its limited capacity for regeneration and self repair, as well as a paucity of therapeutic options, the degeneration of articular cartilage can have severe consequences (Pelttari et al. 2008). Clinical procedures for cartilage defect repair, such as subchondral abrasion arthroplasty, microfracture or autologous chondrocyte implantation, often result in only a temporary partial repair, producing a biomechanically inferior tissue that can ultimately degenerate, leading to osteoarthritis (Schumann et al. 2006). Engineering functional cartilage tissue using MSCs seeded into scaffolds or hydrogels represents a promising alternative treatment for articular cartilage defects. Many adult tissues maintain populations of MSCs that are not terminally differentiated and which could potentially be used for tissue regeneration following trauma, disease or ageing (Pittenger et al. 1999; Fuchs and Segre 2000; Hadjantonakis and Papaioannou 2001; Wickham et al. 2003). Such cells possess the ability to proliferate extensively *ex vivo* while maintaining their differentiation capabilities (Caplan 1991; Pittenger et al. 1999).

BM derived MSCs have been studied extensively *in vitro* for their capacity to differentiate and synthesize markers associated with adipocytes, chondrocytes (CC) and osteoblasts (Maniatopoulos et al. 1988; Caplan 1991; Johnstone et al. 1998; Pittenger et al. 1999) and for their ability to generate a mechanically functional cartilaginous tissue (Mauck et al. 2006; Mauck et al. 2007; Thorpe et al. 2008; Erickson et al. 2009; Huang et al. 2010). However, traditional BM procurement procedures may be painful, time consuming, expensive and risk cell contamination and loss (Pelttari et al. 2008; Khan et al. 2009). An ideal source of autologous cells would both be easy to obtain and would result in minimal patient discomfort during harvesting (Zuk et al. 2001). Recent studies have shown that

MSCs isolated from other connective tissues, such as FP, SM and subcutaneous fat, may possess significant plasticity in their multi-lineage capabilities (De Bari et al. 2001; Zuk et al. 2001; Zuk et al. 2002; Dragoo et al. 2003; Wickham et al. 2003; Huang et al. 2005; English et al. 2007; Khan et al. 2007; Pei et al. 2008). These tissues represent attractive cell sources for tissue engineering because they are generally accessible with minimal donor site morbidity. Comparisons of the chondrogenic potential of different sources of MSCs (synovium, BM, subcutaneous fat and muscle) in a pellet culture system suggest that SM-derived MSCs possess a chondrogenic capability superior to that of other sources (Koga et al. 2008). It has also been reported that human osteoarthritic FP tissue contains highly clonogenic and multipotent MSCs with stable chondrogenic potential *in vitro* (English et al. 2007), which express markers common with other sources of MSCs (Khan et al. 2008). We have recently demonstrated that functional cartilaginous tissue can be engineered using infrapatellar FP-derived MSCs embedded in agarose hydrogel (Buckley et al. 2010). While it has been demonstrated that cartilaginous tissues engineered using BM-derived MSCs embedded in agarose hydrogel possess inferior mechanical properties to those engineered using CC (Mauck et al. 2007; Erickson et al. 2009), it is unclear whether this finding extends to MSCs isolated from sources other than BM. The objective of this study was to compare the biomechanical and biochemical properties of tissues engineered using porcine MSCs isolated from the infrapatellar FP and BM to those derived from CC. A porcine model was chosen as pigs are similar to humans in terms of their genetics, anatomy and physiology (Vacanti et al. 2005). The tripotentiality of porcine MSCs from different tissue sources was first assessed (chondrogenic, adipogenic and osteogenic potential). Next CC and MSCs from each source were encapsulated in agarose hydrogels and cultured in the presence of TGF-β3. Our hypothesis was that MSCs isolated from within the synovial joint would possess a potential to generate functional cartilaginous tissues comparable to that of CC isolated from articular cartilage.

5.2 Materials and methods

5.2.1 Cell isolation and expansion

Four month old porcine MSCs were aseptically harvested from the BM of the femur and from the whole infrapatellar FP (the outer and the inner layers of the FP were not separated, but any fibrous tissue attached to the FP was removed). CC were harvested from the articular surface of the femoro-patellar joint. Cells from each tissue source were

harvested from 2 animals and pooled. BM MSCs were isolated and expanded according to a modified method developed for human MSCs (Lennon and Caplan 2006). Muscle and fascia was removed and bones were sawn close to the femoral head. BM was removed from the medullary canal and transferred to a 50mL tube containing expansion medium consisting of high-glucose DMEM, GlutaMAX, containing 10 % FBS and 1 % penicillin (100 U/mL)-streptomycin (100 µg/mL) (all from GIBCO, Biosciences, Dublin, Ireland). Marrow was aspirated repeatedly to break up large aggregates prior to centrifugation at 650 G for 5 min. The separated fatty layer was discarded and the cell pellet was re-suspended in expansion medium, triturated through a 16 gauge needle and filtered through a 40 µm nylon cell strainer (B.D. Falcon, Unitech, Dublin, Ireland). Red blood cells were lysed using 4 % acetic acid and mononuclear cells were counted using a haemocytometer. Cartilage slices were rinsed with phosphate buffered saline containing penicillin/streptomycin (200 U/ml) and amphotericin B (2.5 µg/ml). Cartilage and FP pieces were incubated with DMEM/F12 containing collagenase type II (125 U/mg) (all from Sigma–Aldrich, Dublin, Ireland) for 16-18 hours under constant rotation at 37°C. The resulting cell suspension was then filtered through a 40 µm pore-size cell sieve (Falcon Ltd, Sarstedt, Ireland) and the filtrate centrifuged and rinsed with PBS twice. CC were seeded at a density of 50,000 cells/cm^2 in 175 cm^2 T flasks and expanded to passage one (P1). Viable cells were counted using a hemacytometer and 0.4 % trypan blue staining. Isolated CC from all joints were pooled and maintained in DMEM/F-12 (Sigma–Aldrich, Arklow, Ireland) supplemented with 10 % v/v FBS and 100U/ml penicillin/streptomycin (GIBCO, Biosciences, Dublin, Ireland) during the expansion phase. MSCs from the BM and FP were seeded at a density of 5,000 cells/cm^2 in 175 cm^2 T flasks and expanded to passage three (P3).

5.2.2 Adipogenesis and osteogenesis

Expanded MSCs from the BM and FP were plated in 9.5 cm^2 six well plates at a density of 10^3 cells/cm^2 and cultured for 7 days in complete medium (DMEM GlutaMAX supplemented with 10 % v/v FBS and 100 U/ml penicillin/streptomycin) and changed to osteogenic or adipogenic medium for 21 days. Osteogenic medium consisted of complete medium supplemented with 100 nM dexamethasone, 10 mM β-glycerolphosphate and 0.05 mM ascorbic acid, while adipogenic medium consisted of complete medium supplemented with 100 nM dexamethasone, 0.5 mM isobutylmethylxanthine and 50 µM indomethacin (all from Sigma-Aldrich, Ireland). After 21 days cells were washed twice with PBS and

fixed with ethanol for ten minutes. The wells were then stained for two minutes as follows: 1 % oil red solution, which stains lipid deposits, was used as a marker of adipogenesis; while 1 % alizarin red solution, which stains calcium deposits, was used as a marker of osteogenesis.

5.2.3 Chondrogenesis

A pellet culture system was used to assess the chondrogenic capacity of MSCs. 500 000 cells were placed in 1.5 ml conical microtubes and centrifuged at 650 G for 5 minutes. The pellets were cultured in a chemically defined chondrogenic medium: DMEM GlutaMAX supplemented with penicillin (100 U/ml)-streptomycin (100 µg/ml) (both GIBCO, Biosciences, Ireland), 100 µg/ml sodium pyruvate, 40 µg/ml L-proline, 50 µg/ml L-ascorbic acid-2-phosphate, 4.7 µg/ml linoleic acid, 1.5 mg/ml BSA, 1× insulin–transferrin–selenium, 100 nM dexamethasone (all from Sigma-Aldrich, Ireland) and 10 ng/ml recombinant human TGF-β3 (Prospec, Israel). For histological evaluation the pellets were embedded in paraffin, cut into 5 µm thick sections and stained with 1 % alcian blue 8 GX (Sigma–Aldrich, Ireland) in 0.1 M HCl to assess glycosaminoglycan content. Immunohistochemistry was used to detect type II collagen.

5.2.4 Cell-agarose constructs

Culture-expanded CC and MSCs were encapsulated in agarose (Type VII) at a final gel concentration of 2 % and a cell density of 15×10^6 cells/ml. The agarose-cell suspension was cast in a stainless steel mould to produce cylindrical constructs (diameter 5mm×3mm thickness). Agarose hydrogel constructs were maintained in the chondrogenic medium described above for 6 weeks. There were 2 samples per well with 2.5 ml of medium per construct. Medium was changed every 3-4 days. The study was designed with 5 gels per cell source at each time point; samples were analyzed at day 0, day 21 and day 42.

5.2.5 Mechanical testing

Constructs were mechanically tested between impermeable platens using a standard materials testing machine (Zwick Roell Z005, Herefordshire, UK) with a 5 N load cell, as previously described (Buckley et al. 2009). Agarose constructs were kept hydrated through immersion in a PBS bath maintained at room temperature. To assess construct functionality, an unconfined stress relaxation test was performed. Contact between the

loading platen and the construct was first visually confirmed following the application of a small pre-load (0.01 N), and then a ramp compression to 10 % strain followed by a hold period until equilibrium of the sample was achieved in a period of 30 minutes. The equilibrium Young's modulus was determined from the equilibrium force. This was followed by a 1 Hz cyclic test at 1 % strain amplitude for 10 cycles in unconfined compression to determine the dynamic modulus. Construct dimensions were measured using a toolmaker's microscope (Mitutoyo UK Ltd., Andover, UK).

5.2.6 Histology and immunohistochemistry

Constructs were fixed in 4 % paraformaldehyde overnight, rinsed in PBS, embedded in paraffin and sectioned to 8 μm thickness. The histological sections were stained with 1 % alcian blue 8 GX (Sigma–Aldrich, Ireland) in 0.1 M HCl to assess GAG content and picrosirius red to detect collagen. Collagen type I, II and X were evaluated with a standard immunohistochemical technique as previously described (Thorpe et al. 2010). Positive and negative controls were included in the immunohistochemistry staining protocol for each batch.

5.2.7 Biochemical analysis

The 5mm diameter constructs were cored using a 3mm biopsy punch. All samples were digested in papain (125 μg/ml) in 0.1 M sodium acetate, 5 mM cysteine HCl, 0.05 M EDTA, pH 6.5 (all from Sigma–Aldrich, Dublin, Ireland) at 60°C under constant rotation for 18 hours. Aliquots of the digested samples were assayed separately for DNA, collagen and sulphated GAG content. DNA content was quantified using the Quant-it Picogreen DNA assay (Invitrogen, UK). The proteoglycan content was estimated by quantifying the amount of sulphated GAG in constructs using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Northern Ireland), with a chondroitin sulphate standard. Total collagen content was determined by measuring the hydroxyproline content, using a hydroxyproline-to-collagen ratio of 1:7.69 (Kafienah and Sims 2004; Ignat'eva et al. 2007). Matrix accumulation (total collagen and GAG) was normalised to the tissue wet weight and to DNA.

5.2.8 Statistical analysis

Mechanical and biochemical properties of engineered constructs are expressed in the form of mean ± standard deviation (SD). Five or six samples were analysed per group at each time point; 3 or 4 samples were used for mechanical and biochemical analyses and 2 samples were used for histology. Differences in mechanical and biochemical properties with cell type and/or time-in-culture were determined by a general linear model for analysis of variance with groups as factors. Statistics were performed with MINITAB 15.1 (Minitab Ltd., Coventry, UK) software package. Tukey's test for multiple comparisons was used to compare conditions. Commercially available software (GraphPadPrism 4, San Diego, USA) was also used for a correlation analyses and to compare correlation slopes between conditions. A level of $p<0.05$ was considered significant. Each experimental arm (CC, FP and BM) was replicated at least once using MSCs isolated from 2 different porcine donors to the original donors, with the same trends observed in replicate experiments. The results of the first replicate are presented below. The data from the second replicate is included in the correlation analysis of mechanical properties with biochemical composition (see below).

5.3.1 MSC Tripotentiality

After 21 days in culture, pellets in chondrogenic conditions became spherical and increased in size, in particular the FP pellets. Alcian blue staining for GAG was stronger for FP pellets compared to BM pellets (Fig. 5.1). Pellets were also stained for type II collagen; the immunoreaction revealed strong staining for FP pellets in comparison with weaker staining for BM pellets (data not shown).

After 3 weeks in culture with osteogenic medium BM cells deposited calcium, as evident by staining with alizarin red. In comparison, weak staining for alizarin red was observed for the FP group (Fig. 5.1). All the cell sources demonstrated deposition of fat generating oil droplets that stained with oil red. MSCs cultured in control conditions did not stain with either alizarin red or oil red.

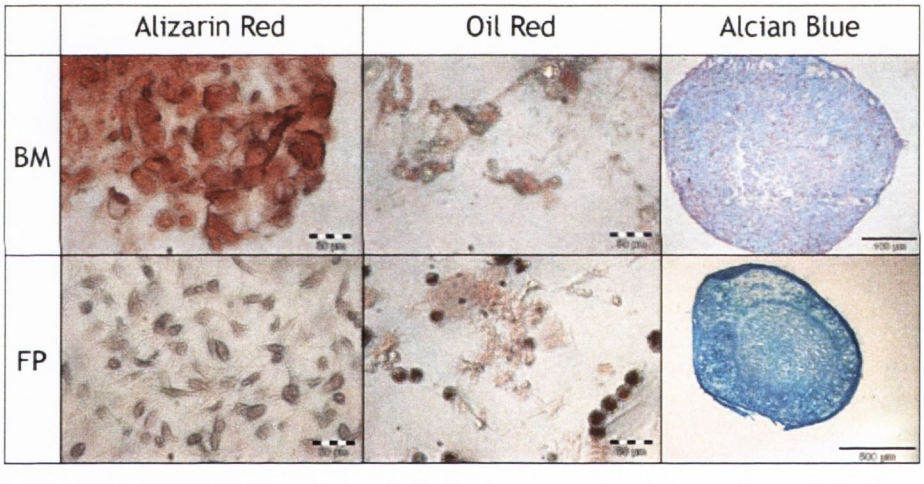


Figure 5.1: Osteogenic potential of stem cells: colonies staining positive for alizarin red staining (left column). Adipogenic potential of stem cells: colonies staining positive for oil red staining (middle column). Chondrogenic potential of stem cells: pellet staining positive for glycosaminoglycan with alcian blue (right column).

5.3.2 Agarose hydrogel culture

Histological evaluation and immunohistochemical analysis (Fig. 5.2) revealed CC, FP and BM groups stained positively for sulphated proteoglycan and collagen accumulation. Immunohistochemical analysis also revealed the presence of type II collagen in CC, FP and BM constructs. Only weak staining for type I and type X collagen was observed (Fig. 5.2).

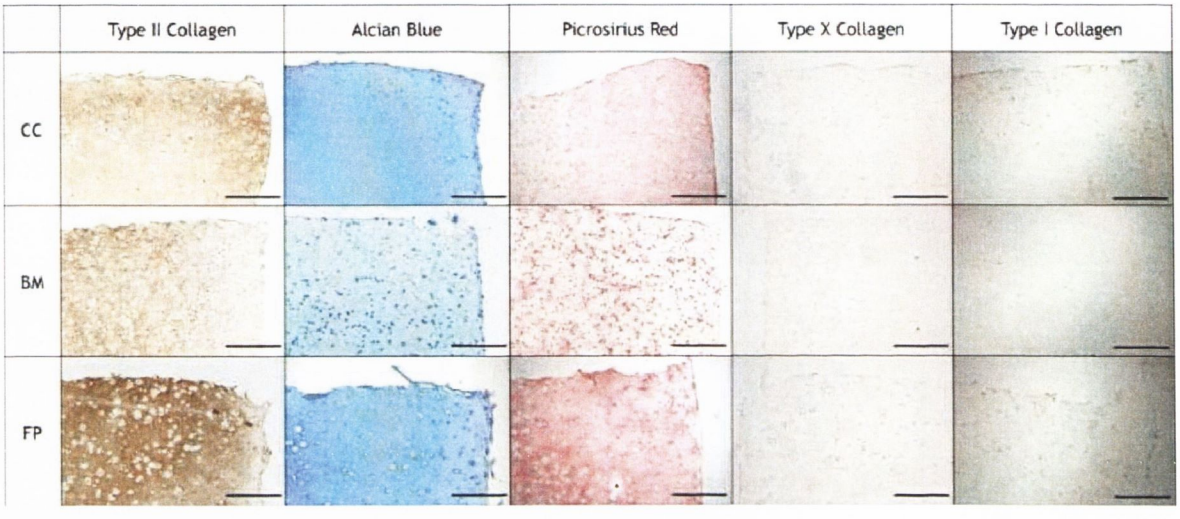


Figure 5.2: Agarose gels seeded with either CC or stem cells at day 42 stained for type II collagen, alcian blue (stains glycosaminoglycans) picrosirius red (stains collagen), type X collagen and type I collagen. Scale bar: 500μm.

For CC-seeded hydrogels, the annular region exhibited stronger type II collagen staining compared to the core, with an opposite effect observed for the MSC groups. Matrix staining appeared more diffuse for CC and FP constructs, while a more pericellular staining was observed for BM constructs. FP constructs stained strongly for alcian blue with evidence of cell clustering and development of lacunae.

Significantly greater total GAG accumulation was observed in CC-seeded hydrogels in comparison to both MSC groups, while the highest collagen accumulation was found in FP constructs (Fig. 5.3). Overall analysis of variance results showed that GAG, total collagen and DNA deposition for each cell seeded agarose hydrogel was dependent on time in culture ($p < 0.0001$), cell type ($p < 0.0001$) and region of construct (annulus or core) ($p < 0.0001$). At day 42, GAG accumulation for CC constructs reached 1.50 % w/w (± 0.05) compared to 0.95 % w/w (± 0.04) for FP constructs and 0.43 % w/w (± 0.03) for BM constructs. At day 42, collagen accumulation reached 0.88 % w/w (± 0.02) for FP, 0.54 % w/w (± 0.01) for CC and 0.48 % w/w (± 0.02) for BM. DNA content generally increased

with time for all the groups, where an increase in DNA content was observed between day 0 and day 42. Normalisation of the GAG content to DNA generally revealed a similar trend as the normalisation to wet weight.

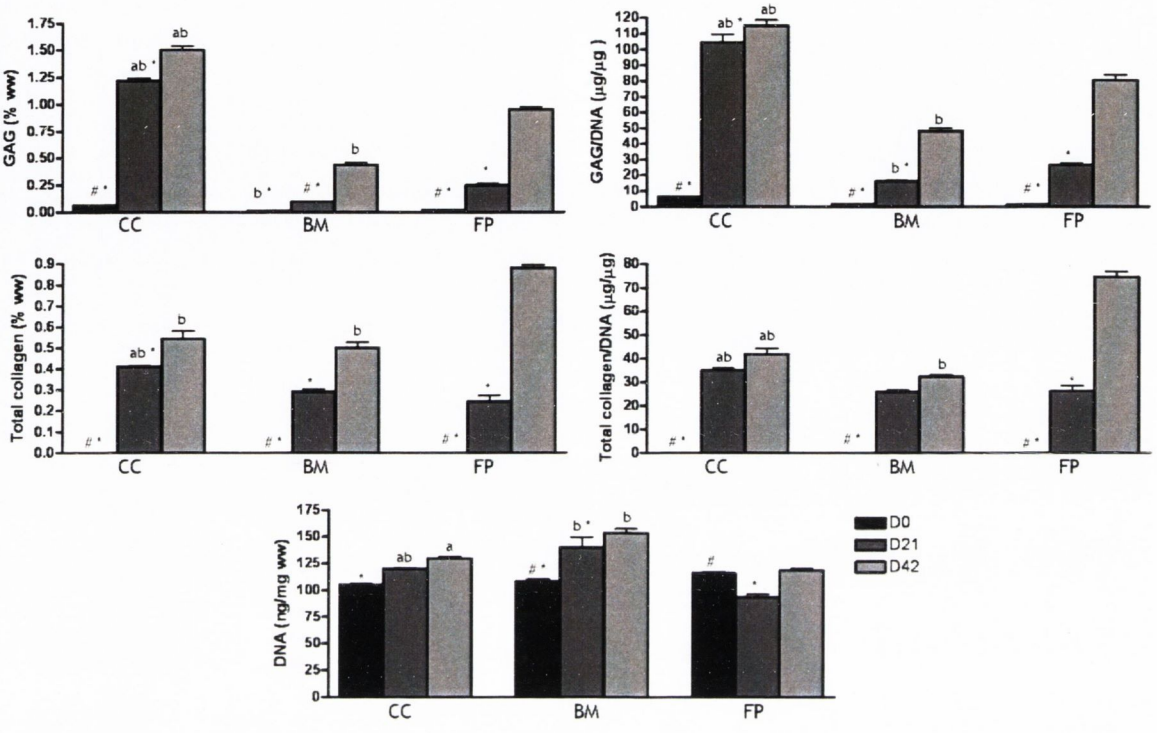


Figure 5.3: GAG, total collagen and DNA content in engineered tissues at day 0, 21 and 42 expressed in percentages of wet weight and normalized by DNA. Data represent the mean ± SD of three samples. A significance vs bone marrow, b vs fat pad, # vs day 21 and * vs day 42. $p < 0.05$.

GAG accumulation was higher in the core region of the construct for the two MSCs groups, compared to greater GAG accumulation in the annular region for CC constructs (Fig. 5.4). By day 42, in terms of percentage of wet weight the core regions of BM and FP scaffolds showed significantly greater GAG accumulation compared to their corresponding annular region (BM annulus = 0.27 ± 0.04 %w/w, BM core = 0.79 ± 0.06 %w/w, FP annulus = 0.61 ± 0.06 %w/w and FP core = 1.48 ± 0.11 %w/w).

Similar differences were observed in core-annulus accumulation when normalised by DNA content, but with higher GAG/DNA in the core regions of CC constructs compared to their annuli (Fig. 5.4). FP cores contained similar amounts of GAG per DNA (112.85 µg/µg ± 8.58) as the core (127.71 µg/µg ± 4.45) and annular (110.32 µg/µg ± 6.16) region of CC constructs. Total collagen accumulation in MSCs constructs was greater in the core

compared to the annulus (BM annulus= 0.43 ± 0.01 %w/w, BM core= 0.66 ± 0.02 %w/w, FP annulus= 0.56 ± 0.02 %w/w and FP core= 1.383 ± 0.1 %w/w). CC collagen content was lower in the core (0.38 ± 0.04 %w/w) in comparison with the annulus (0.66 ± 0.09 %w/w). When normalised by DNA, collagen values were higher in the core of the FP constructs compared to other groups. For BM constructs, no significant differences were observed in the DNA content between annular or core regions. DNA content was higher within core regions (131.87 ng/mg w/w ± 7.12) for FP constructs in comparison with the annulus (109.50 ng/mg w/w ± 1.17). The opposite trend was observed in CC constructs, with DNA content of 107.68 ng/mg w/w (± 9.14) for the core region and 131.87 ng/mg w/w (± 7.12) for the annulus.

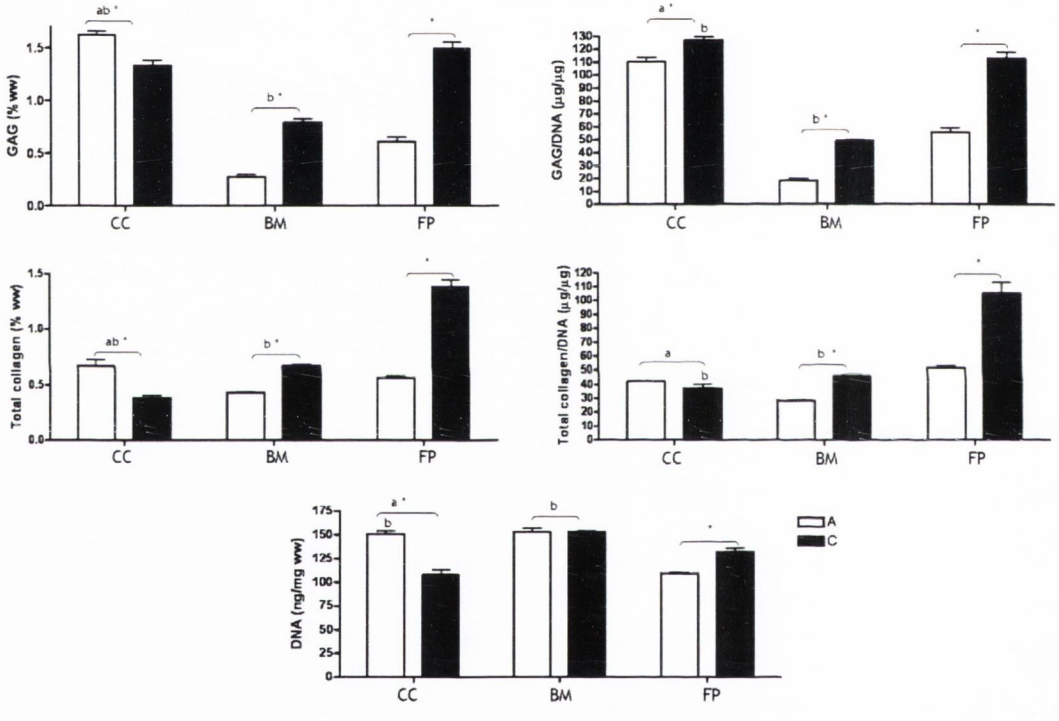


Figure 5.4: GAG, total collagen and DNA content in engineered tissues at day 42 expressed in percentages of wet weight and normalised by DNA in construct annulus (A) and core (C). Data represent the mean ± SD of three samples. a significance vs bone marrow, b vs fat pad, * annulus vs core. $p < 0.05$.

Time and cell type were significant factors in both mechanical measures ($p<0.05$). After 42 days of culture CC constructs exhibited higher equilibrium modulus (39.4 kPa ± 0.3) compared to MSCs constructs ($p< 0.001$) (Fig. 5.5). Within the MSCs groups, FP gels demonstrated comparable equilibrium modulus (16.9 kPa ± 1.6) to that of BM constructs (15.6 kPa ± 0.61). The dynamic modulus significantly increased ($p<0.05$) with time in

culture for all groups. In the FP group the modulus increased from (29.0 kPa ± 0.62) at day 0 to (133.7 kPa ± 8.4) at week 6, in the BM group from (33.6 kPa ± 1.62) to (112.6 kPa ± 13.7) and in the CC group from (38.1 kPa ± 0.19) to (256.8 kPa ± 9.9). The dynamic modulus of FP constructs was significantly higher than BM constructs after 6 weeks of culture ($p<0.05$).

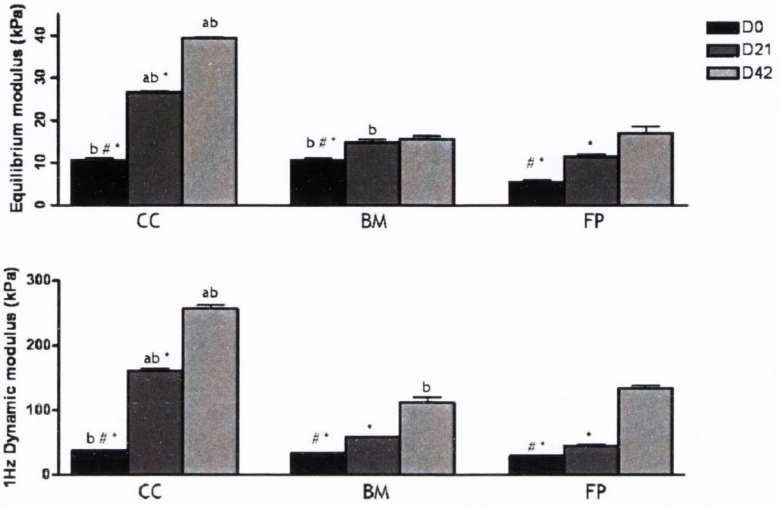


Figure 5.5: Equilibrium and 1 Hz dynamic modulus at day 0, day 21 and day 42. Data represent the mean ± SD of three samples. a significance vs bone marrow, b vs fat pad, # vs day 21 and * vs day 42 . $p< 0.05$.

5.3.3 Structure-function correlation analysis

The equilibrium modulus and dynamic modulus at day 42 were plotted against the percentage of wet weight of total collagen and GAG content for CC, FP and BM constructs (Fig. 5.6). All correlations were found to be statistically significant (Table 2), except the relation between total collagen content and the equilibrium modulus in the FP group. Comparisons between groups were not statistically significant.

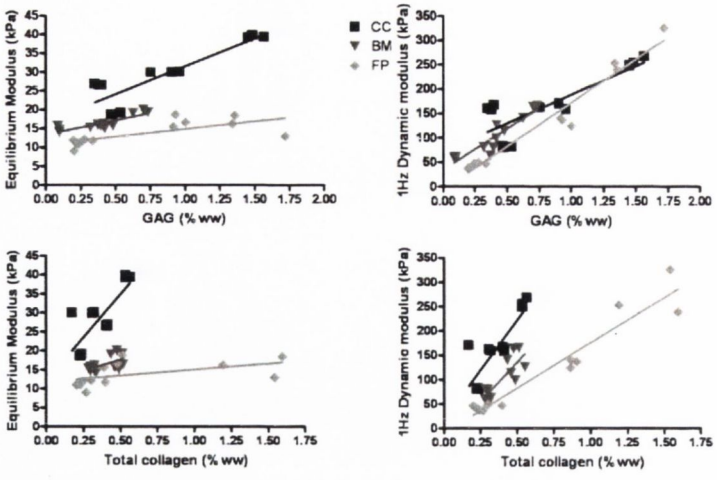


Figure 5.6: Correlation plots relating measured mechanical properties to biochemical constituents. Note that replicate data from separate experiments are included in the analysis.

Cell source	*Comparison*	*Slope*	*R^2*	*P*	*vs CC*	*vs BM*
CC	Eq Modulus *vs.* GAG	14.7	0.7797	P=0.0001		
	Eq Modulus *vs.* Total collagen	45.5	0.6346	P<0.01		
	Dyn Modulus *vs.* GAG	117.7	0.6939	P<0.001		
	Dyn Modulus vs. Total collagen	412.2	0.7289	P<0.001		
BM	Eq Modulus *vs.* GAG	7.6	0.7535	P<0.001	NS	
	Eq Modulus vs. Total collagen	11.8	0.3293	P=0.05	NS	
	Dyn Modulus vs. GAG	168.2	0.862	P<0.0001	NS	
	Dyn Modulus vs. Total collagen	327.3	0.7003	P<0.001	NS	
FP	Eq Modulus vs. GAG	4.0	0.4911	P=0.01	NS	NS
	Eq Modulus vs. Total collagen	3.1	0.2693	NS	NS	NS
	Dyn Modulus vs. GAG	179.2	0.9584	P<0.0001	NS	NS
	Dyn Modulus vs. Total collagen	185.9	0.9232	P<0.0001	NS	NS

Table 2: Correlation of mechanical properties and biochemical content for different sources of MSCs and chondrocyte constructs. Correlation coefficients relating measured mechanical properties with concentration of GAG and total collagen. NS: not significant. Note that replicate data from separate experiments are included in this analysis.

5.4 Discussion

We evaluated the potential of porcine BM and infrapatellar FP MSCs embedded in agarose hydrogels to generate functional cartilaginous tissue, and compared the properties of these tissues to those generated using CC isolated from articular cartilage. Our objective was not to identify the optimal isolation, expansion and chondrogenic differentiation conditions for different MSCs sources, but rather to compare composition-function relations of

cartilaginous tissues generated using a number of different cell sources under near identical culture conditions. Understanding how the functional properties of cartilaginous tissue evolve is an essential pre-requisite to the development of any MSCs-based therapy regardless of the cell source. While porcine BM and FP derived MSCs underwent robust chondrogenesis in agarose culture, construct properties were overall inferior to CC seeded constructs. This agrees in part with a previous study using a hyaluronan-based scaffold that demonstrated that cartilaginous constructs generated using human CC contained higher fractions of GAG compared to FP derived cells and other cell sources (Marsano et al. 2007). Interestingly, in the present study core levels of total GAG and collagen accumulation in the FP group were comparable to that found in the CC group, suggesting that under the environmental conditions created in the core of these constructs, the chondrogenic capability of FP MSCs is comparable to CC. Therefore while we were unable to fully corroborate our initial hypothesis that FP derived MSCs possess a potential to generate functional cartilaginous tissues comparable to CC, these are a source of chondro-progenitor cells worthy of further investigation for cartilage regeneration therapies.

The infrapatellar FP is composed of adipocytes and adipose connective tissue containing collagen and glycosaminoglycans, covered by a layer of SM which articulates with the trochlear cartilage of the distal femur (Mochizuki et al. 2006). MSCs from the FP are presumably derived either from the perivascular region of vessels and capillaries invading the tissue (Khan et al. 2008), and/or from the synovial layer (De Bari et al. 2001) that covers the tissue. It has recently been demonstrated that MSCs isolated from intra-articular tissues and CC share similar gene expression profiles distinct from other mesenchymal tissue-derived cells such as BM and adipose derived MSCs (Segawa et al. 2009). Differences in gene expression profiles, possibly due to differences in their respective stem cell niches, provide one possible explanation for the apparent superior functional properties of FP constructs compared to BM constructs in this study. However, direct comparisons of different MSCs sources must, for a number of reasons, be made with caution. Firstly, the initial fraction of MSCs within the different tissues has not been measured and therefore the exact number of population doublings that have occurred in each group during the expansion phase cannot be accurately quantified. This may be important as the chondrogenic capacity of porcine BM derived MSCs is known to diminish during prolonged passaging (Vacanti et al. 2005). It should also be noted that BM derived MSCs cultured in our laboratory under similar conditions to that described here have generated

cartilaginous tissues with comparable ECM content and mechanical properties to that measured here for FP derived MSCs (Thorpe et al. 2008).

Variability in the chondrogenic capability of BM MSCs has been documented, compared to reports of relatively consistent chondrogenesis of synovium derived MSCs (Sakaguchi et al. 2005). Therefore, before concluding that any one source of MSCs is superior for functional cartilage tissue engineering, it will be necessary to first optimise the isolation, expansion and differentiation conditions for each cell type. For example, it has been demonstrated that the initial monolayer culture conditions used to expand adipose derived stem cells determine their subsequent chondrogenic capacity in agarose hydrogel culture (Estes et al. 2008). Future studies will investigate how variables associated with MSCs expansion protocols such as plating density and growth factor supplementation (e.g. FGF-2, TGF-β) will influence the subsequent functional properties of engineered cartilaginous tissues, as these factors have been shown to regulate proliferation and subsequent chondrogenic differentiation of MSCs isolated from different sources (Colter et al. 2000; Erickson et al. 2002; Bianchi et al. 2003; Solchaga et al. 2005; Estes et al. 2006; Yanada et al. 2006; Marsano et al. 2007; Stewart et al. 2007; Khan et al. 2008). GAG and collagen accumulation in MSCs groups was greater in the core, while CC constructs had a higher concentration of extracellular matrix in the annular region. A similar inhomogeneous distribution of the extracellular matrix synthesised by MSCs embedded in agarose hydrogels was also observed in our previous studies (Thorpe et al. 2008; Buckley et al. 2010; Thorpe et al. 2010). The development of an inhomogeneous cartilaginous tissue following encapsulation of CC in agarose has also previously been reported (Kelly et al. 2006; Buckley et al. 2009; Kelly et al. 2009; Buckley et al. 2010). This distribution seems to be specific to CC in agarose gels and may be due to spatial gradients of nutrients, oxygen and growth factors. The reason for the specific matrix distributions has not yet been elucidated, but it does suggest that CC and MSCs respond differently to the gradients in regulating factors present within the developing tissue.

The mechanical properties of MSCs seeded hydrogels were lower than those of CC seeded hydrogels after 21 and 42 days in culture under identical conditions. It has previously been reported that immature MSCs may possess a more limited chondrogenic capacity compared to CC in an agarose hydrogel model (Mauck et al. 2006; Vinardell et al. 2009). The results of this study extend this finding to other sources of MSCs. Composition-function relations (Fig. 5.6) revealed that even in cases where total GAG and collagen

content was comparable or higher in FP hydrogels, the equilibrium modulus of the engineered tissue was higher for CC seeded constructs. This result is unexpected, as the equilibrium modulus is strongly dependant on the GAG and collagen content. Differences in the basic structure and properties of proteoglycans synthesised by MSCs and CC may partially explain these results, although it has been suggested that the BM MSCs-produce aggrecan with a phenotype more characteristic of young tissue than chondrocyte-produced aggrecan (Kopesky et al. 2010). The types of proteoglycan synthesised by engineered tissues from different cell sources and their ability to assemble, aggregate and form a stable tissue, may also play a key role (Connelly et al. 2008; Babalola and Bonassar 2010).

The spatial composition and organization of engineered cartilage tissues will also influence the apparent mechanical properties of the construct (Kelly and Prendergast 2004). Mechanical tests such as that utilized in this study to quantify construct properties make the assumption of tissue homogeneity. The spatial distribution of GAG within CC seeded constructs is typically more homogenous than MSCs groups, where the majority of GAG is accumulated within the construct core. Such an inhomogeneous distribution of matrix components will lower the apparent equilibrium modulus of the tissue compared to a more homogenous construct, even if the total matrix accumulation is identical. This spatial distribution of matrix would also appear to influence the correlation between the dynamic modulus of the engineered tissue and its composition. The relationship between the total GAG content and the dynamic modulus of CC and MSCs seeded constructs is quite similar, despite the spatial differences in tissue composition. However the collagen content of an engineered cartilaginous construct will also have a strong bearing on the dynamic properties of a tissue. For example, comparing the relationship between the dynamic modulus and the biochemical content in CC and FP MSCs constructs, its appears that greater amounts of collagen are required in FP MSCs constructs to achieve comparable values of dynamic modulus to CC constructs, which again may be due in part to the greater heterogeneity of the tissues engineered using MSCs.

It has been demonstrated that bovine CC in agarose hydrogels produce a more mechanically functional tissue when TGF-β is withdrawn from the media after two weeks of culture (Byers et al. 2008), while we have demonstrated that porcine FP MSCs respond more favourably to continued growth factor supplementation (Buckley et al. 2009). In this study, all cell types received continued TGF-β3 supplementation, so it is possible that the results of this study may be underestimating the superior functional cartilage tissue

forming ability of CC compared to MSCs. Clearly more optimal expansion and differentiation conditions exist for all cell types. While not reported in this study, we have observed that porcine subcutaneous fat derived MSCs undergo poor chondrogenesis following encapsulation in agarose hydrogels, with significant cell death occurring. Other authors have also observed decreases in cell viability following encapsulation of adipose derived MSCs in agarose hydrogels (Estes et al. 2008) and poorer chondrogenesis of adipose derived MSCs in agarose hydrogels compared to BM MSCs (Kisiday et al. 2008). A number of other studies also suggest a poorer chondrogenic capacity for adipose derived MSCs compared to other sources (Winter et al. 2003; Awad et al. 2004; Huang et al. 2005; Im et al. 2005; Afizah et al. 2007; Koga et al. 2008; Peng et al. 2008; Vidal et al. 2008; Danisovic et al. 2009; Hildner et al. 2009; Diekman et al. 2010). Future studies will investigate whether the mixed population of cells from the synovial layer and adipose tissue of FP biopsies has a positive effect on chondrogenesis, or whether they should be used in isolation for cartilage tissue engineering.

The results of this study demonstrate that differences in the chondrogenic capacity of different MSCs sources in pellet culture reported here and in previous studies translate into differences in the functional properties of cartilage engineered using these different cell populations. Given its anatomical location, a large number of MSCs can be obtained from a FP biopsy during arthroscopy for tissue engineering therapies. There have been reports stating that removing the entire FP during total knee replacement procedures can produce postoperative pain for the patient (Meneghini et al. 2007). On the other hand, removal of the FP leads to significant improvement in symptoms and function after the surgery in cases of impingement (Ogilvie-Harris and Giddens 1994). In general, there is little clinical evidence to suggest that taking biopsies of FP could be seriously detrimental to the patient. Therefore harvesting a biopsy of FP tissue has a lower degree of invasiveness compared to BM aspiration, and causes minimal complications during joint arthroscopy, especially if the goal is to treat a chondral or osteochondral defect in the joint. In conclusion, functional cartilaginous tissues can be engineered using MSCs isolated from different sources. The development of these tissues is different to those generated using CC, suggesting that MSCs and CC respond differently to the regulatory factors present within developing cartilaginous constructs. Using the same expansion and differentiation conditions, MSCs isolated from the FP would appear to possess a potential to generate functional cartilaginous tissues at least comparable to BM MSCs, although CC still represent a

standard to which all MSCs-based cartilage tissue engineering strategies should be compared.

6 A comparison of the functionality and in vivo phenotypic stability of cartilaginous tissues engineered from different stem cells sources

6.1 Introduction

The long term outcome in cartilage repair studies *in vivo* is often disappointing (Jakobsen et al. 2005), which may be at least partially attributable to the known limitations of current treatment options. Cell based therapies, such as ACI, involve the creation of an additional cartilage injury in order to biopsy tissue and dedifferentiation of CC is known to occur during monolayer expansion (Benya et al. 1978; Barbero et al. 2004; Diaz-Romero et al. 2005). In order to obtain an optimum cartilage repair tissue, the chondrocyte like phenotype needs to be adopted by the transplanted cells and the long term stability has to be warranted (Pelttari et al. 2006). Joint derived stem cells, including infrapatellar FP and SM derived stem cells are a promising alternative cell source for cartilage repair therapies that may overcome many of the problems associated with the use of primary CC (De Bari et al. 2001; English et al. 2007; Jones and McGonagle 2008; Khan et al. 2008; Pei et al. 2008; Hermida-Gomez et al. 2011). When compared to MSCs derived from other tissues, joint tissue derived stem cells have demonstrated superior capacity for chondrogenesis (Sakaguchi et al. 2005; Shirasawa et al. 2006). This has led to increased interest in the regenerative medicine industry to develop a proprietary stem cell platform based on SM stem cells for the treatment of damaged and diseased cartilage, bone, meniscus, tendon and muscle tissue (Pascual 2011).

A central concern associated with the use of MSCs is their ability to form stable cartilage resistant to hypertrophy or fibrous dedifferentiation. To test the phenotypic stability and the capacity to form stable cartilage an ectopic model is commonly used, were the cells alone, or seeded into scaffolds, are implanted under the skin or into the muscle of transgenic mice (Pelttari et al. 2006; Cui et al. 2007). CC are known to form stable ectopic cartilage (Dell'Accio et al. 2001), however cartilaginous tissues derived from SM stem cells have been shown to undergo fibrous dedifferentiation or complete degeneration when

implanted in an ectopic model (De Bari et al. 2004; Park et al. 2005; Marsano et al. 2007; Dickhut et al. 2009). BM derived MSCs are known to expand rapidly in culture while retaining their capacity to differentiate making them ideally suitable for a wide spectrum of clinical applications for repair of damaged or defective tissue (Caplan 1991; Pittenger et al. 1999), but previous studies have observed that BM MSCs express type X collagen, a marker of hypertrophy. This fact might therefore threaten the future of BM MSCs as a candidate for replacing culture-expanded CC in cell-based repair of cartilage lesions (Pelttari et al. 2006; Karlsson et al. 2007).

The hydrogel or scaffold in which MSCs are encapsulated may play a key role in determining their *in vivo* phenotypic stability. For example, adipose derived stem cells have been shown to undergo hypertrophy and calcification associated with vascularization in spheroids after ectopic transplantation (Hennig et al. 2007), however when encapsulated in Matrigel™, a gelatinous protein mixture, chondrogenesis and suppression of the calcification was observed, making them an attractive scaffold for MSC-based tissue engineering approaches in cartilage repair (Dickhut et al. 2008). *In vivo*, agarose has been shown to stabilize the chondrocyte phenotype (Selmi et al. 2008) and to prevent vascularization leading to stable cartilage (Emans et al. 2010). Furthermore, when joint derived stem cells were encapsulated in agarose, they were shown to generate cartilaginous tissues the mechanical functionality of which improved with time in culture (Buckley et al. 2010; Buckley et al. 2010; Vinardell et al. 2010; Sampat et al. 2011).

The objective of this study was to compare the *in vitro* functionality and *in vivo* phenotypic stability of cartilaginous tissues engineered using BM derived MSCs, with joint derived stem cells. Our initial hypothesis was that joint tissue derived stem cells seeded in agarose hydrogels would form a stable cartilaginous tissue *in vivo*. In the first phase of the study, we compared the *in vitro* functionality of cartilaginous tissues engineered using different joint derived stem cells to BM MSCs. The influence of exposing these engineered tissues to factors known to promote hypertrophy *in vitro* was also assessed. In the second phase of the study, the *in vivo* stability of these engineered cartilaginous tissues was compared in a subcutaneous nude mouse model.

6.2 Materials and methods

6.2.1 Cell isolation and expansion

CC, BM MSCs were harvested from immature (4 month old) porcine tissue, isolated and expanded as described previously (Vinardell et al. 2010). SM and FP tissue was harvested from the femoropatellar joint, sliced and rinsed with PBS (Sigma–Aldrich, Dublin, Ireland) containing penicillin/streptomycin (200 U/ml) (GIBCO, Biosciences, Dublin, Ireland) and amphotericin B (2.5 μg/ml) (Sigma–Aldrich, Dublin, Ireland). SM and FP pieces were incubated with DMEM GlutaMAX (GIBCO, Biosciences, Dublin, Ireland) containing collagenase type II (315 U/mg) (Worthington, Langanbach Services, Ireland) for 3-4 hours under constant rotation at 37°C. The resulting cell suspension was then filtered through a 40μm pore-size cell sieve (Falcon Ltd, Sarstedt, Ireland) and the filtrate centrifuged and rinsed with PBS twice.

CC and stem cells were seeded at a density of 5×10^3 cells/cm^2 in 175 cm^2 T flasks and expanded to passage two (11 population doublings for CC, 14 for BM MSCs and 16 population doublings for joint derived stem cells) in a humidified atmosphere at 37°C and 5 % CO_2. Viable cells were counted using a hemacytometer and 0.4 % trypan blue staining. Isolated cells from different tissues were maintained in DMEM GlutaMAX supplemented with 10 % v/v FBS (GIBCO, Biosciences, Dublin, Ireland) and 100 U/ml penicillin/streptomycin during the expansion phase with 5ng/mL of human FGF-2 (Prospec, Israel). At the first passage colonies were stained with crystal violet (Sigma-Aldrich, Arklow, Ireland) and counted to obtain the colony-forming cell fraction.

6.2.2 Agarose gel constructs formation

Following isolation and expansion stem cells were suspended in DMEM and mixed with 4 % agarose (Type VII, Sigma-Aldrich, Arklow, Ireland) in PBS at a 1:1 ratio. This produced a 2 % agarose gel containing 20 x 10^6 cells/mL. The gel was cast in a stainless steel mould to produce a 3 mm thick agarose sheet, from which cylindrical constructs (Ø5×3 mm) were created using a biopsy punch. The gels were cultured in a chemically defined chondrogenic medium consisting of DMEM GlutaMAX supplemented with 100 U/ml penicillin/streptomycin, 100 μg/ml sodium pyruvate, 40 μg/ml L-proline, 50 μg/ml L-ascorbic acid-2-phosphate, 4.7 μg/ml linoleic acid, 1.5 mg/ml BSA, 1×insulin–transferrin–selenium, 100 nM dexamethasone (all from Sigma-Aldrich, Ireland) and 10ng/ml of

human TGF-β3 (Prospec, Israel). On day 21 an additional group was exposed to hypertrophic medium for 28 days, by changing the following parameters: TGF-β3 withdrawal, addition of 1 nM L-thyroxine (Sigma-Aldrich, Ireland) and reduction of the level of dexamethasone to 1 nM. Medium was exchanged every 3 or 4 days.

6.2.3 In vivo subcutaneous transplantation

After 21 days of *in vitro* culture under chondrogenic conditions, stem cell-seeded hydrogels were transplanted subcutaneously into the back of transgenic mice and CC seeded agarose hydrogels were also implanted as a control group. Nude mice (Balb/c; Harlan, UK) were utilized in this study. Animals were weighed and anesthetized with an intraperitoneal injection of 10 mg/kg xylazine (Chanazine® 2 %, Chanelle, UK) and 100 mg/kg ketamine (Narketan®, Vetoquinol, UK). Under sterile conditions, two skin incisions (1.5 cm long) were made along the central line of the spine, one at the shoulders and one at the hips. A subcutaneous dissection was made, and a subcutaneous pocket was created on both sides of the wound. Two to three constructs were inserted in each subcutaneous pocket. Incisions were closed with 4-0 Vicryl plus (Ethicon, Johnson&Johnson, Ireland) sutures and tissue glue (Vetloc xcel, Germany) was applied. Animals were allowed to recover in a warm environment. Mice were euthanized 4 weeks after the surgery by CO_2 inhalation and the constructs were analyzed for gross appearance, histological, immunohistochemical and biochemical properties. The animal protocol was reviewed and approved by the ethics committee of Trinity College Dublin.

6.2.4 Mechanical, biochemical and histological analysis

Agarose constructs were mechanically evaluated using a standard materials testing machine (Zwick Roell Z005, Herefordshire, UK) with a 5 N load cell, as previously described (Buckley et al. 2009; Vinardell et al. 2010). The equilibrium Young's modulus was determined from the equilibrium force following unconfined compression testing to 10 % strain. The dynamic modulus was determined by applying an additional 1 % dynamic strain at 1Hz. Construct dimensions were measured using a toolmaker's microscope (Mitutoyo UK Ltd., Andover, UK). Biochemistry was performed to determine DNA, collagen and sulphated GAG content. DNA content was quantified using the Hoechst Bisbenzimide 33258 dye assay. The proteoglycan content was determined using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Northern Ireland), and

total collagen content was determined by measuring the hydroxyproline content (Kafienah and Sims 2004; Ignat'eva et al. 2007). Alkaline phosphatase activity was analysed in the media, determined using SensoLyte® pNPP Alkaline Phosphatase Assay Colorimetric kit (AnaSpec, Inc., San Jose, CA, USA) and the absorbance was measured at 405 nm. For histological evaluation gels were fixed in 4 % paraformaldehyde and embedded in paraffin, cut into 5 μm thick sections, and stained with 1 % alcian blue 8 GX (Sigma–Aldrich, Ireland) in 0.1 M HCl to assess GAG content and alizarin red to detect calcium deposition. Type I, II and X collagen content were evaluated with a standard immunohistochemical technique as previously described (Vinardell et al. 2010). Positive and negative controls were included in the immunohistochemistry staining protocol for each batch.

6.2.5 Micro-computed tomography

Micro-computed tomography (μCT) scans were carried out on the *in vivo* specimens to quantify mineralization within the engineered constructs. A Scanco Medical 40 μCT system (Scanco Medical, Bassersdorf, Switzerland) was used for the evaluation with 70 kVp X-ray source and 114 μA.

6.2.6 Statistical analysis

Five to nine samples were analysed per group at each time point, with three to seven samples used for biochemical/mechanical analyses and two samples were used for histology. Differences in mechanical and biochemical properties with cell type and/or time-in-culture were determined by analysis of variance with time and cell source as independent factors, followed by Tukey's post-hoc test (95 % confidence interval). Statistics were preformed with MINITAB 15.1 (Minitab, Coventry, UK) and GraphPadPrism 4 software package (San Diego, USA). Biochemical and mechanical properties of constructs are expressed in the form of mean ± standard error of the mean (SEM). Statistical significance was declared at $p \leq 0.05$.

6.3.1 In vitro development of engineered tissues maintained in either chondrogenic or hypertrophic media

The mechanical properties of the all stem cell seeded hydrogels increased significantly with time in culture, with SM stem cells seeded constructs exhibiting the highest equilibrium (144.9 kPa ± 2.2) and dynamic modulus (668.4 kPa ± 1.0) following 49 days of *in vitro* culture (Fig. 6.1).

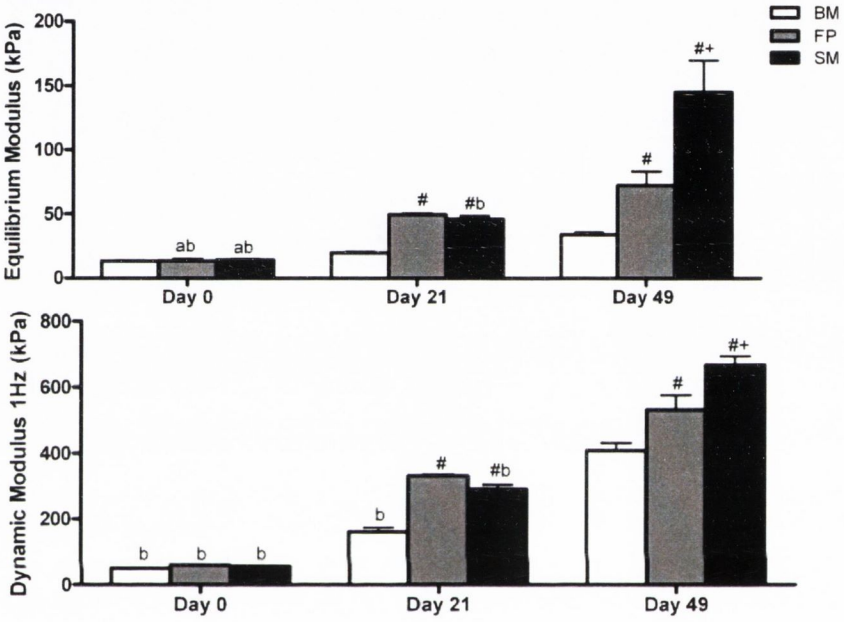


Figure 6.1: Equilibrium and 1 Hz dynamic modulus of stem cells seeded agarose gels cultured *in vitro* with chondrogenic media at days 0, 21 and 49. Data represent the mean ± SEM. Significance vs. (a) vs. day 21; (b) vs. day 49; #vs. BM, +vs. FP ($p < 0.05$).

GAG accumulation was significantly higher ($p < 0.0001$) in SM constructs (2.8 %w/w ± 0.1) when compared to either FP (1.49 %w/w ± 0.3) or BM MSCs (0.65 %w/w ± 0.1) seeded constructs. Collagen accumulation was also highest in SM constructs, reaching 1.77 %w/w (± 0.1) compared to FP 0.93 %w/w (± 0.1) or BM MSCs 1.5 %w/w (± 0.1) (Fig. 6.2). The addition of hypertrophic factors at day 21 was observed to significantly reduce GAG accumulation in joint derived seeded constructs and significantly reduce collagen accumulation in all groups (Fig. 6.2).

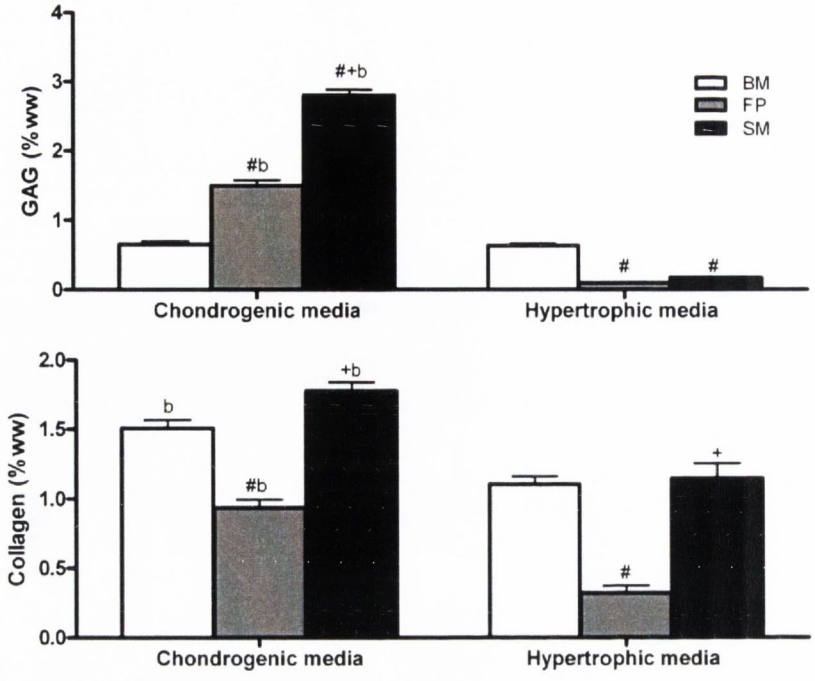
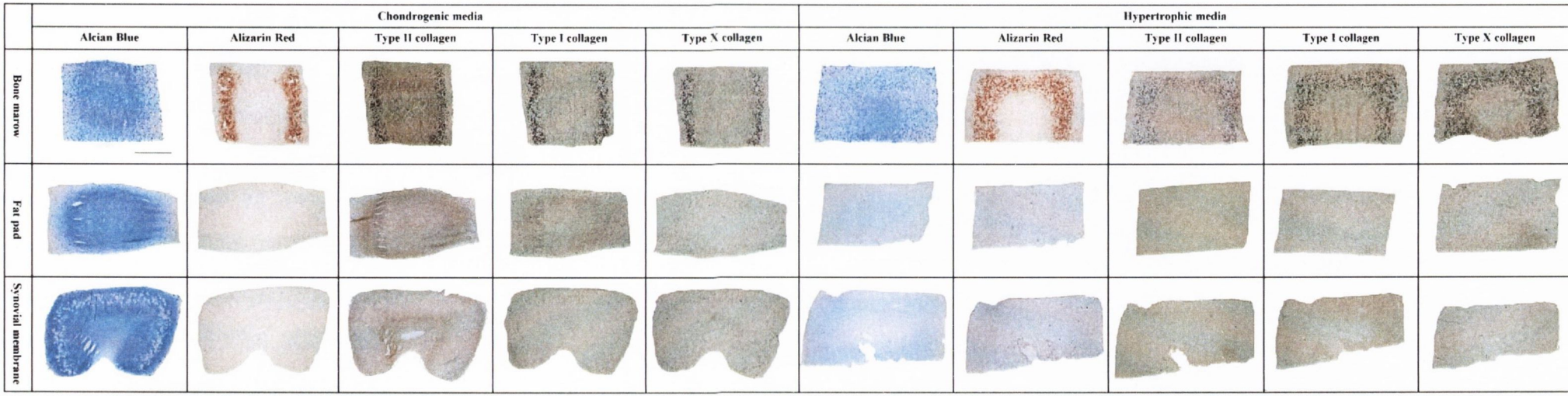


Figure 6.2: GAG and collagen content measured in agarose gels seeded with stem cells cultured with chondrogenic media for 49 days or with hypertrophic media (chondrogenic media for 21 days followed by 28 days of hypertrophic media). Data is expressed in percentages of wet weight and represent the mean $\pm$ SEM. Significance: b vs. hypertrophic media; #vs. BM, +vs. FP ($p < 0.05$).

Histological analysis revealed an intense staining for GAG in all stem cell seeded constructs continuously maintained in chondrogenic media. Alcian blue staining was more intense in the core of BM MSCs and FP constructs, with more homogenous staining observed in SM constructs. Some cell clustering was also observed in SM constructs. Calcium deposition was detected towards the periphery of the BM group as evidenced by the positive staining for alizarin red. Immunohistochemical staining also indicated the presence of type I and X collagen staining in the BM hydrogels, which appeared to co-localise to regions of alizarin red staining (Fig. 6.3).

Figure 6.3: Agarose gels seeded with stem cells from BM, FP and SM cultured with chondrogenic medium for 49 days or with hypertrophic media (chondrogenic media for 21 days followed by 28 days of hypertrophic media), stained for alcian blue (stains GAG), alizarin red (stains calcium depositions), type II collagen, type I collagen and type X collagen. Scale bar: 1 mm.



With the addition of hypertrophic factors to the media, more intense staining for alizarin red, type I collagen and type X collagen was observed in BM seeded gels (Fig. 6.3). Only weak staining for type X collagen was observed in the SM and FP constructs. Alcian blue staining was weaker in all groups when supplemented with hypertrophic factors, particularly the SM and FP constructs. Analysis of the media from all groups revealed negligible levels of alkaline phosphatase activity except for the BM MSCs group in both chondrogenic and hypertrophic media (data not shown).

6.3.2 In vivo phenotypic stability of cartilaginous tissues engineered using different sources of stem cells

At the time of implantation (21 days of *in vitro* culture), all constructs contained GAG and collagen, with GAG accumulation highest in SM constructs at 1.3 %w/w (± 0.2) (Fig. 6.4); post-implantation (21 days of chondrogenic medium followed by 28 days *in vivo*) GAG content significantly decreased for the different stem cell sources ($p < 0.005$), while no significant decrease was observed in the chondrocyte seeded constructs (Fig. 6.4). Total collagen content did not significantly change for any group during the *in vivo* cultivation phase (Fig. 6.4).

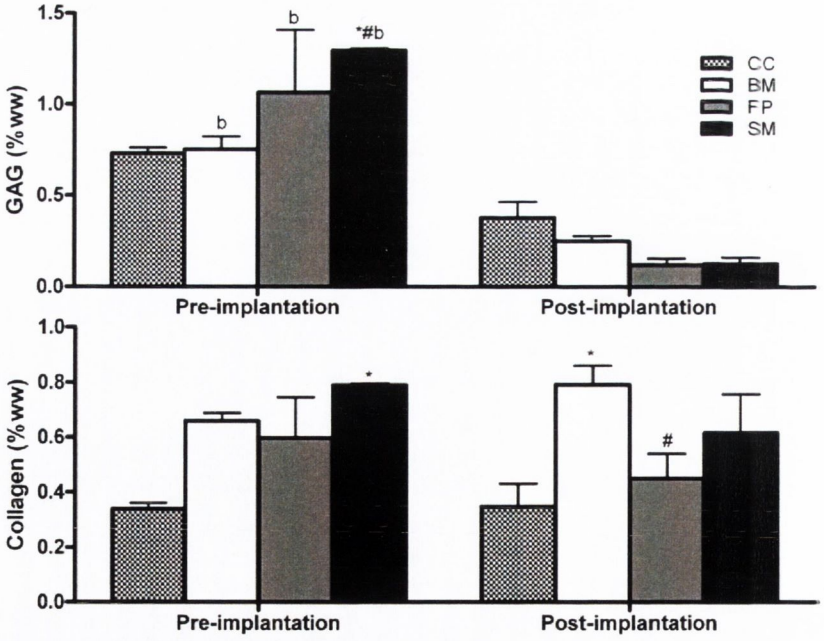


Figure 6.4: GAG and collagen content measured in the CC and stem cells gels at the *in vitro* pre-implantation time point (21 days of chondrogenic media) and post-implantation

(21 days of chondrogenic media followed by 28 days of *in vivo* culture). Data is expressed in percentages of wet weight and represent the mean ± SEM. Significance: b vs. post-implantation; #vs. BM MSCs, *vs. CC ($p < 0.05$).

Prior to implantation, BM MSCs constructs stained positive for the hypertrophic marker type X collagen, particularly towards the periphery of the construct with evidence of early calcium production also observed in these regions of the construct. None of the other cell types (FP, SM neither CC) stained positive for calcium deposition and only stained weakly for type X collagen (Fig. 6.5). Post-implantation type X collagen and alizarin red was produced throughout the whole construct in the BM group, whether the other cells showed an increase in type X collagen staining but inexistent calcium deposits. Type II and I collagen were present in the different sources of stem cells post-implantation and CC stained for type II but no staining was observed for type I (Fig. 6.5).

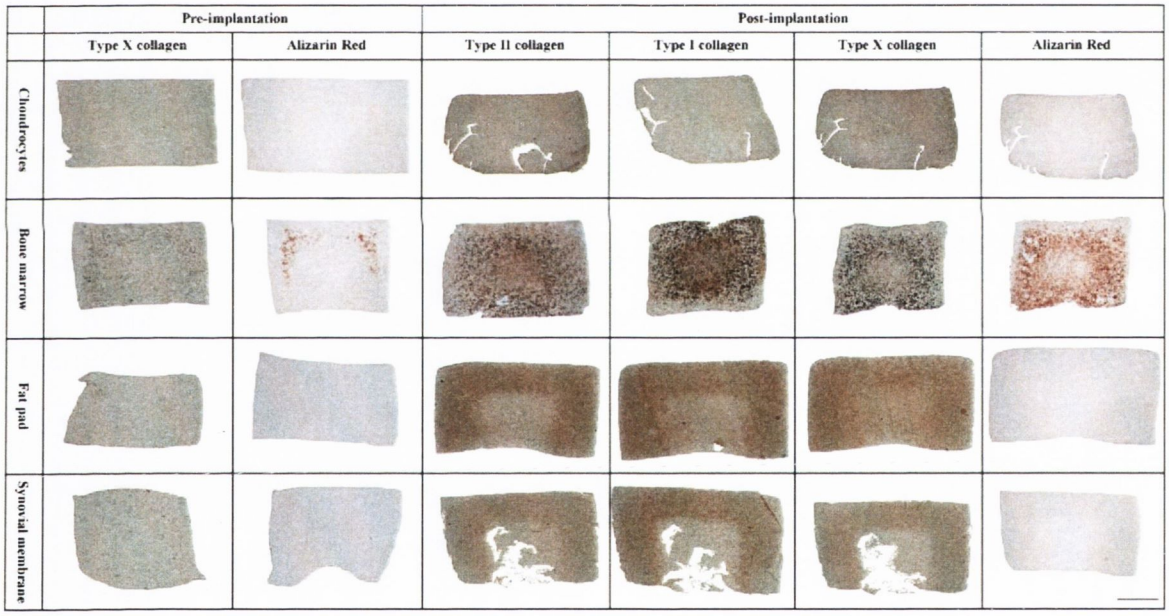


Figure 6.5: Agarose gels seeded with either CC or BM, FP and SM stem cells stained for type X collagen and alizarin red (stains calcium deposition) at the *in vitro* pre-implantation time point (21 days of chondrogenic media). Post-implantation gels (21 days of chondrogenic media followed by 28 days of *in vivo* culture) stained for type II collagen, type I collagen, type X collagen and alizarin red (stains calcium deposition). Scale bar: 1 mm.

As evident by the spatially distinct regions of BM MSCs seeded constructs staining positive for either alcian blue or alizarin red, clearly a chondrogenic or an

osteogenic/endochondral phenotype is being supported depending on the different regions of the engineered tissue. In order to further investigate this, the temporal and spatial patterns of matrix staining were compared during chondrogenic differentiation (Fig. 6.6). When BM MSCs gels were cultured in chondrogenic or hypertrophic media, they showed an intense deposition and/or core effect of GAG as observed by the strong alcian blue staining and matrix calcification deposits as detected with the alizarin red. The calcium deposits appeared from the early time points, intensified with time in culture and became more evident after implantation. When the alcian blue and the alizarin red staining were superposed in a composite image (right column), regions of calcium deposits appeared to correlate with regions that stained weakly for alcian blue. After *in vivo* implantation more homogeneous staining was observed (Fig. 6.6).

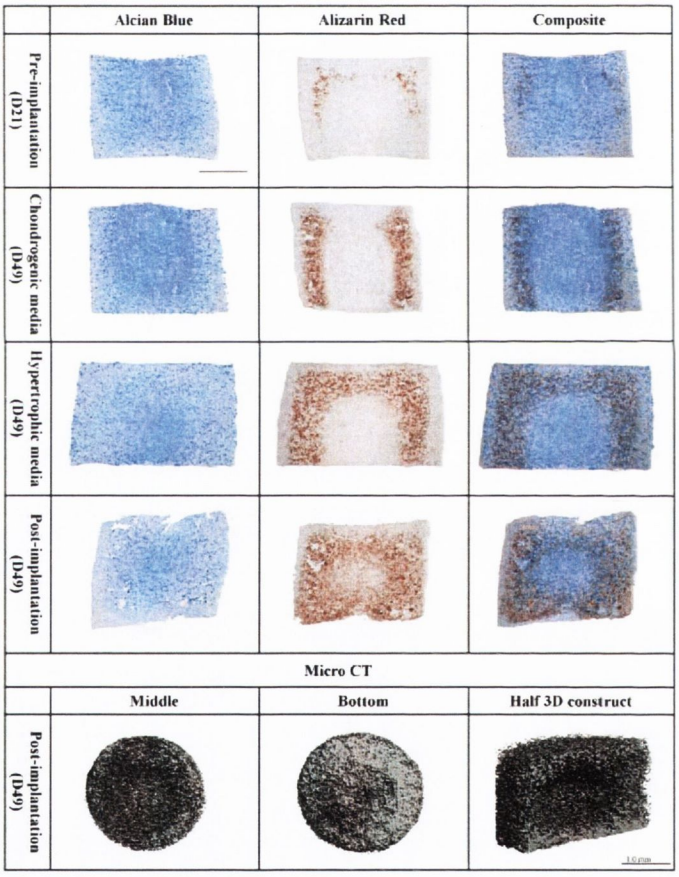


Figure 6.6: Agarose gels seeded with BM MSCs stained for alcian blue (stains glycosaminoglycans), alizarin red (stains calcium depositions) and a composite image of both. Different time points of the study are shown in the figure: pre-implantation (21 days of chondrogenic media supplementation *in vitro*), supplemented with chondrogenic media for 49 days *in vitro*, supplemented with hypertrophic media *in vitro* (chondrogenic media for 21 days followed by 28 days of hypertrophic media) and post-implantation (21 days of

chondrogenic media followed by 28 days of *in vivo* culture). Bottom of the image: μCT scans of the middle (left image), bottom (middle image) and half section (right image) of BM MSCs seeded hydrogel harvested post-implantation. Scale bar: 1mm.

μCT images in figure 6 confirmed alizarin red staining, with more mineral deposition detected around the periphery. 9 % of the volumetric composition of the BM gels was mineral whether all the other cell sources did not show any mineral deposition. The cross-sectional image of the bottom of the gel showed an increase in the density in comparison with the middle cross section of the gel. This mineral density was correlated with the calcium deposition detected with the alizarin red staining (Fig. 6.6).

6.4 Discussion

The aim of this study was to compare the *in vitro* chondrogenic potential of SM, FP and BM stem cells following encapsulation in agarose hydrogels *in vitro* and their subsequent *in vivo* phenotypic stability following subcutaneous implantation in a nude mouse. We demonstrated that SM derived stem cells encapsulated in these hydrogels accumulated GAG levels approaching that of native articular cartilage, although the mechanical properties and collagen content did not reach the same relative levels. Potentially of more concern was the observation that the *in vitro* chondrocyte like phenotype generated in all stem cell was transient and lost once implanted in the back of nude mice. FP and SM stem cells appeared to undergo fibrous dedifferentiation or resorption with a dramatic loss in GAG content, while BM MSCs followed a more endochondral pathway as evident by type X collagen staining, calcium deposition and type I collagen production.

In agreement with previous studies, FP derived stem cells were able to generate cartilaginous constructs at least as mechanical functional as BM MSCs (Vinardell et al. 2010). Furthermore, here it has been demonstrated that SM stem cells generated the most mechanically functional tissue. Comparable or even greater levels of matrix accumulation have been observed in similar studies using immature bovine SM derived stem cells (Sampat et al. 2011). In their study removal of TGF-β from the culture media after 21 days was found to enhance subsequent chondrogenesis. After 56 days in culture both the equilibrium modulus and GAG content approached native cartilage values, although the collagen content was still low. We have previously shown that FP agarose gels needed a continual TGF-β3 supplementation in order to maintain high levels of ECM synthesis

(Buckley et al. 2010). Overall, TGF-β3 withdrawal seems to be an interesting protocol to explore as it has been shown that continuous exposure to TGF-β3 is not needed and that the addition in the first week is the most critical to the chondrogenesis of MSCs (Buxton et al. 2011). It remains unclear how such *in vitro* media supplementation conditions will impact the *in vivo* phenotypic stability of the engineered tissue. It may be that co-encapsulation of growth factor and/or hormone releasing microspheres into the hydrogels may be required to suppress hypertrophy and help maintain a chondrogenic phenotype (Bian et al. 2011).

BM MSCs and joint tissue derived stem cells appeared to initiate along unique differentiation pathways in response to TGF-β3 stimulation as observed in the histology sections for alcian blue and alizarin red. BM MSCs have been shown to be phenotypically different to other stem cell sources, producing typical features for hypertrophic cartilage and catabolic enzymes (Winter et al. 2003; Hennig et al. 2007; Segawa et al. 2009). In our study mineral deposits were observed near the periphery of BM constructs at 3 week time point, so it could be tempting to speculate that aspects of bone development are being recapitulated in this *in vitro* culture system. Given that these mineral deposits do not appear in the more chondrogenic regions of the constructs it suggests that a sub-population of stem cells located towards the periphery of the hydrogel are proceeding along an intra-membranous ossification pathway (Gray 1974), with the environment in this region of the construct supporting a more osteogenic phenotype. For example, the oxygen tension in this region would be higher than the low oxygen region in the core. Furthermore, the stiffing chondrogenic core may provide more appropriate substrate stiffness for osteogenic differentiation, as stiffer substrates have been shown to support a more osteogenic phenotype (Engler et al. 2006; Park et al. 2011). Alternatively, or perhaps in conjunction, stem cells that have undergone chondrogenic differentiation in the core of the construct are releasing factors that support mineralisation of the periphery, mimicking aspects of bone development, such as the intra-cartilaginous ossification, ie: formation of a bone collar around cartilaginous template (Gray 1974). This characteristic pattern was observed only for BM MSCs. Joint derived stem cells showed no sign of mineral deposition, similar to what it has been observed in CC seeded hydrogels.

When subjected to hypertrophic media, joint derived stem cells reacted with a loss of chondrogenic phenotype and a loss of proteoglycan. In comparison, BM MSCs produced markers associated with accelerated hypertrophy and bone production, as evident by

increased alizarin red, type I and X collagen staining and increased ALP activity (data not shown). Previous studies have demonstrated the tendency of BM MSCs to undergo hypertrophy with typical cytomorphologic changes accompanied by an increase in ALP activity, matrix mineralization (Mueller and Tuan 2008; Scotti et al. 2010; Farrell et al. 2011). Those studies supported the idea that BM MSCs may undergo endochondral ossification *in vivo* after chondrogenic induction, suggesting that BM MSCs seems already predetermined towards adapting this route regardless of the fact that they are encapsulated in a hydrogel known to support chondrogenesis.

In this study the addition of hypertrophic media was also predictive of the phenotypic changes observed following subcutaneous implantation, Joint derived stem cells failed to from stable ectopic cartilage *in vivo*. Previous studies have shown that SM stem cells chondrogenically primed *in vitro* fail to form stable cartilage in an ectopic environment (De Bari et al. 2004; Park et al. 2005; Marsano et al. 2007; Dickhut et al. 2009). CC maintained a stable ectopic phenotype as previously described by other authors (Dell'Accio et al. 2001). We initially hypothesized that the encapsulation of stem cells within agarose might provide them with the necessary environment to support a more stable chondrogenic phenotype, but this approach was not successful.

A number of different approaches have been proposed in the literature to promote a more stable chondrogenic phenotype *in vivo*. For example, co-culture of BM MSCs with CC *in vivo*, as they may create a chondrogenic niche to direct chondrogenesis of MSCs (Liu et al. 2010), or the co-delivery of parathyroid hormone related protein with TGF-β3 in alginate microspheres (Bian et al. 2011). Alternatively a longer *in vitro* culture period might support a more stable cartilaginous phenotype (Dickhut et al. 2008; Liu et al. 2008), but an extension of seven weeks of the *in vitro* induction did not prevent calcification or vascular invasion of BM MSCs (Pelttari et al. 2006). Encapsulation in a appropriate biomaterial might also suppress endochondral ossification (Dickhut et al. 2008). Of course, it may be that this differentiation pathway may not proceed in an orthotopic environment. Physiological joint loading and the joint biochemical environment may suppress the endochondral phenotype and support a more stable chondrogenic phenotype (O'Driscoll et al. 1988; Wakitani et al. 1994; Carter et al. 1998; Salisbury Palomares et al. 2010).

In conclusion, while the results of this study demonstrate that SM derived stem cells generate a more mechanically functional tissue *in vitro*, cartilaginous constructs generated

by seeding different sources of MSCs into agarose hydrogels all failed to form stable cartilaginous tissue *in vivo*. Future studies are needed to assess the ability of joint derived stem cells seeded in agarose gels to regenerate articular cartilage defects *in vivo*.

7 Hydrostatic pressure acts to stabilise a chondrogenic phenotype in joint tissue derived stem cells

7.1 Introduction

Articular cartilage lesions do not heal adequately leading to intense effort towards developing new therapies to repair and regenerate articular cartilage (Buckwalter 1998; Elder and Athanasiou 2009). The variety of treatment options available implies that there is no single, ideal, reliable and predictable therapy (Hildner et al. 2011). The use of MSCs as part of a cell based therapy for cartilage repair is very appealing. MSCs have a high potential for proliferation and differentiation (Pittenger et al. 1999) and overcome many of the drawbacks associated with the use of autologous CC including the creation of a new defect in the articular surface. Stem cells isolated from synovial joint tissues such as SM (De Bari et al. 2001; Pei et al. 2008; Pei et al. 2008) and infrapatellar FP (Dragoo et al. 2003; Wickham et al. 2003; English et al. 2007; Khan et al. 2007; Khan et al. 2008) are a particularly promising alternative to CC as they are phenotypically similar and accessible during arthroscopy (Segawa et al. 2009). This has led to increased interest in using joint tissue derived stem cells for engineering functional cartilaginous grafts (Buckley et al. 2010; Buckley et al. 2010; Vinardell et al. 2010). To fully realise their potential in this regard requires a more thorough understanding of how biochemical and biophysical factors will influence their phenotypic stability and biosynthetic activities during *in vitro* cultivation and following implantation into the joint environment.

The pellet culture model is commonly used to explore chondrogenic differentiation of MSCs (Johnstone et al. 1998). In this system, cell-cell interactions within the MSCs condensation coupled with the extrinsic signal provided by the addition of TGF-β1 has been shown to induce chondrogenesis of MSCs. Chondrogenesis was found to be dependent on the concentration of TGF-β1 within the culture media, with more robust differentiation observed at higher concentrations of TGF-β1 (Johnstone et al. 1998). Chondrogenesis of SM derived stem cells and FP derived stem cells has also been demonstrated using the pellet culture system (Mochizuki et al. 2006; Marsano et al. 2007; Vinardell et al. 2010). Additional growth factors have been used in combination with TGF-

β stimulation with synergistic effects observed (Lee et al. 2008; Pei et al. 2008). While cellular condensation coupled with endogenous TGF-β stimulation have been shown to induce chondrogenic differentiation *in vitro*, other environmental signals, including biophysical stumuli, will play a key role in determining stem cell fate.

Biophysical stimuli such as tension, compression, fluid flow and HP are thought to regulate chondrogenesis of stem cells (Stoddart et al. 2009; Kelly and Jacobs 2010). Finite element models have been used to support the hypothesis that mechanical loading can regulate tissue differentiation and that HP can promote cartilage formation and potentially suppress endochondral ossification (Carter et al. 1998; Loboa et al. 2001). *In vitro* studies have demonstrated that physiological frequencies and magnitudes of HP (3 to 10 MPa at frequencies of 1 Hz) increase CC matrix synthesis (Parkkinen et al. 1993; Toyoda et al. 2003; Toyoda et al. 2003; Elder et al. 2006; Elder and Athanasiou 2008; Elder and Athanasiou 2009). In either the absence or presence of TGF, cyclic HP also seems to enhance chondrogenesis of BM MSCs at the gene and/or protein level (Angele et al. 2003; Miyanishi et al. 2006; Finger et al. 2007; Wagner et al. 2008; Meyer et al. 2011). HP has also been shown to enhance chondrogenesis of SM stem cells (Sakao et al. 2008; Wu et al. 2008), however the interaction between HP and members of the TGF-β family of growth factors in regulating the initiation and maintenance of a chondrogenic phenotype in joint tissue derived stem cells remains poorly understood.

MSCs offer tremendous potential for the treatment of damaged and diseased articular cartilage. The success of new therapies utilizing joint tissue derived stem cells will require a fundamental understanding of how local environmental factors regulate their phenotype and subsequent synthetic activities. While both TGF-β stimulation and HP have been identified as key determinants of stem cell differentiation, little is known about how these signals interact to regulate their fate. Given that both stimuli are key components of the regenerative environment, the goal of this study was to investigate the interaction between HP and TGF-β3 in regulating the induction and maintenance of a chondrogenic phenotype in joint tissue derived stem cells. To this end this study explored the combined effects of HP and different concentrations of TGF-β3 (0, 1 and 10 ng/ml) on chondrogenic gene expression and matrix synthesis for SM stem cells and FP stem cells and compared this response to CC isolated from articular cartilage from the same joint.

7.2 Materials and methods

7.2.1 Cell isolation and expansion

CC and joint tissue derived stem cells were aseptically harvested from four month old pigs (two donors). CC, FP and SM of the femoropatellar joint were used in this study. CC were isolated and expanded as described previously (Vinardell et al. 2010). SM and FP tissues were harvested, sliced and rinsed with PBS (Sigma–Aldrich, Dublin, Ireland) containing penicillin/streptomycin (200 U/ml) (GIBCO, Biosciences, Dublin, Ireland) and amphotericin B (2.5 μg/ml) (Sigma–Aldrich, Dublin, Ireland). SM and FP pieces were incubated with DMEM GlutaMAX (GIBCO, Biosciences, Dublin, Ireland) containing collagenase type II (315 U/mg) (Worthington, Langanbach Services, Ireland) for 3-4 hours under constant rotation at 37°C. The resulting cell suspension was then filtered through a 40 μm pore-size cell sieve (Falcon Ltd, Sarstedt, Ireland) and the filtrate centrifuged and rinsed with PBS twice. CC, SM stem cells and FP stem cells were seeded at a density of 5×10^3 cells/cm^2 in 175 cm^2 T flasks and expanded to passage one in a humidified atmosphere at 37°C and 5 % CO_2. Viable cells were counted using a hemacytometer and 0.4 % trypan blue staining. Isolated cells from different tissues were maintained in DMEM GlutaMAX supplemented with 10 % v/v FBS (GIBCO, Biosciences, Dublin, Ireland) and 100 U/ml penicillin/streptomycin during the expansion phase with 5 ng/ml of human FGF-2 (Prospec, Israel).

7.2.2 Pellet formation

250 000 cells were placed in 1.5 ml conical microtubes and centrifuged at 650 G for 5 minutes. The pellets were cultured in a chemically defined chondrogenic medium: DMEM GlutaMAX supplemented with 100 U/ml penicillin/streptomycin, 100 μg/ml sodium pyruvate, 40 μg/ml L-proline, 50 μg/ml L-ascorbic acid-2-phosphate, 4.7 μg/ml linoleic acid, 1.5 mg/ml BSA, 1× insulin–transferrin–selenium, 100 nM dexamethasone (all from Sigma-Aldrich, Ireland). The pellets were cultured with different concentrations of human TGF-β3 (Prospec, Israel) 0, 1 and 10 ng/ml. Medium was exchanged every 3 or 4 days with 1 ml samples of media taken for biochemical analysis.

7.2.3 Application of hydrostatic pressure

Pellets (10 or 12 per group) were sealed in sterile plastic bags with 4 ml of chondrogenic medium with different concentrations of TGF-β3. The pellets were assigned to receive either mechanical stimulation (HP group), or were left unloaded (Free swelling: FS group). The loaded group was exposed to 10 MPa of HP at a frequency of 1 Hz for 4 h per day, for 14 days, while the unloaded controls were kept in bags in an incubator immersed in water at 37°C during the loading period. After loading all pellets were returned to culture dishes. A stainless steel pressure chamber (Parr instruments company, Moline, IL, USA), connected to a piston (phd Inc., Irish Pneumatics, Kildare, Ireland), driven by an Instron 8801 materials testing machine was used to generate the required levels of HP. Further details are available elsewhere (Meyer et al. 2011).

7.2.4 Histology and immunohistochemistry

For histological evaluation pellets were fixed in 4 % paraformaldehyde and embedded in paraffin, cut into 5 μm thick sections, and stained with 1 % alcian blue 8 GX (Sigma–Aldrich, Ireland) in 0.1 M HCl to assess GAG content and picrosirius red to detect collagen. Type I, II and X collagen content were evaluated with a standard immunohistochemical technique; briefly, sections were treated with peroxidase (between steps slides were washed in PBS) followed by treatment with chondroitinase ABC (Sigma-Aldrich, Ireland) in a humidified environment at 37°C to enhance permeability of the extracellular matrix. After incubation with goat serum to block non-specific sites, the primary antibody (mouse monoclonal, Abcam, Cambridge, UK) was applied on the sections for 1 hour at room temperature. Next, the secondary antibody (Anti–Mouse IgG biotin conjugate, Sigma-Aldrich, Ireland) was added for one hour, followed by incubation with ABC reagent (Vectastain PK-4000, Vector Labs, Petersbough, UK) for 45 min. Finally, the slides were developed with DAB peroxidase (Vector Labs, Petersbough, UK) for five minutes. Positive and negative controls were included in the immunohistochemistry staining protocol for each batch.

7.2.5 Biochemical analysis

All samples were digested in papain (125 μg/ml) in 0.1 M sodium acetate, 5 mM cysteine HCl, 0.05 M EDTA, pH 6.0 (all from Sigma-Aldrich, Ireland) at 60°C under constant rotation for 18 hours. Aliquots of the digested samples were assayed separately for DNA,

collagen and sulphated GAG content. DNA content was quantified using the Quant-it Picogreen DNA assay (Invitrogen, UK). The proteoglycan content was estimated by quantifying the amount of GAG in constructs using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Northern Ireland), with a chondroitin sulphate standard. Total collagen content was determined by measuring the hydroxyproline content, using a hydroxyproline-to-collagen ratio of 1:7.69.(Kafienah and Sims 2004; Ignat'eva et al. 2007) Alkaline phosphatase activity was determined using SensoLyte® pNPP Alkaline Phosphatase Assay Colorimetric kit (AnaSpec, Inc., San Jose, CA, USA) and the absorbance was measured at 405 nm. At each media exchange samples of media were taken for analysis of alkaline phosphatise, GAG and total collagen secreted into the media.

7.2.6 RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was used to determine the relative gene expression changes in chondrogenic specific genes over time and subsequent to the application of loading. Total RNA was extracted from SM stem cells and FP stem cells pellets following 14 days of culture. Day zero groups were harvested following centrifugation and formation of cellular pellets. Loaded and unloaded groups were harvested directly after the application of loading at day 14. Pellet construct pooling was performed for all groups, with a total of 5 pellets pooled per group. Total cellular RNA was extracted from each pellet group by physical homogenisation using a motor driven pestle and mortar system (Sigma–Aldrich, Dublin, Ireland) in 1ml of TRIZOL reagent (Invitrogen, UK), followed by a chloroform (Sigma–Aldrich, Dublin, Ireland) extraction with PureLink™ RNA Mini kit (Invitrogen, UK) as per manufacturer's instructions. RNA was re-suspended in RNase free water prior to reverse transcription.

Total RNA concentrations were determined using the Quant-iT™ RNA assay and a Qubit® fluorometer (Invitrogen, UK) and adjusted to a standard concentration prior to cDNA synthesis. To quantify mRNA expression, 100 ng of total RNA was reverse transcribed into cDNA using iScript™ cDNA systhesis kit (BioRad, UK) as per manufacturer's instructions. TaqMan® gene expression Assays (Applied Biosystems, UK) which contain forward and reverse primers, and a FAM-labelled TaqMan probe for porcine (Sus Scrofa) *Sox9* (Ss03392406m1), *aggrecan* (Ss03374822m1), *collagen type I alpha I* (Ss03373340m1), *collagen type II alpha 1* (Ss03373344g1), *collagen type X alpha 1* (Ss03391766m1), *Indian Hedgehog* (Ss03373541m1) and *Glyceraldehyde-three-phosphate*

dehydrogenase (GAPDH) (Ss03373286u1) were used in this study. Each assay was chosen based on the amplicon spanning an exon junction. qRT-PCR was performed using an ABI 7500 Sequence Detection system (Applied Biosystems, UK). A 20 µl volume was added to each well (5µl of cDNA preparation diluted 1:5 with RNase free water, 1 µl of gene assay, 10 µl of TaqMan Universal PCR Master mix (Applied Biosystems, UK) and 4 µl RNase free water). Samples were assayed in triplicate in one run (40 cycles). qRT-PCR data were analysed using relative quantification and the C^t method as described previously (Livak and Schmittgen 2001) with the FS day zero samples used as the calibrator and *GAPDH* as the endogenous control gene. Relative quantification values are presented as fold changes in gene expression plus/minus the standard error of the mean relative to the control group, which was normalised to one.

7.2.7 Statistical analysis

For each cell source, at least two replicates of the entire study were performed. For each of these replicates, twelve samples were analysed per group at each time point, with 5 samples used for biochemical analysis of matrix components, 5 pooled samples for qRT-PCR and 2 samples were used for histology. The data from all replicates was then pooled for subsequent statistical analysis. Differences in gene expression, mechanical and biochemical properties with cell type and/or time-in-culture were determined by t-tests and by analysis of variance with time or loading as independent factors, followed by Tukey's post-hoc test (95 % confidence Interval). Statistics were preformed with MINITAB 15.1 (Minitab, Coventry, UK) and the GraphPadPrism 4 software package (San Diego, USA). Data are expressed in the form of mean ± standard error of the mean (SEM). Statistical significance was declared at $p \leq 0.05$.

7.3 Results

7.3.1 Effect of HP on pellet morphology

After 14 days in culture, pellets were mostly spherical and stained positive for proteoglycan and collagen deposition when supplemented with TGF-β3, while minimum staining was observed in both the non-supplemented FS and HP groups (Fig. 7.1). In general an increase in matrix staining was observed for FP stem cells and SM stem cells when supplemented with higher concentrations of TGF-β3.

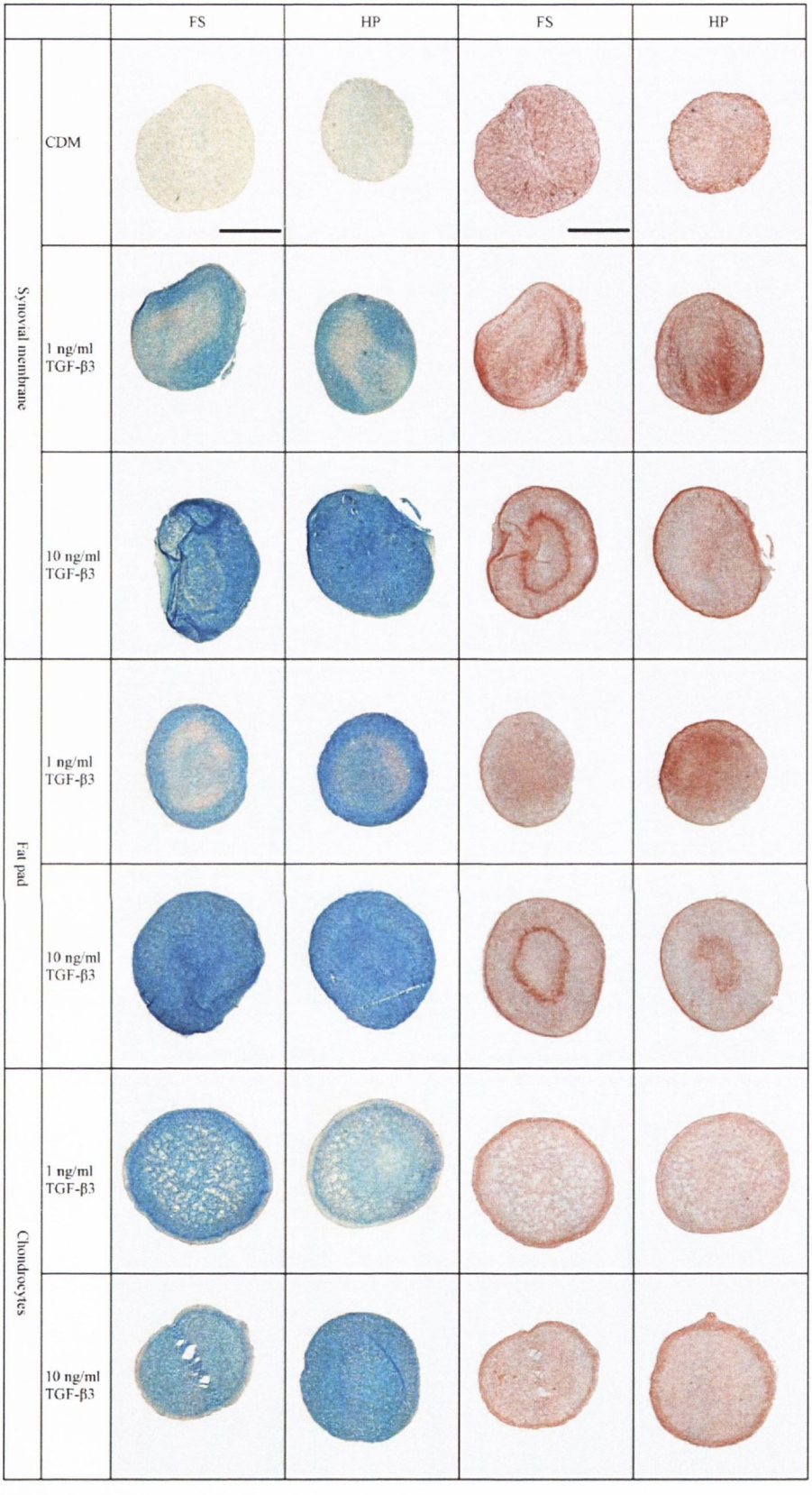


Figure 7.1: Histology sections of SM, FP and CC pellets kept in free swelling (FS) or exposed to hydrostatic pressure (HP) following 14 days of culture. Pellets were stained with alcian blue (stains GAG, right columns) and picro-sirius red (stains collagen, left columns); scale bar 500 μm.

Moreover, HP appeared to influence the organization of the neo-tissue, as evident by the appearance of a core region in the FS pellets compared to more homogeneous distribution of extracellular matrix in the HP group (Fig. 7.1). CC pellets supplemented with 1 ng/ml of TGF-β3 presented some evidence of cell clustering, with more intense staining observed in the FS group in comparison with pellets subjected to HP. Immunohistochemistry confirmed the presence of type II collagen in the SM pellets (Fig. 7.2). The same pattern of staining was observed in FP stem cells and CC pellets (data not shown). Weak staining for type I and X collagen was observed.

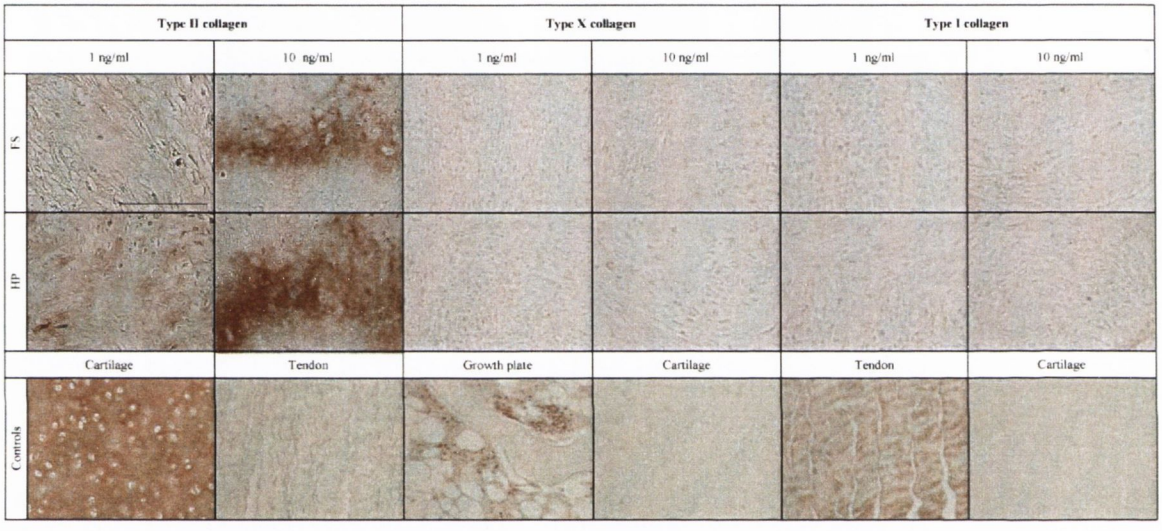


Figure 7.2: SM pellets immunostained for type II, X and I collagen with subsequent controls (Type II collagen, positive control: cartilage, negative control: ligament; type X collagen, positive control: growth plate, negative control: cartilage; type I collagen positive control: ligament, negative control: cartilage). Free swelling (FS); hydrostatic pressure (HP). Scale bar 100 μm.

7.3.2 Effect of HP on matrix production

HP had no effect on DNA content for any cell type at both 1 and 10 ng/ml of TGF-β3 (Fig. 7.3a and b).

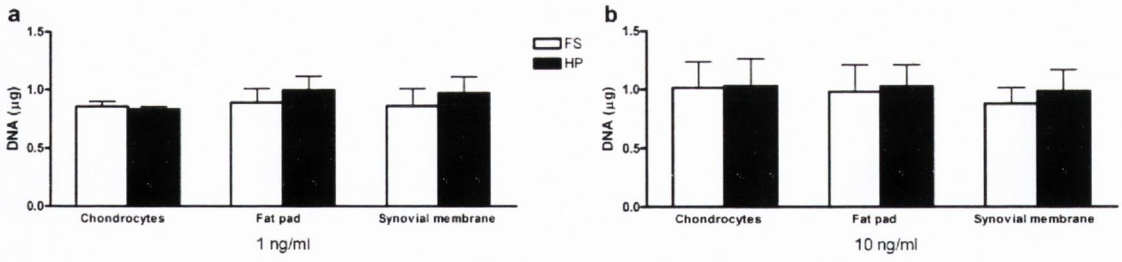


Figure 7.3: DNA content of CC and joint tissue derived stem cells cultured in chondrogenic medium with (a) 1 ng/ml of TGF-β3 or (b) 10 ng/ml of TGF-β3 after 14 days in culture. Free swelling (FS); hydrostatic pressure (HP). Data is expressed in µg per pellet and represent the mean ± SEM.

HP significantly increased GAG accumulation for both FP stem cells and SM stem cells supplemented with 1 ng/ml of TGF-β3 (FP HP: 10.5 µg (± 1.1) *vs* FP FS: 7.01 µg (± 0.6); SM HP: 8.35 µg (± 1.3) *vs* SM FS: 5.5 µg (± 0.5)). In contrast, HP decreased GAG accumulation in the CC group when supplemented with 1 ng/ml of TGF-β3 (FS: 27.7 µg (± 2.3) *vs* HP: 14.3 µg (± 1.7)) (Fig. 7.4a). A similar result was observed for total amount of GAG synthesised (addition of GAG retained in the pellet and the amount of GAG secreted to the media). HP was also observed to inhibit collagen accumulation for CC supplemented with 1 ng/ml of TGF-β3 (FS: 14.8 µg (± 0.25) *vs* HP: 9.34 µg (± 0.05)), but did not affect collagen synthesis for the 2 types of stem cells (Fig. 7 .4a).

When CC were supplemented with 10 ng/ml of TGF-β3, HP increased both GAG (FS: 13.7 µg (± 1.1) *vs* HP: 23.9 µg (± 1.6)) and collagen (FS: 10.1 µg (± 0.4) *vs* HP: 12.8 µg (± 0.7)) accumulation. In contrast HP had no effect on GAG synthesis for both FP and SM stem cells when supplemented with 10 ng/ml of TGF-β3 (Fig. 7.4b). Analysis of the media from all groups revealed negligible levels of alkaline phosphatase activity (data not shown).

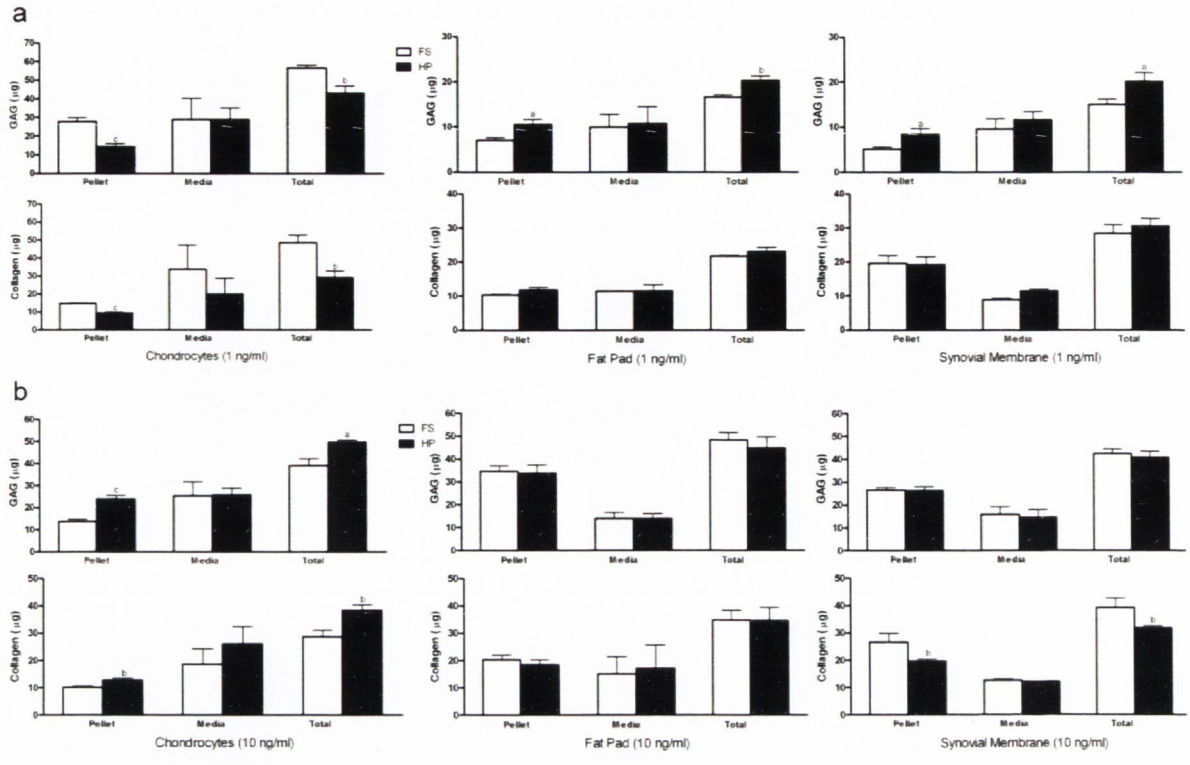


Figure 7.4: GAG and collagen content measured in the pellets and culture media of CC and joint tissue derived stem cells with (a) 1ng/ml of TGF-β3 or (b) 10ng/ml of TGF-β3. Free swelling (FS); hydrostatic pressure (HP). The total content represents the sum of matrix components being retained in the pellet and released to the media. a: $p<0.05$; b: $p<0.001$; c: $p<0.0001$.

7.3.3 Influence of TGF-β3 and HP on gene expression

SM stem cells responded to the addition of 10 ng/ml of TGF-β3 with a greater than 19-fold increase in *Sox9* expression, 2,400-fold increase in *Aggrecan* expression and a 140,000-fold increase in *type II collagen* expression following 14 days in FS conditions compared to day 0 control levels (Fig. 7.5a, b, c). The addition of 1 ng/ml of TGF-β3 resulted in a 1,200-fold (D14 FS: 1202.7 (±858.6) *vs* D0 FS: 0.92 (±0.04), $p\leq0.0001$) increase in *Aggrecan* relative gene expression compared to day 0 control levels (Fig. 7.5b). No difference was observed for *type I collagen* with and without TGF-β3 supplementation following 14 days in FS culture (Fig. 7.5d). *Type X collagen* and *Indian Hedgehog* were not expressed at day 0. By day 14, the expression of *type X collagen and Indian Hedgehog* was 100 fold and 5 fold higher respectively in pellets supplemented with 10 ng/ml compared to 1 ng/ml of TGF-β3 (data not shown).

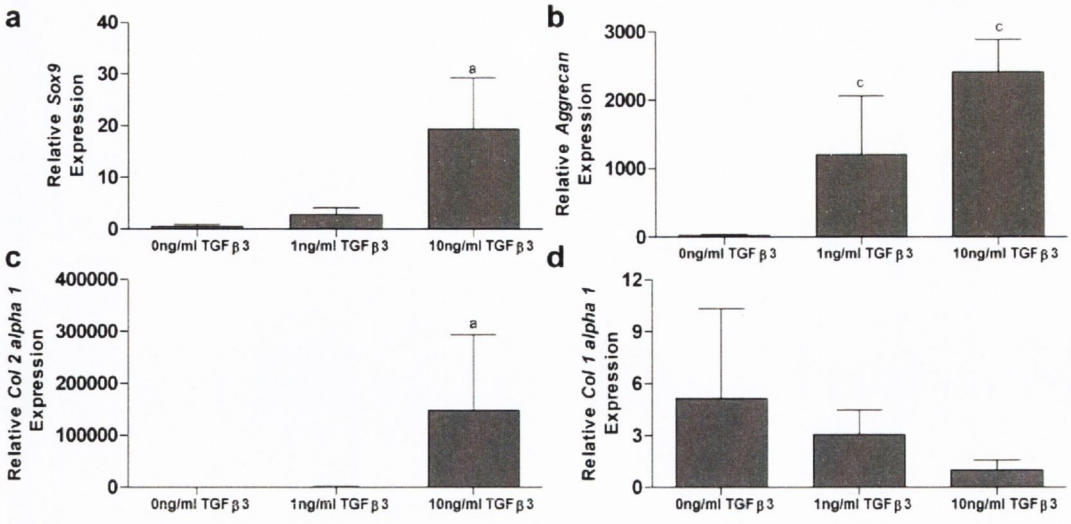


Figure 7.5: Chondrogenic and extracellular matrix-specific relative gene expression in synovial membrane pellets cultured in free swelling conditions with 0, 1 or 10ng/ml TGF-β3 over 14 days. Calibration performed according to expression of the endogenous control gene GAPDH and normalised to day 0 free swelling controls. Sox9 (a), Aggrecan (b), Collagen type II alpha 1 (c) and Collagen type I alpha 1 (d) expression. Data is expressed as relative expression per group and represented as mean ±SEM. n=3 samples per group. a: $p<0.05$, b: $p<0.0001$ vs gene expression at day 0.

The application of HP had no significant effect on the expression of *Sox9, Aggrecan* and *type II collagen* in the absence of growth factor supplementation at day 14, but did lead to significant decrease in *type I collagen* gene expression (HP: 0.13 (±0.07) *vs* FS: 1.02 (±0.002), $p\leq0.01$) (data not shown). Applying HP in the presence of 1ng/ml of TGF-β3 resulted in a 3-fold increase in the mRNA levels of *Sox9* (HP: 3.19 (±0.45) *vs* FS: 1.21 (±0.09), $p\leq0.05$) in comparison to FS controls (Fig. 7.6a). A similar trend was observed in the FP stem cells group following the application of HP and 1 ng/ml of TGF-β3 stimulation with a 3.5-fold increase in *Sox9* (HP: 3.53 (±0.45) *vs* FS: 1.05 (±0.03), $p\leq0.1$) relative gene expression in comparison to FS controls (data not shown). No significant difference was observed for the extracellular matrix specific genes *aggrecan*, *type II collagen* and *type I collagen* following the application of HP combined with either 1 or 10 ng/ml TGF-β3 (Fig. 7.6b, c, d). Applying HP to SM pellets in the presence of TGF-β3 at 1 and 10 ng/ml also lead to decreases in the mRNA levels of the hypertrophic marker *type X collagen* and the developmental regulatory gene *Indian Hedgehog* (Fig. 7.6e, f).

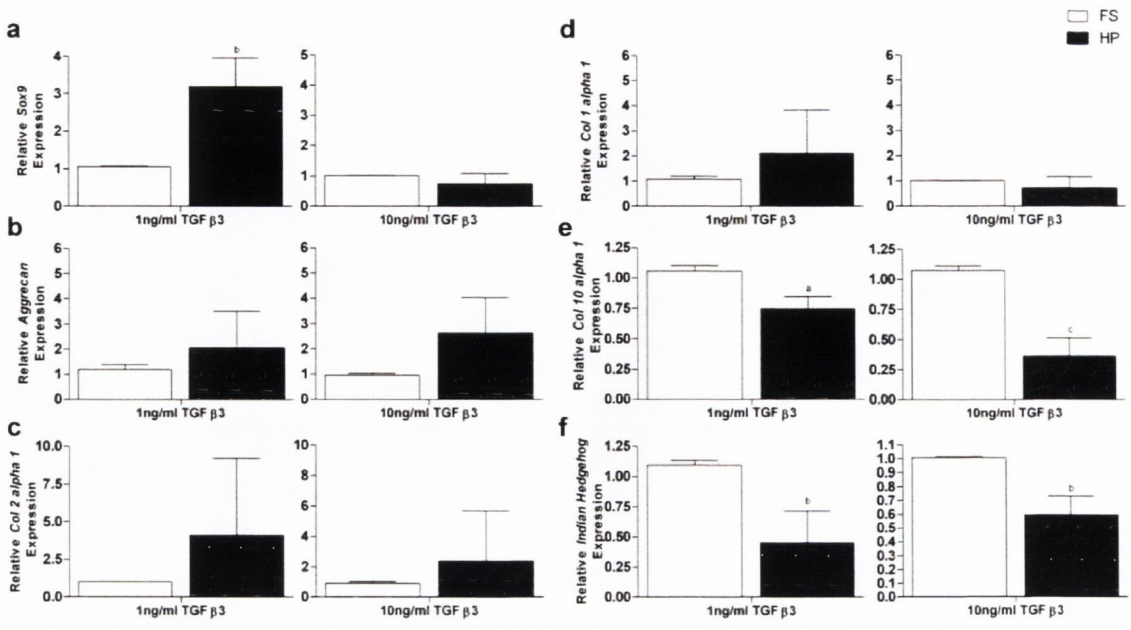


Figure 7.6: The influence of hydrostatic pressure on gene expression in synovial membrane pellets at day 14 cultured in free swelling (FS) and hydrostatic pressure (HP) conditions with 1 or 10ng/ml TGF-β3. Calibration of each gene to GAPDH expression levels and normalised to free swelling controls. Sox9 (a), Aggrecan (b), Collagen type II alpha 1 (c), Collagen type I alpha 1 (d), Collagen type X alpha 1 (e) and Indian Hedgehog (Ihh) (f) expression. Data is expressed as relative expression per group and represent mean ±SEM. n=3 per group. a: $p<0.1$, b: $p<0.05$, c: $p<0.01$. vs gene expression at day 14 for FS control.

7.4 Discussion

Understanding how various environmental cues interact to regulate chondrogenesis of stem cells will be critical to the development of new regenerative medicine therapies for treating damaged and diseased articular cartilage. The objective of this study was to investigate the interaction between HP and TGF-β3 in regulating the induction and maintenance of a chondrogenic phenotype in joint tissue derived stem cells. HP alone did not induce robust chondrogenesis of joint derived stem cells, however when applied with low concentrations of TGF-β3, it acted synergistically to increase *Sox-9* gene expression, promote GAG synthesis and inhibit *Indian Hedgehog* and *type X collagen* gene expression, the latter two being markers of pre-hypertrophy and terminal differentiation. At high magnitudes of TGF-β3, HP had no additional synergistic effect on matrix synthesis, suggesting an upper limit on the stimulatory effects these two chondrogenic cues can have on matrix production, although it continued to act to suppress *Indian Hedgehog* and *type X collagen*

gene expression. Interestingly, CC responded differently to both TGF-β3 stimulation and HP than joint tissue derived stem cells, with synthesis of GAG and collagen inhibited at higher concentrations of TGF-β3.

HP is one of the main mechanical stimuli exerted on articular cartilage and understanding its effects on stem cells and CC is a central challenge in the field of articular cartilage tissue engineering and regeneration (Elder and Athanasiou 2009). The loading regime chosen for this study was based on previous reports demonstrating that 10MPa of HP applied for 4h daily at a frequency of 1 Hz enhanced chondrogenesis (Angele et al. 2003; Ikenoue et al. 2003; Miyanishi et al. 2006). Exposure to 10 MPa of HP influenced the organization of the tissue, resulting in the formation of a more homogenous pellet. A similar observation has been reported using human BM MSCs, where pellets exposed to 10 MPa of HP exhibited a more compact and uniform structure than FS controls (Miyanishi et al. 2006; Miyanishi et al. 2006). Such alterations in the distribution of extracellular matrix components might be expected to lead to improvements in construct mechanical functionality (Kelly and Prendergast 2004) should the use of HP be extended to engineering cartilaginous grafts using scaffolds seeded with joint tissue derived MSCs.

Chondrocytes and joint tissue derived stem cells were found to differentially respond to altered TGF-β3 concentrations. Johnstone *et al.* evaluated the effect of different concentrations of TGF-β (0.5 to 10 ng/ml) on BM derived MSCs, concluding that lowering the TGF-β concentration decreased their chondrogenic response (Johnstone et al. 1998). A similar response to TGF-β3 stimulation was observed in this study for joint tissue derived stem cells. FP stem cells and SM stem cells responded similarly to TGF-β3 stimulation, corroborating previous reports where these two stem cell sources demonstrate a comparable level of chondrogenesis in response to cytokine stimulation (Mochizuki et al. 2006; Marsano et al. 2007). Interestingly when CC were supplemented with higher concentrations of TGF-β3, lower levels of GAG and collagen accumulation were observed, which has also been reported for bovine CC where supplementation with 10 ng/ml of TGF-β3 was observed to suppress chondrogenesis (Erickson et al. 2011). This suppression of CC chondrogenesis at high concentrations of TGF-β3 was partially overcome when cells were subjected to HP. It is possible that HP is activating an alternative pathway to that of TGF-β3 stimulation, one that acts independently or perhaps cross-talks intracellularly, or alternatively that HP is directly influencing TGF-β signalling. It has been suggested that HP can directly affect the conformation of transmembrane proteins (Kornblatt and

Kornblatt 2002; Elder and Athanasiou 2009), raising the possibility that such extrinsic mechanical loading can physically alter TGF-β receptors on the cell surface and hence their binding affinity for TGF-β3.

HP in the absence of TGF-β3 did not appear to induce chondrogenesis of joint derived stem cells. In the case of BM derived MSCs, some uncertainty exists in the literature as to the ability of HP in inducing chondrogenesis in the absence of growth factors with some reporting that HP has little or no effect on chondrogenic gene expression and matrix production (Zeiter et al. 2009), while others report increases in *Sox-9*, *type II collagen* and *aggrecan* gene expression but no increase in pellet wet weight due to loading (Miyanishi et al. 2006). HP has also been shown to promote *Sox-9* gene expression and protein production in SM stem cells (Sakao et al. 2008; Wu et al. 2008), although such increases in the expression of regulatory genes due to HP does not necessarily lead to changes in the expression of *aggrecan* and *type II collagen* (Finger et al. 2007). In the present study we found that HP acts synergistically with low concentrations of TGF-β3 to enhance chondrogenesis of joint tissue derived MSCs, as evidenced by a 3-fold increase in *Sox9* expression and enhanced GAG synthesis. As both biochemical and biophysical stimuli are present within the *in vivo* joint environment, further work is necessary to fully understand how these various cues interact to regulate stem cell fate.

In agreement with previous studies (Zeiter et al. 2009), we also observed that HP had no beneficial effect on cartilage-specific matrix production when stem cells were supplemented with 10 ng/ml of TGF-β3. In contrast, a number of other studies have reported a synergistic effect for BM MSCs supplemented with 10 ng/ml of TGF-β3 (Miyanishi et al. 2006; Wagner et al. 2008). Similar results have been observed for adipose derived stem cells (Ogawa et al. 2009). Given that synovial fibroblasts have been shown to produce TGF-β when subjected to HP (Wu et al. 2008), it may be that HP exerts its effects on joint tissue derived stem cells, at least in part, *via* this pathway and that the additional signal provided by HP is lost when saturation levels of TGF-β are present. Previous studies have demonstrated that mechanical loading influences chondrogenesis of human BM MSCs, and was promoted through the TGF pathway by up-regulating TGF gene expression and protein synthesis (Li et al. 2010).

It has been demonstrated that cartilaginous tissues engineered using SM and FP stem cells do not form stable cartilage following subcutaneous implantation (De Bari et al. 2004;

Marsano et al. 2007; Dickhut et al. 2009). A major difference between the ectopic and orthotopic environment is the absence of mechanical signals such as HP, which may play a key role in achieving a type X collagen negative permanent cartilage phenotype (Steck et al. 2009). Joint loading has been shown to be an essential requirement for the homeostasis of normal articular cartilage (Palmoski et al. 1980; Kiviranta et al. 1987) and definition of articular tissues during development (Nowlan et al. 2010; Roddy et al. 2011). As chondrogenically primed BM MSCs proceed to terminal differentiation, they express *type X collagen* (Winter et al. 2003) and *alkaline phosphatase* (Pelttari et al. 2006), indicative of a hypertrophic phenotype (Dickhut et al. 2009). SM stem cells have been shown to deposit similar levels of type X collagen to BM and adipose derived stem cells after 5 weeks of chondrogenic induction *in vitro* (Dickhut et al. 2009), although they have reduced tendency to undergo ectopic calcification, instead undergoing fibrous degeneration or complete degeneration. The hypertrophic markers analysed in this study: *Indian hedgehog*, which is expressed in the pre-hypertrophic zone during bone development (Kronenberg 2003; Day and Yang 2008) and *type X collagen*, a marker of the terminally differentiated (hypertrophic) chondrocyte phenotype, were both down-regulated due to the application of HP; suggesting that mechanical signals play a key role in the formation of a permanent or stable chondrocyte phenotype. Further support for such a hypothesis can be found in the results of previous studies demonstrating that HP down-regulates the expression of *MMP-13*, a proteolytic enzyme in hypertrophic cartilage required for vascularisation of calcified cartilage (Wong et al. 2003). Given our finding that HP in the absence of TGF-β stimulation had little effect on cartilage-specific matrix accumulation, but that it did suppress the expression of genes associated with hypertrophy regardless of the concentration of TGF-β3, provides support for the idea that HP may play a more important role in the maintenance rather than the induction of a chondrogenic phenotype in joint derived stem cells. Mechanobiological models have previously suggested that HP could inhibit endochondral ossification during skeletal regeneration (Carter et al. 1998) and here we provide experimental data in support of this hypothesis.

In conclusion, this study demonstrates that HP and TGF-β3 interact to regulate chondrogenesis of joint tissue derived stem cells. The data demonstrates that HP can act synergistically with low concentrations of TGF-β3 to up-regulate *Sox9* expression and the synthesis of cartilage-specific matrix molecules. Furthermore HP acts to down-regulate the expression of genes associated with pre-hypertrophy and terminal differentiation (*Indian hedgehog* and *type X collagen*). In the context of stem cell based therapies for articular cartilage repair, the results of this study demonstrate the importance of considering how

both biochemical and biophysical joint specific environmental factors might interact to regulate not only the initiation of chondrogenesis, but also the development of a stable cartilage repair tissue.

8 Discussion

8.1 Introduction

The main goal of this thesis was to compare the chondrogenic potential of different stem cell sources in the context of articular cartilage repair. Five separate studies were undertaken as part of this thesis in order to address this goal (Figure 8.1).

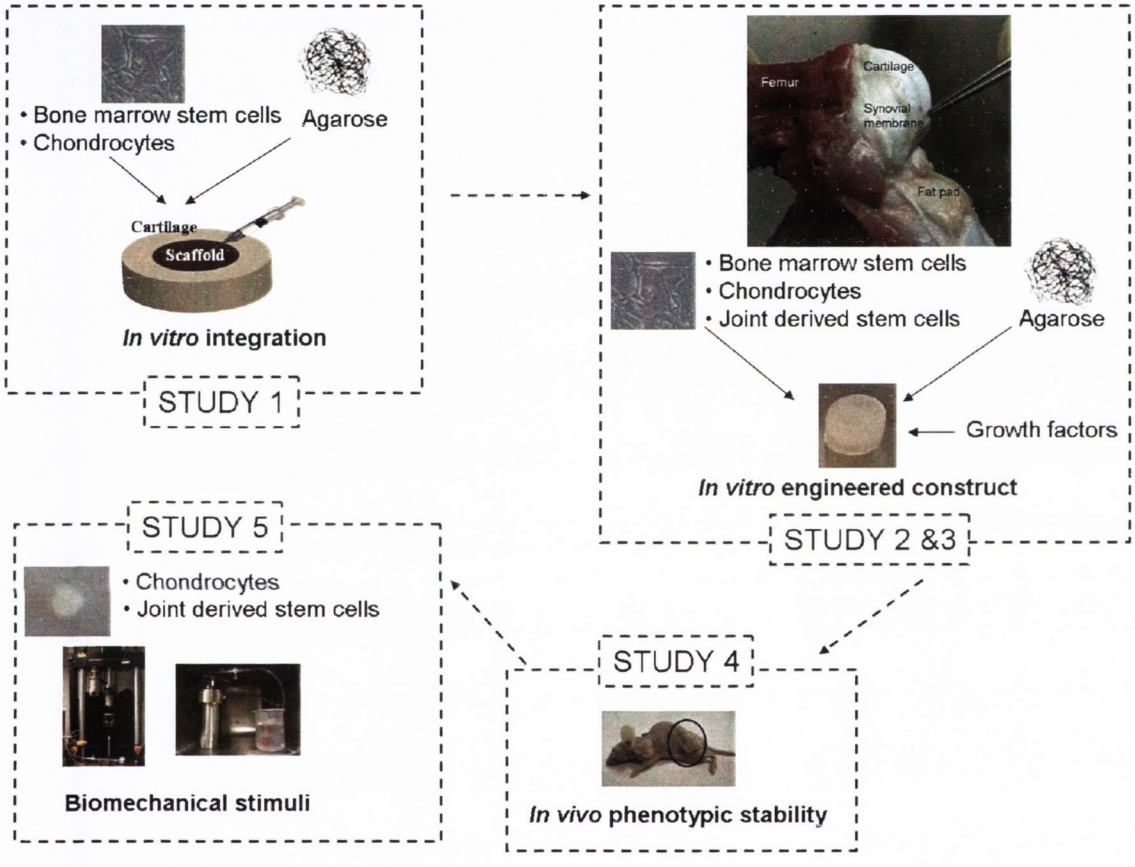


Figure 8.1: Tissue regeneration approach taken in this thesis.

The first study explored the *in vitro* integration of cartilaginous tissues generated by BM MSCs in comparison with CC in a cartilage defect model. Those results were not satisfactory regarding the outcome using BM derived stem cells, which partially motivated the decision to explore the use of joint tissue derived stem cells for cartilage tissue engineering and the effects of biochemical and biophysical cues on these cells. The second study explored the chondrogenic effects of TGF-β3 on FP cells seeded in agarose gels. Based on the promising mechanical properties and chondrogenesis obtained, FP stem cells were next compared to BM MSCs and CC in the same culture conditions. In this third study, MSCs isolated from the FP appeared to possess a potential to generate functional

cartilaginous tissues at least comparable to BM MSCs, although CC retained a stronger cartilaginous phenotype. The next study considered the phenotypic stability of FP, BM and SM stem cells, the latter having previously been shown to have a similar chondrogenic potential as FP stem cells (Sakaguchi et al. 2005; Mochizuki et al. 2006). All three cell types were compared *in vitro* and in an *in vivo* ectopic environment. SM stem cells showed the potential to generate functional cartilaginous tissues superior to the other stem cell sources *in vitro*. In this study CC were kept as a control and the final outcome revealed that joint derived stem cells fail to form stable ectopic cartilage *in vivo*, BM MSCs underwent the hypertrophic/calcified route and CC were the only stable cell source. The last study undertaken in this thesis was to explore how mechanical load will influence the chondrogenic stability of joint derived stem cells. This study demonstrated that HP acted synergistically with TGF-β3 to increase chondrogenic markers and appeared to inhibit hypertrophy and terminal differentiation. Based on the results obtained in this thesis, the initial hypothesis was proven and joint derived stem cells have the potential to generate functional cartilaginous tissues compared to other MSC sources. In the context of stem cell based therapies for articular cartilage repair, the results of this thesis demonstrate the importance of considering how both biochemical and biophysical joint specific environmental factors might interact to regulate not only the initiation of chondrogenesis, but also the development of a stable cartilage repair tissue.

In this chapter, a discussion of the main results obtained based on cell types will be stated. This will be followed by the assumptions and limitations of the methods presented in this thesis. Finally, the *in vivo* articular repair therapeutic approaches will be discussed throughout and the future work addressing the limitations indicated in this thesis will be stated.

8.2 Is there an optimal cell source for cartilage tissue engineering?

8.2.1 Chondrocytes

In general CC seemed to integrate and accumulate greater amounts of cartilaginous matrix than BM MSCs in a cartilage explant model. When compared to FP and BM agarose seeded gels *in vitro* they showed a higher equilibrium modulus, and once implanted in an ectopic *in vivo* model, they were the only cell source able to form stable cartilage.

Taken together the results show that CC are still the gold standard to which all stem cells-based cartilage tissue engineering strategies should be compared. Other studies have shown similar results when comparing stem cells to cartilage, where CC formed a more hyaline cartilage phenotype and MSCs presented a mixed cartilage phenotype (Karlsson et al. 2007; Polacek et al. 2010), but a true comparison of CC and stem cells is challenging, as the optimal culture conditions are clearly different for each cell type. For example, CC seem to respond differently to different concentrations of TGF-β3 as observed in Chapter 7 and as has been reported in the literature (Kopesky et al. 2010; Erickson et al. 2011). There are however a number of key limitations still associated with the use of CC; they dedifferentiate during monolayer expansion (Benya et al. 1978; Diaz-Romero et al. 2005) and a cartilage biopsy is needed in order to harvest them, creating an additional injury to the joint surface and increasing the potential risk of developing OA (Schumann et al. 2006). Therefore more studies need to continue in order to obtain a desired stable phenotypic cell source for cartilage repair.

8.2.2 Bone marrow derived stem cells

This thesis showed that BM MSCs failed to integrate and accumulate comparable cartilaginous extracellular matrix to CC in a cartilage explant model, although their tripotentiality, capacity to form fat, oil droplets and GAG, and their chondrogenic potential when encapsulated in agarose gels was demonstrated in previous chapters. When compared to FP seeded hydrogels they shared similar mechanical properties but synthesised less GAG than FP gels. When implanted in the *in vivo* ectopic model, BM showed markers related with hypertrophy, mineralization and type I and X collagen production. Even if it is acknowledged that BM MSCs expand rapidly in culture while retaining their capacity to differentiate (Caplan 1991; Pittenger et al. 1999), the finding that cartilaginous tissues engineered using BM MSCS tend to undergo hypertrophy may threaten their future as a candidate for replacing culture-expanded CC in cell-based repair of cartilage lesions (Pelttari et al. 2006; Karlsson et al. 2007). Co-culturing the BM stem cells *in vitro* with CC have been suggested by different authors in order to overcome the dedifferentiation problem observed with BM MSCs, but the problem of harvesting CC remains unsolved (Liu et al. 2010; Bian et al. 2011).

8.2.3 Joint derived stem cells

The main limitations associated with the use of CC and BM derived MSCs for cartilage repair were discussed in the previous sections. Joint derived stem cells could potentially overcome many of the problems associated with CC and BM stem cells for cartilage repair. In this thesis FP stem cells required the continuous supplementation with TGF-β3 when encapsulated in agarose gels in order to produce chondrogenic markers and achieve high mechanical properties. Their tri-lineage potential to exhibit adipo-, chondro-, and osteogenesis as a measure of multipotency was also shown. FP stem cells underwent comparable chondrogenesis to BM stem cells but generated cartilaginous constructs with inferior mechanical properties to CC. When expanded, they did not produced hypertrophic markers or type I collagen but they failed to form stable ectopic cartilage *in vivo*. Robust chondrogenesis was also observed for SM derived stem cells *in vitro*. Even if those cells failed to form stable cartilage *in vivo*, when subjected to HP and low concentrations of TGF-β3, an enhancement of the chondrogenesis and inhibition of the markers associated with hypertrophy and mineralization was observed. This suggests that a more stable cartilaginous tissue may form in an orthotopic environment where physiological levels of biophysical stimuli exist. A real potential exist with SM stem cells as compared to FP, a lower amount of tissue is needed in order to obtain the same quantity of stem cells, and they have demonstrated a higher colony-forming, proliferative and chondrogenic potential. Furthermore they have been shown to secrete greater levels of superficial zone protein secretion (a boundary lubricant of articular cartilage in joints) after chondrogenic induction compared with FP stem cells (Lee et al. 2010). Another advantage of using synovium as a cell source for cartilage repair is their potential for self-regeneration after removal from synovial joints (Bentley et al. 1975). Therefore implanting SM derived stem cells in an *in vivo* environment and submitting them to joint loading may lead to the formation of a stable hyaline cartilage repair tissue.

8.3 Limitations of the studies

The author acknowledges that a pure population of MSCs was not used in this thesis. No analyses of surface markers were used to assess the purity of the population and neither was a gradient separation system (e.g. Percoll^{TM}) used to select the cells. There is still a widely held perception that MSCs represent a phenotypically heterogeneous population of cells, it has been recognized that not all CFU-Fs were highly proliferative and multipotential and many different groups have used distinct phenotypic markers or

magnetic enrichment systems to identify MSCs (Jones and McGonagle 2008). However these techniques do not provide a completely pure cell population either. The purity of stem cells used in this study was comparable to that used by many others and sufficient for the hypotheses tested.

The studies presented in the thesis were performed with porcine cells and cartilage explants. Similarities of porcine MSCs with human counterpart allow them to be considered as a valuable model system for *in vitro* studies and preclinical assessments (Rho et al. 2009). Actually most of the studies based on MSCs and cartilage repair are still in the experimental trial phase and have been performed using animal models. Only a few clinical trials have been performed on humans with unclear results (Mobasheri et al. 2009).

In all experiments, 10 % FBS was added to the cell culture medium during the expansion phase, to enhance cell survival and proliferation. Different serum lots were used; therefore a variation may have had an effect on results and could explain the presence of hypertrophy in some hydrogels. In this thesis a serum-free culture approach was taken when the tissue engineered constructs were elaborated and cultured in order to eliminate the presence of undefined factors and the possibility of inter-batch variation (Heng et al. 2004); efforts are being actually made towards the use of autologous serum to expand cells, specially human derived stem cells (Kuroda et al. 2007; Lindroos et al. 2009).

8.4 In vivo therapeutic approaches for cartilage repair using joint derived stem cells

The main *in vivo* therapeutic approaches for cartilage repair reported in the literature use BM derived stem cells. Few studies have focused on joint derived stem cells; their outcome and their future use in cartilage repair therapies will be discussed in this section.

As shown by Barry and collaborators when stem cells are injected in the knee joint, they have the ability to migrate towards damaged sites (Barry et al. 2001). Similar results have been observed when SM stem cells were injected into rats with a massive meniscal defect; cells adhered to the lesion, differentiated into meniscal cells directly and promoted meniscal regeneration (Horie et al. 2009). Even if good results have been observed for meniscus repair, when it comes to cartilage repair the injected cells seems to spread throughout the joint space (Agung et al. 2006). Consequently, other authors have created a

system to track the mobilization of those MSCs. An intra-articular magnet was used in a study to confirm the chondrogenic potential of magnetically labelled SM stem cells, and to examine whether they could be delivered to the articular cartilage defect created in the rat osteochondral defect and subsequently repair the lesion. 12 weeks after treatment the regeneration of the articular cartilage was achieved, but it will be technically challenging if such diagnostic tools are to be used in a clinical context (Hori et al. 2011).

Experimentally, SM tissue was observed to contribute to the repair of the cartilage, when partial-thickness defects in the articular cartilage of rabbits were formed (Hunziker and Rosenberg 1996), and with appropriate chemical stimuli they tend to differentiate toward chondrocytes, although the author concluded that an artificial matrix seemed to be required as a guide to properly repair the defect (Hunziker 2001). Based on those promising results a number of studies have investigated repairing cartilage defects with SM stem cell seeded scaffolds. After 6 months of *in vivo* culture in a rabbit knee defect model, an osteochondral graft seeded with SM stem cells demonstrated good integration between the implanted tissue and the surrounding native cartilage but an overgrowth was observed and the bone graft matrix did not re-establish normal subchondral bone architecture (Pei et al. 2009). *In vitro*, the same authors observed contaminated macrophages affecting the quality of SM constructs, suggesting that the effects of macrophages on the *in vivo* tissue regeneration and integration necessitate further investigation. Another study using SM derived stem cells demonstrated full thickness repair of articular cartilage defects in rabbits with hyaline cartilage within 12–24 weeks without differentiation into hypertrophic cartilage. At 24 weeks, a thinning of the articular cartilage and a rising of the tidemark over the majority of the repair zone was observed; these outcomes could lead to a long-term failure of the repair procedure (Koga et al. 2007). Later on the same authors compared the chondrogenic potential of SM, BM, adipose and muscle stem cells seeded in a collagen gel and implanted in a cartilage defect model *in vivo*. SM and BM stem cells had greater *in vivo* chondrogenic potential than adipose and muscle stem cells, and SM showed the advantage of a greater proliferation potential. However, the transplanted composites were not able to extend sufficiently to fill the defects, probably attributable to the type of scaffold used in this model (Koga et al. 2008).

The only *in vivo* study using FP stem cells to date is based on a direct intra-articular injection of FP cells in an experimental animal model of OA in rabbits. At 16 weeks, there was no statistically difference between treated group and control group, but 20 weeks after

surgery a decrease in the degree of cartilage degeneration, osteophyte formation, and subchondral sclerosis was observed in the treatment group (Toghraie et al. 2011). These results suggest that FP stem cells require time to differentiate and proliferate.

The results obtained with these joint derived stem cells seems promising but the main limitation of all these *in vivo* models of cartilage damage is the fact that they do not mimic the exact environment of a joint that has a focal cartilage lesion or an advanced state of OA. Therefore larger groups and longer pre- and post-treatment periods may provide additional support for using this therapeutic approach as a new way of cartilage engineering.

In conclusion, the management of articular cartilage lesions is a challenging problem. While a number of surgical approaches have been described, it remains difficult to compare the efficacy of these techniques because of a paucity of well-designed randomized controlled trials in the literature. The current evidence suggests that BM stimulation procedures and whole-tissue transplantation of allografts or autografts can achieve favorable outcomes when used for the management of focal chondral defects of the knee, although they typically only result in temporary repair. Stem cell based techniques performed with or without a scaffold have demonstrated early promise in animal and basic-science models, but several questions still need to be addressed, such as understanding and optimizing the graft repair technique, finding the optimal cell source for engineering cartilage grafts, and discovering how these stem cells will respond to physical stimulation in order to create stable cartilage tissue. The future is exciting but additional studies need to be completed in order to validate the efficacy of stem cell based therapies in achieving successful human clinical outcomes.

8.5 Future work

- To use a serum-free medium during expansion in order to eliminate unspecified growth factors.
- To determine how the application of dynamic compressive loading and HP influences cartilage specific matrix production of SM and FP stem cells in long-term agarose culture *in vitro*.
- To evaluate the performance of joint derived stem cells seeded in agarose gels in an animal knee defect model, with or without pre-chondrogenic induction.
- To compare if a better outcome is observed by transplanting agarose seeded hydrogels that have been previously mechanical stimulated, chemically stimulated or both in an articular cartilage defect *in vivo*.
- To explore the use of growth factor delivery systems as a means to prevent hypertrophy and dedifferentiation of cartilaginous tissues engineered using stem cells.
- To eventually move from the use of porcine cells to human cells.

9 References

Adams, J. C. and D. L. Hamblen (1995). Affections of joints. Outline of orthopaedics. M. Parkinson. New York, Churchill Livingstone: 108-131.

Adkisson, H. D., M. P. Gillis, et al. (2001). "In vitro generation of scaffold independent neocartilage." Clin Orthop Relat Res(391 Suppl): S280-94.

Afizah, H., Z. Yang, et al. (2007). "A comparison between the chondrogenic potential of human bone marrow stem cells (BMSCs) and adipose-derived stem cells (ADSCs) taken from the same donors." Tissue Eng **13**(4): 659-66.

Agung, M., M. Ochi, et al. (2006). "Mobilization of bone marrow-derived mesenchymal stem cells into the injured tissues after intraarticular injection and their contribution to tissue regeneration." Knee Surg Sports Traumatol Arthrosc **14**(12): 1307-14.

Ahsan, T. and R. L. Sah (1999). "Biomechanics of integrative cartilage repair." Osteoarthritis Cartilage **7**(1): 29-40.

Albro, M. B., N. O. Chahine, et al. (2008). "Dynamic loading of deformable porous media can induce active solute transport." J Biomech **41**(15): 3152-3157.

Almqvist, K. F., A. A. Dhollander, et al. (2009). "Treatment of cartilage defects in the knee using alginate beads containing human mature allogenic chondrocytes." Am J Sports Med **37**(10): 1920-9.

Ando, W., K. Tateishi, et al. (2007). "Cartilage repair using an in vitro generated scaffold-free tissue-engineered construct derived from porcine synovial mesenchymal stem cells." Biomaterials **28**(36): 5462-70.

Ando, W., K. Tateishi, et al. (2008). "In vitro generation of a scaffold-free tissue-engineered construct (TEC) derived from human synovial mesenchymal stem cells: biological and mechanical properties and further chondrogenic potential." Tissue Eng Part A **14**(12): 2041-9.

Angele, P., D. Schumann, et al. (2004). "Cyclic, mechanical compression enhances chondrogenesis of mesenchymal progenitor cells in tissue engineering scaffolds." Biorheology **41**(3-4): 335-46.

Angele, P., J. U. Yoo, et al. (2003). "Cyclic hydrostatic pressure enhances the chondrogenic phenotype of human mesenchymal progenitor cells differentiated in vitro." J Orthop Res **21**(3): 451-7.

Awad, H. A., M. Q. Wickham, et al. (2004). "Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds." Biomaterials **25**(16): 3211-3222.

Babalola, O. M. and L. J. Bonassar (2010). "Effects of seeding density on proteoglycan assembly of passaged mesenchymal stem cells." Cell and Molec Bioeng **3**(3): 197-206.

Bahuleyan, B., S. H. Cheung, et al. (2009). "Role of Biomechanical Force in Stem Cell-Based Therapy for Cartilage Repair." Current Rheumatology Reviews **5**(1): 34-39.

Barbero, A., S. Grogan, et al. (2004). "Age related changes in human articular chondrocyte yield, proliferation and post-expansion chondrogenic capacity." Osteoarthritis Cartilage **12**(6): 476-484.

Barry, F., R. E. Boynton, et al. (2001). "Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components." Exp Cell Res **268**(2): 189-200.

Barry, F. P. and J. M. Murphy (2004). "Mesenchymal stem cells: clinical applications and biological characterization." Int J Biochem Cell Biol **36**(4): 568-84.

Bentley, G., A. Kreutner, et al. (1975). "Synovial regeneration and articular cartilage changes after synovectomy in normal and steroid-treated rabbits." J Bone Joint Surg Br **57**(4): 454-62.

Benya, P. D., S. R. Padilla, et al. (1978). "Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture." Cell **15**(4): 1313-21.

Bian, L., D. Y. Zhai, et al. (2011). "Coculture of human mesenchymal stem cells and articular chondrocytes reduces hypertrophy and enhances functional properties of engineered cartilage." Tissue Eng Part A **17**(7-8): 1137-45.

Bian, L., D. Y. Zhai, et al. (2011). "Enhanced MSC chondrogenesis following delivery of TGF-beta3 from alginate microspheres within hyaluronic acid hydrogels in vitro and in vivo." Biomaterials **32**(27): 6425-34.

Bianchi, G., A. Banfi, et al. (2003). "Ex vivo enrichment of mesenchymal cell progenitors by fibroblast growth factor 2." Exp Cell Res **287**(1): 98-105.

Bonassar, L. J., A. J. Grodzinsky, et al. (2001). "The effect of dynamic compression on the response of articular cartilage to insulin-like growth factor-I." J Orthop Res **19**(1): 11-7.

Boon, C. H., T. Cao, et al. (2004). "Directing stem cell differentiation into the chondrogenic lineage in vitro." Stem Cells **22**(7): 1152-1167.

Bos, P. K., J. DeGroot, et al. (2002). "Specific enzymatic treatment of bovine and human articular cartilage: implications for integrative cartilage repair." Arthritis Rheum **46**(4): 976-85.

Bosnakovski, D., M. Mizuno, et al. (2004). "Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells in pellet cultural system." Exp Hematol **32**(5): 502-9.

Bosnakovski, D., M. Mizuno, et al. (2006). "Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells (MSCs) in different hydrogels: influence of collagen type II extracellular matrix on MSC chondrogenesis." Biotechnol Bioeng **93**(6): 1152-63.

Brittberg, M. (2010). "Cell carriers as the next generation of cell therapy for cartilage repair: a review of the matrix-induced autologous chondrocyte implantation procedure." Am J Sports Med **38**(6): 1259-71.

Brittberg, M., A. Lindahl, et al. (1994). "Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation." N Engl J Med **331**(14): 889-95.

Bruder, S. P., N. Jaiswal, et al. (1997). "Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation." J Cell Biochem **64**(2): 278-294.

Buckley, C. T., S. D. Thorpe, et al. (2009). "Engineering of large cartilaginous tissues through the use of microchanneled hydrogels and rotational culture." Tissue Eng Part A **15**(11): 3213-20.

Buckley, C. T., S. D. Thorpe, et al. (2009). "The effect of concentration, thermal history and cell seeding density on the initial mechanical properties of agarose hydrogels." J Mech Behav Biomed Mater **2**(5): 512-21.

Buckley, C. T., T. Vinardell, et al. (2010). "Oxygen tension differentially regulates the functional properties of cartilaginous tissues engineered from infrapatellar fat pad derived MSCs and articular chondrocytes." Osteoarthritis Cartilage **18**(10): 1345-54.

Buckley, C. T., T. Vinardell, et al. (2010). "Functional properties of cartilaginous tissues engineered from infrapatellar fat pad-derived mesenchymal stem cells." J Biomech **43**(5): 920-6.

Buckwalter, J. A. (1998). "Articular cartilage: injuries and potential for healing." J Orthop Sports Phys Ther **28**(4): 192-202.

Buschmann, M. D., Y. A. Gluzband, et al. (1995). "Mechanical compression modulates matrix biosynthesis in chondrocyte/agarose culture." J Cell Sci **108 (Pt 4)**: 1497-508.

Buschmann, M. D., Y. A. Gluzband, et al. (1992). "Chondrocytes in agarose culture synthesize a mechanically functional extracellular matrix." J Orthop Res **10**(6): 745-58.

Buxton, A. N., C. S. Bahney, et al. (2011). "Temporal exposure to chondrogenic factors modulates human mesenchymal stem cell chondrogenesis in hydrogels." Tissue Eng Part A **17**(3-4): 371-80.

Byers, B. A., R. L. Mauck, et al. (2008). "Transient exposure to transforming growth factor beta 3 under serum-free conditions enhances the biomechanical and biochemical maturation of tissue-engineered cartilage." Tissue Eng Part A **14**(11): 1821-34.

Campbell, J. J., D. A. Lee, et al. (2006). "Dynamic compressive strain influences chondrogenic gene expression in human mesenchymal stem cells." Biorheology **43**(3-4): 455-470.

Caplan, A. I. (1991). "Mesenchymal stem cells." J Orthop Res **9**(5): 641-650.

Caplan, A. I. (2007). "Adult mesenchymal stem cells for tissue engineering versus regenerative medicine." J Cell Physiol **213**(2): 341-7.

Carter, D. R., G. S. Beaupre, et al. (1998). "Mechanobiology of skeletal regeneration." Clin Orthop Relat Res(355 Suppl): S41-55.

Celeste, C., M. Ionescu, et al. (2005). "Repeated intraarticular injections of triamcinolone acetonide alter cartilage matrix metabolism measured by biomarkers in synovial fluid." J Orthop Res **23**(3): 602-10.

Centeno, C. J., D. Busse, et al. (2008). "Increased knee cartilage volume in degenerative joint disease using percutaneously implanted, autologous mesenchymal stem cells." Pain Physician **11**(3): 343-53.

Centeno, C. J., J. Kisiday, et al. (2006). "Partial regeneration of the human hip via autologous bone marrow nucleated cell transfer: A case study." Pain Physician **9**(3): 253-6.

Chapel, A., J. M. Bertho, et al. (2003). "Mesenchymal stem cells home to injured tissues when co-infused with hematopoietic cells to treat a radiation-induced multi-organ failure syndrome." J Gene Med **5**(12): 1028-38.

Cheng, N. C., B. T. Estes, et al. (2009). "Chondrogenic differentiation of adipose-derived adult stem cells by a porous scaffold derived from native articular cartilage extracellular matrix." Tissue Eng Part A **15**(2): 231-41.

Chiang, H. and C. C. Jiang (2009). "Repair of articular cartilage defects: review and perspectives." J Formos Med Assoc **108**(2): 87-101.

Clair, B. L., A. R. Johnson, et al. (2009). "Cartilage repair: current and emerging options in treatment." Foot Ankle Spec **2**(4): 179-88.

Coleman, R. M., N. D. Case, et al. (2007). "Hydrogel effects on bone marrow stromal cell response to chondrogenic growth factors." Biomaterials **28**(12): 2077-86.

Colter, D. C., R. Class, et al. (2000). "Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow." Proc Natl Acad Sci U S A **97**(7): 3213-8.

Connelly, J. T., C. G. Wilson, et al. (2008). "Characterization of proteoglycan production and processing by chondrocytes and BMSCs in tissue engineered constructs." Osteoarthritis Cartilage **16**(9): 1092-100.

Cowin, S. C. and S. B. Doty (2007). Cartilage. Tissue Mechanics. New York, Springer**:** 471-505.

Cui, J. H., S. R. Park, et al. (2007). "Preconditioning of mesenchymal stem cells with low-intensity ultrasound for cartilage formation in vivo." Tissue Eng **13**(2): 351-60.

Cui, L., Y. Wu, et al. (2009). "Repair of articular cartilage defect in non-weight bearing areas using adipose derived stem cells loaded polyglycolic acid mesh." Biomaterials **30**(14): 2683-93.

Danisovic, L., I. Varga, et al. (2009). "Comparison of in vitro chondrogenic potential of human mesenchymal stem cells derived from bone marrow and adipose tissue." Gen Physiol Biophys **28**(1): 56-62.

Darling, E. M. and K. A. Athanasiou (2003). "Biomechanical strategies for articular cartilage regeneration." Ann Biomed Eng **31**(9): 1114-24.

Darling, E. M. and K. A. Athanasiou (2005). "Rapid phenotypic changes in passaged articular chondrocyte subpopulations." J Orthop Res **23**(2): 425-32.

Day, T. F. and Y. Yang (2008). "Wnt and hedgehog signaling pathways in bone development." J Bone Joint Surg Am **90 Suppl 1**: 19-24.

De Bari, C., F. Dell'Accio, et al. (2004). "Failure of in vitro-differentiated mesenchymal stem cells from the synovial membrane to form ectopic stable cartilage in vivo." Arthritis Rheum **50**(1): 142-50.

De Bari, C., F. Dell'Accio, et al. (2001). "Multipotent mesenchymal stem cells from adult human synovial membrane." Arthritis Rheum **44**(8): 1928-42.

de Crombrugghe, B., V. Lefebvre, et al. (2000). "Transcriptional mechanisms of chondrocyte differentiation." Matrix Biol **19**(5): 389-94.

Dell'Accio, F., C. De Bari, et al. (2001). "Molecular markers predictive of the capacity of expanded human articular chondrocytes to form stable cartilage in vivo." Arthritis Rheum **44**(7): 1608-19.

Demarteau, O., D. Wendt, et al. (2003). "Dynamic compression of cartilage constructs engineered from expanded human articular chondrocytes." Biochem Biophys Res Commun **310**(2): 580-8.

Dewire, P. and T. A. Einhorn (2001). The joint as an organ. Osteoarthritis: Diagnosis and medical/surgical management. R. W. Moskowitz, D. S. Howell, R. D. Altman, J. A. Buckwalter and V. M. Goldberg. Philadelphia, W.B.Saunders Company. **1:** 49-68.

Dhert, W. J., C. C. Verheyen, et al. (1992). "A finite element analysis of the push-out test: influence of test conditions." J Biomed Mater Res **26**(1): 119-30.

Diaz-Romero, J., J. P. Gaillard, et al. (2005). "Immunophenotypic analysis of human articular chondrocytes: changes in surface markers associated with cell expansion in monolayer culture." J Cell Physiol **202**(3): 731-42.

Dickhut, A., E. Gottwald, et al. (2008). "Chondrogenesis of mesenchymal stem cells in gel-like biomaterials in vitro and in vivo." Front Biosci **13**: 4517-28.

Dickhut, A., K. Pelttari, et al. (2009). "Calcification or dedifferentiation: requirement to lock mesenchymal stem cells in a desired differentiation stage." J Cell Physiol **219**(1): 219-26.

Diekman, B. O., C. R. Rowland, et al. (2010). "Chondrogenesis of adult stem cells from adipose tissue and bone marrow: Induction by growth factors and cartilage derived matrix." Tissue Eng Part A **16**(2): 523-33.

Djouad, F., L. Rackwitz, et al. (2009). "ERK1/2 activation induced by inflammatory cytokines compromises effective host tissue integration of engineered cartilage." Tissue Eng Part A **15**(10): 2825-35.

Dobratz, E. J., S. W. Kim, et al. (2009). "Injectable cartilage: using alginate and human chondrocytes." Arch Facial Plast Surg **11**(1): 40-7.

Dragoo, J. L., B. Samimi, et al. (2003). "Tissue-engineered cartilage and bone using stem cells from human infrapatellar fat pads." J Bone Joint Surg Br **85**(5): 740-7.

Dzierzak, E. and C. Robin (2010). "Placenta as a source of hematopoietic stem cells." Trends Mol Med **16**(8): 361-7.

Egrise, D., D. Martin, et al. (1992). "The number of fibroblastic colonies formed from bone marrow is decreased and the in vitro proliferation rate of trabecular bone cells increased in aged rats." Bone **13**(5): 355-61.

Elder, B. D. and K. A. Athanasiou (2008). "Effects of confinement on the mechanical properties of self-assembled articular cartilage constructs in the direction orthogonal to the confinement surface." J Orthop Res **26**(2): 238-46.

Elder, B. D. and K. A. Athanasiou (2008). "Synergistic and additive effects of hydrostatic pressure and growth factors on tissue formation." PLoS One **3**(6): e2341.

Elder, B. D. and K. A. Athanasiou (2009). "Hydrostatic pressure in articular cartilage tissue engineering: from chondrocytes to tissue regeneration." Tissue Eng Part B Rev **15**(1): 43-53.

Elder, S. H., S. A. Goldstein, et al. (2001). "Chondrocyte differentiation is modulated by frequency and duration of cyclic compressive loading." Ann Biomed Eng **29**(6): 476-82.

Elder, S. H., S. W. Sanders, et al. (2006). "Chondrocyte response to cyclic hydrostatic pressure in alginate versus pellet culture." J Orthop Res **24**(4): 740-7.

Emans, P. J., L. W. van Rhijn, et al. (2010). "Autologous engineering of cartilage." Proc Natl Acad Sci U S A **107**(8): 3418-23.

Engler, A. J., S. Sen, et al. (2006). "Matrix elasticity directs stem cell lineage specification." Cell **126**(4): 677-89.

English, A., E. A. Jones, et al. (2007). "A comparative assessment of cartilage and joint fat pad as a potential source of cells for autologous therapy development in knee osteoarthritis." Rheumatology (Oxford) **46**(11): 1676-83.

Erickson, G. R., J. M. Gimble, et al. (2002). "Chondrogenic potential of adipose tissue-derived stromal cells in vitro and in vivo." Biochem Biophys Res Commun **290**(2): 763-9.

Erickson, I. E., A. H. Huang, et al. (2009). "Differential maturation and structure-function relationships in mesenchymal stem cell- and chondrocyte-seeded hydrogels." Tissue Eng Part A **15**(5): 1041-52.

Erickson, I. E., S. C. van Veen, et al. (2011). "Cartilage Matrix Formation by Bovine Mesenchymal Stem Cells in Three-dimensional Culture Is Age-dependent." Clin Orthop Relat Res: DOI:10.1007/s11999-011-1869-z.

Estes, B. T., B. O. Diekman, et al. (2008). "Monolayer cell expansion conditions affect the chondrogenic potential of adipose-derived stem cells." Biotechnol Bioeng **99**(4): 986-95.

Estes, B. T., A. W. Wu, et al. (2006). "Potent induction of chondrocytic differentiation of human adipose-derived adult stem cells by bone morphogenetic protein 6." Arthritis Rheum **54**(4): 1222-32.

Fan, J., R. R. Varshney, et al. (2009). "Synovium-derived mesenchymal stem cells: a new cell source for musculoskeletal regeneration." Tissue Eng Part B Rev **15**(1): 75-86.

Farrell, E., S. K. Both, et al. (2011). "In-vivo generation of bone via endochondral ossification by in-vitro chondrogenic priming of adult human and rat mesenchymal stem cells." BMC Musculoskelet Disord **12**: 31.

Finger, A. R., C. Y. Sargent, et al. (2007). "Differential effects on messenger ribonucleic acid expression by bone marrow-derived human mesenchymal stem cells seeded in agarose constructs due to ramped and steady applications of cyclic hydrostatic pressure." Tissue Eng **13**(6): 1151-8.

Fitzsimmons, J. S., A. Sanyal, et al. (2004). "Serum-free media for periosteal chondrogenesis in vitro." J Orthop Res **22**(4): 716-25.

Forraz, N. and C. P. McGuckin (2011). "The umbilical cord: a rich and ethical stem cell source to advance regenerative medicine." Cell Prolif **44 Suppl 1**: 60-9.

Fowler, M. R., C. O. Nathan, et al. (2002). "Synovial metaplasia, a specialized form of repair." Arch Pathol Lab Med **126**(6): 727-30.

Friedenstein, A. J. (1976). "Precursor cells of mechanocytes." Int Rev Cytol **47**: 327-59.

Frisbie, D. D. (2006). Synovial Joint Biology and Pathobiology. Equine Surgery. J. A. Auer and J. A. Stick. St.Louis, Saunders**:** 1036-1055.

Frisbie, D. D., S. Morisset, et al. (2006). "Effects of calcified cartilage on healing of chondral defects treated with microfracture in horses." Am J Sports Med **34**(11): 1824-31.

Fuchs, E. and J. A. Segre (2000). "Stem cells: a new lease on life." Cell **100**(1): 143-55.

Gabay, O., C. Sanchez, et al. (2010). "Update in cartilage bio-engineering." Joint Bone Spine **77**(4): 283-6.

Ge, Z., Y. Hu, et al. (2006). "Osteoarthritis and therapy." Arthritis Rheum **55**(3): 493-500.

Gelse, K., K. von der Mark, et al. (2003). "Articular cartilage repair by gene therapy using growth factor-producing mesenchymal cells." Arthritis Rheum **48**(2): 430-41.

Getgood, A., T. P. S. Bhullar, et al. (2009). "Current concepts in articular cartilage repair." Orthop Trauma **23**(3): 189-200.

Gilbert, S. J., S. K. Singhrao, et al. (2009). "Enhanced tissue integration during cartilage repair in vitro can be achieved by inhibiting chondrocyte death at the wound edge." Tissue Eng Part A **15**(7): 1739-49.

Gimble, J. M., A. J. Katz, et al. (2007). "Adipose-derived stem cells for regenerative medicine." Circ Res **100**(9): 1249-60.

Goldring, M. B. (2000). "Osteoarthritis and cartilage: the role of cytokines." Curr Rheumatol Rep **2**(6): 459-65.

Goodrich, L. R. and A. J. Nixon (2006). "Medical treatment of osteoarthritis in the horse - a review." Vet J **171**(1): 51-69.

Grad, S., D. Eglin, et al. (2011). "Physical Stimulation of Chondrogenic Cells In Vitro: A Review." Clin Orthop Relat Res: DOI: 10.1007/s11999-011-1819-9.

Granero-Molto, F., J. A. Weis, et al. (2008). "Role of mesenchymal stem cells in regenerative medicine: application to bone and cartilage repair." Expert Opin Biol Ther **8**(3): 255-68.

Gratz, K. R., V. W. Wong, et al. (2006). "Biomechanical assessment of tissue retrieved after in vivo cartilage defect repair: Tensile modulus of repair tissue and integration with host cartilage." J Biomech **39**(1): 138-146.

Gray, H. (1974). The bone. Gray's anatomy. T. Pickering Pick and R. Howden. Philadelphia, Courage books**:** 1096-1106.

Groppe, J., C. S. Hinck, et al. (2008). "Cooperative assembly of TGF-beta superfamily signaling complexes is mediated by two disparate mechanisms and distinct modes of receptor binding." Mol Cell **29**(2): 157-68.

Hadjantonakis, A. and V. Papaioannou (2001). "The stem cells of early embryos." Differentiation **68**(4-5): 159-66.

Hannouche, D., H. Terai, et al. (2007). "Engineering of implantable cartilaginous structures from bone marrow-derived mesenchymal stem cells." Tissue Eng **13**(1): 87-99.

Hao, J., R. R. Varshney, et al. (2008). "TGF-beta3: A promising growth factor in engineered organogenesis." Expert Opin Biol Ther **8**(10): 1485-93.

Heng, B. C., T. Cao, et al. (2004). "An overview and synopsis of techniques for directing stem cell differentiation in vitro." Cell Tissue Res **315**(3): 291-303.

Hennig, T., H. Lorenz, et al. (2007). "Reduced chondrogenic potential of adipose tissue derived stromal cells correlates with an altered TGFbeta receptor and BMP profile and is overcome by BMP-6." J Cell Physiol **211**(3): 682-91.

Hermida-Gomez, T., I. Fuentes-Boquete, et al. (2011). "Quantification of cells expressing mesenchymal stem cell markers in healthy and osteoarthritic synovial membranes." J Rheumatol **38**(2): 339-49.

Hildner, F., C. Albrecht, et al. (2011). "State of the art and future perspectives of articular cartilage regeneration: a focus on adipose-derived stem cells and platelet-derived products." J Tissue Eng Regen Med **5**(4): 36-51.

Hildner, F., A. Peterbauer, et al. (2009). "Minipig derived mesenchymal stem cells from bone marrow and adipose tissue: comparison of adipogenic, osteogenic and chondrogenic differentiation potential." Eur Cell Mater **18**(Suppl 1): 40.

Holtzer, H., J. Abbott, et al. (1960). "The Loss of Phenotypic Traits by Differentiated Cells in Vitro, I. Dedifferentiation of Cartilage Cells." Proc Natl Acad Sci U S A **46**(12): 1533-42.

Honn, K. V., J. A. Singley, et al. (1975). "Fetal bovine serum: a multivariate standard." Proc Soc Exp Biol Med **149**(2): 344-7.

Horas, U., D. Pelinkovic, et al. (2003). "Autologous chondrocyte implantation and osteochondral cylinder transplantation in cartilage repair of the knee joint. A prospective, comparative trial." J Bone Joint Surg - Series A **85**(2): 185-192.

Hori, J., M. Deie, et al. (2011). "Articular cartilage repair using an intra-articular magnet and synovium-derived cells." J Orthop Res **29**(4): 531-8.

Horie, M., I. Sekiya, et al. (2009). "Intra-articular Injected synovial stem cells differentiate into meniscal cells directly and promote meniscal regeneration without mobilization to distant organs in rat massive meniscal defect." Stem Cells **27**(4): 878-87.

Hou, L. L., M. Zheng, et al. (2003). "[Migration and differentiation of human bone marrow mesenchymal stem cells in the rat brain]." Sheng Li Xue Bao **55**(2): 153-9.

Hu, J. C. and K. A. Athanasiou (2006). "The effects of intermittent hydrostatic pressure on self-assembled articular cartilage constructs." Tissue Eng **12**(5): 1337-44.

Huang, A. H., M. J. Farrell, et al. (2010). "Long-term dynamic loading improves the mechanical properties of chondrogenic mesenchymal stem cell-laden hydrogel." Eur Cell Mater **19**: 72-85.

Huang, A. H., M. J. Farrell, et al. (2010). "Mechanics and mechanobiology of mesenchymal stem cell-based engineered cartilage." J Biomech **43**(1): 128-36.

Huang, A. H., A. Stein, et al. (2009). "Transient exposure to transforming growth factor beta 3 improves the mechanical properties of mesenchymal stem cell-laden cartilage constructs in a density-dependent manner." Tissue Eng Part A **15**(11): 3461-72.

Huang, A. H., M. Yeger-McKeever, et al. (2008). "Tensile properties of engineered cartilage formed from chondrocyte- and MSC-laden hydrogels." Osteoarthritis Cartilage **16**(9): 1074-1082.

Huang, C. Y. C., K. L. Hagar, et al. (2004). "Effects of cyclic compressive loading on chondrogenesis of rabbit bone-marrow derived mesenchymal stem cells." Stem Cells **22**(3): 313-323.

Huang, C. Y. C., P. M. Reuben, et al. (2004). "Chondrogenesis of Human Bone Marrow-Derived Mesenchymal Stem Cells in Agarose Culture." Anatomical Record - Part A Discoveries in Molecular, Cellular, and Evolutionary Biology **278**(1): 428-436.

Huang, J. I., N. Kazmi, et al. (2005). "Chondrogenic potential of progenitor cells derived from human bone marrow and adipose tissue: a patient-matched comparison." J Orthop Res **23**(6): 1383-9.

Hunter, C. J. and M. E. Levenston (2002). "The influence of repair tissue maturation on the response to oscillatory compression in a cartilage defect repair model." Biorheology **39**(1-2): 79-88.

Hunter, C. J. and M. E. Levenston (2004). "Maturation and integration of tissue-engineered cartilages within an in vitro defect repair model." Tissue Eng **10**(5-6): 736-46.

Hunziker, E. B. (2001). "Growth-factor-induced healing of partial-thickness defects in adult articular cartilage." Osteoarthritis Cartilage **9**(1): 22-32.

Hunziker, E. B. (2002). "Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects." Osteoarthritis Cartilage **10**(6): 432-63.

Hunziker, E. B. and L. C. Rosenberg (1996). "Repair of partial-thickness defects in articular cartilage: cell recruitment from the synovial membrane." J Bone Joint Surg Am **78**(5): 721-33.

Ignat'eva, N. Y., N. A. Danilov, et al. (2007). "Determination of hydroxyproline in tissues and the evaluation of the collagen content of the tissues." J Anal Chem **62**(1): 51-57.

Ikenoue, T., M. C. Trindade, et al. (2003). "Mechanoregulation of human articular chondrocyte aggrecan and type II collagen expression by intermittent hydrostatic pressure in vitro." J Orthop Res **21**(1): 110-6.

Im, G. I., D. Y. Kim, et al. (2001). "Repair of cartilage defect in the rabbit with cultured mesenchymal stem cells from bone marrow." J Bone Joint Surg Br **83**(2): 289-94.

Im, G. I., Y. W. Shin, et al. (2005). "Do adipose tissue-derived mesenchymal stem cells have the same osteogenic and chondrogenic potential as bone marrow-derived cells?" Osteoarthritis Cartilage **13**(10): 845-53.

Iwai, R., M. Fujiwara, et al. (2011). "Ex vivo cartilage defect model for the evaluation of cartilage regeneration using mesenchymal stem cells." J Biosci Bioeng **111**(3): 357-64.

Jakobsen, R. B., L. Engebretsen, et al. (2005). "An analysis of the quality of cartilage repair studies." J Bone Joint Surg Am **87**(10): 2232-9.

Jo, C. H., H. J. Ahn, et al. (2007). "Surface characterization and chondrogenic differentiation of mesenchymal stromal cells derived from synovium." Cytotherapy **9**(4): 316-27.

Johnson, T. S., J. W. Xu, et al. (2004). "Integrative repair of cartilage with articular and nonarticular chondrocytes." Tissue Eng **10**(9-10): 1308-1315.

Johnstone, B., T. M. Hering, et al. (1998). "In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells." Exp Cell Res **238**(1): 265-272.

Jones, E. and D. McGonagle (2008). "Human bone marrow mesenchymal stem cells in vivo." Rheumatology (Oxford) **47**(2): 126-31.

Jurgens, W. J., A. van Dijk, et al. (2009). "Freshly isolated stromal cells from the infrapatellar fat pad are suitable for a one-step surgical procedure to regenerate cartilage tissue." Cytotherapy **11**(8): 1052-64.

Kadiyala, S., R. G. Young, et al. (1997). "Culture expanded canine mesenchymal stem cells possess osteochondrogenic potential in vivo and in vitro." Cell Transplantation **6**(2): 125-134.

Kafienah, W. and T. J. Sims (2004). "Biochemical methods for the analysis of tissue-engineered cartilage." Methods Mol Biol **238**: 217-30.

Karlsson, C., C. Brantsing, et al. (2007). "Differentiation of human mesenchymal stem cells and articular chondrocytes: analysis of chondrogenic potential and expression pattern of differentiation-related transcription factors." J Orthop Res **25**(2): 152-63.

Karlsson, C. and A. Lindahl (2009). "Articular cartilage stem cell signalling." Arthritis Res Ther **11**(4): 121.

Kawcak, C. E., C. W. McIlwraith, et al. (2001). "The role of subchondral bone in joint disease: a review." Equine Vet J **33**(2): 120-6.

Kelly, D. J. and C. R. Jacobs (2010). "The role of mechanical signals in regulating chondrogenesis and osteogenesis of mesenchymal stem cells." Birth Defects Res C Embryo Today **90**(1): 75-85.

Kelly, D. J. and P. J. Prendergast (2004). "Effect of a degraded core on the mechanical behaviour of tissue-engineered cartilage constructs: a poro-elastic finite element analysis." Med Biol Eng Comput **42**(1): 9-13.

Kelly, T. A., K. W. Ng, et al. (2009). "Analysis of radial variations in material properties and matrix composition of chondrocyte-seeded agarose hydrogel constructs." Osteoarthritis Cartilage **17**(1): 73-82.

Kelly, T. A., K. W. Ng, et al. (2006). "Spatial and temporal development of chondrocyte-seeded agarose constructs in free-swelling and dynamically loaded cultures." J Biomech **39**(8): 1489-97.

Kelly, T. A., C. C. Wang, et al. (2004). "Role of cell-associated matrix in the development of free-swelling and dynamically loaded chondrocyte-seeded agarose gels." Biorheology **41**(3-4): 223-37.

Khan, I. M., S. J. Gilbert, et al. (2008). "Cartilage integration: evaluation of the reasons for failure of integration during cartilage repair. A review." Eur Cell Mater **16**: 26-39.

Khan, W. S., A. B. Adesida, et al. (2007). "Hypoxic conditions increase hypoxia-inducible transcription factor 2alpha and enhance chondrogenesis in stem cells from the infrapatellar fat pad of osteoarthritis patients." Arthritis Res Ther **9**(3): R55.

Khan, W. S., A. B. Adesida, et al. (2009). "The epitope characterisation and the osteogenic differentiation potential of human fat pad-derived stem cells is maintained with ageing in later life." Injury **40**(2): 150-7.

Khan, W. S., S. R. Tew, et al. (2008). "Human infrapatellar fat pad-derived stem cells express the pericyte marker 3G5 and show enhanced chondrogenesis after expansion in fibroblast growth factor-2." Arthritis Res Ther **10**(4): R74.

Kim, H. J. and G. I. Im (2009). "Chondrogenic differentiation of adipose tissue-derived mesenchymal stem cells: greater doses of growth factor are necessary." J Orthop Res **27**(5): 612-9.

Kim, H. J. and G. I. Im (2009). "Combination of transforming growth factor-beta2 and bone morphogenetic protein 7 enhances chondrogenesis from adipose tissue-derived mesenchymal stem cells." Tissue Eng Part A **15**(7): 1543-51.

Kim, M. K., S. W. Choi, et al. (2009). "Autologous chondrocyte implantation in the knee using fibrin." Knee Surg Sports Traumatol Arthrosc.

Kim, Y. J., R. L. Sah, et al. (1988). "Fluorometric assay of DNA in cartilage explants using Hoechst 33258." Anal Biochem **174**(1): 168-76.

Kisiday, J. D., D. D. Frisbie, et al. (2009). "Dynamic compression stimulates proteoglycan synthesis by mesenchymal stem cells in the absence of chondrogenic cytokines." Tissue Eng Part A **15**(10): 2817-24.

Kisiday, J. D., P. W. Kopesky, et al. (2008). "Evaluation of adult equine bone marrow- and adipose-derived progenitor cell chondrogenesis in hydrogel cultures." J Orthop Res **26**(3): 322-31.

Kiviranta, I., J. Jurvelin, et al. (1987). "Weight bearing controls glycosaminoglycan concentration and articular cartilage thickness in the knee joints of young beagle dogs." Arthritis Rheum **30**(7): 801-9.

Koga, H., L. Engebretsen, et al. (2009). "Mesenchymal stem cell-based therapy for cartilage repair: a review." Knee Surg Sports Traumatol Arthrosc.

Koga, H., T. Muneta, et al. (2007). "Synovial stem cells are regionally specified according to local microenvironments after implantation for cartilage regeneration." Stem Cells **25**(3): 689-96.

Koga, H., T. Muneta, et al. (2008). "Comparison of mesenchymal tissues-derived stem cells for in vivo chondrogenesis: suitable conditions for cell therapy of cartilage defects in rabbit." Cell Tissue Res **333**(2): 207-15.

Kon, E., M. Delcogliano, et al. (2008). "Second generation issues in cartilage repair." Sports Med Arthrosc **16**(4): 221-9.

Kopesky, P. W., H. Y. Lee, et al. (2010). "Adult equine bone marrow stromal cells produce a cartilage-like ECM mechanically superior to animal-matched adult chondrocytes." Matrix Biol **29**(5): 427-38.

Kornblatt, J. A. and M. J. Kornblatt (2002). "The effects of osmotic and hydrostatic pressures on macromolecular systems." Biochim Biophys Acta **1595**(1-2): 30-47.

Krasnokutsky, S., M. Attur, et al. (2008). "Current concepts in the pathogenesis of osteoarthritis." Osteoarthritis Cartilage **16 Suppl 3**: S1-3.

Kronenberg, H. M. (2003). "Developmental regulation of the growth plate." Nature **423**(6937): 332-336.

Kuroda, R., K. Ishida, et al. (2007). "Treatment of a full-thickness articular cartilage defect in the femoral condyle of an athlete with autologous bone-marrow stromal cells." Osteoarthritis Cartilage **15**(2): 226-231.

Leddy, H. A. and F. Guilak (2003). "Site-specific molecular diffusion in articular cartilage measured using fluorescence recovery after photobleaching." Ann Biomed Eng **31**(7): 753-760.

Lee, S. Y., T. Nakagawa, et al. (2008). "Induction of chondrogenesis and expression of superficial zone protein (SZP)/lubricin by mesenchymal progenitors in the infrapatellar fat pad of the knee joint treated with TGF-beta1 and BMP-7." Biochem Biophys Res Commun **376**(1): 148-53.

Lee, S. Y., T. Nakagawa, et al. (2010). "Mesenchymal progenitor cells derived from synovium and infrapatellar fat pad as a source for superficial zone cartilage tissue engineering: analysis of superficial zone protein/lubricin expression." Tissue Eng Part A **16**(1): 317-25.

Lennon, D. P. and A. I. Caplan (2006). "Isolation of human marrow-derived mesenchymal stem cells." Exp Hematol **34**(11): 1604-5.

Li, Z., L. Kupcsik, et al. (2010). "Mechanical load modulates chondrogenesis of human mesenchymal stem cells through the TGF-beta pathway." J Cell Mol Med **14**(6A): 1338-46.

Li, Z., S. J. Yao, et al. (2010). "Chondrogenesis of human bone marrow mesenchymal stem cells in fibrin-polyurethane composites is modulated by frequency and amplitude of dynamic compression and shear stress." Tissue Eng Part A **16**(2): 575-84.

Lima, E. G., L. Bian, et al. (2007). "The beneficial effect of delayed compressive loading on tissue-engineered cartilage constructs cultured with TGF-beta3." Osteoarthritis Cartilage **15**(9): 1025-33.

Lindroos, B., S. Boucher, et al. (2009). "Serum-free, xeno-free culture media maintain the proliferation rate and multipotentiality of adipose stem cells in vitro." Cytotherapy **11**(7): 958-72.

Liu, K., G. D. Zhou, et al. (2008). "The dependence of in vivo stable ectopic chondrogenesis by human mesenchymal stem cells on chondrogenic differentiation in vitro." Biomaterials **29**(14): 2183-92.

Liu, X., H. Sun, et al. (2010). "In vivo ectopic chondrogenesis of BMSCs directed by mature chondrocytes." Biomaterials **31**(36): 9406-14.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-8.

Loboa, E. G., G. S. Beaupre, et al. (2001). "Mechanobiology of initial pseudarthrosis formation with oblique fractures." J Orthop Res **19**(6): 1067-72.

Lu, Z. F., B. Zandieh Doulabi, et al. (2007). "Differentiation of adipose stem cells by nucleus pulposus cells: Configuration effect." Biochem Biophys Res Commun **359**(4): 991-996.

Manfredini, M., F. Zerbinati, et al. (2007). "Autologous chondrocyte implantation: a comparison between an open periosteal-covered and an arthroscopic matrix-guided technique." Acta Orthop Belg **73**(2): 207-18.

Maniatopoulos, C., J. Sodek, et al. (1988). "Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats." Cell Tissue Res **254**(2): 317-330.

Mankin, H. J., A. J. Grodzinsky, et al. (2007). Articular Cartilage and Osteoarthritis. Orthopaedic Basic Science. Foundations of clinical practice. T. A. Einhorn, R. J. O'Keefe and J. A. Buckwalter. Rosemont. IL, AAOS**:** 161-174.

Manning, W. K. and W. M. Bonner, Jr. (1967). "Isolation and culture of chondrocytes from human adult articular cartilage." Arthritis Rheum **10**(3): 235-9.

Marcacci, M., E. Kon, et al. (2007). "Arthroscopic second generation autologous chondrocyte implantation." Knee Surg Sports Traumatol Arthrosc **15**(5): 610-619.

Maroudas, A. (1970). "Distribution and diffusion of solutes in articular cartilage." Biophys J **10**(5): 365-379.

Marsano, A., S. J. Millward-Sadler, et al. (2007). "Differential cartilaginous tissue formation by human synovial membrane, fat pad, meniscus cells and articular chondrocytes." Osteoarthritis Cartilage **15**(1): 48-58.

Martel-Pelletier, J. (2004). "Pathophysiology of osteoarthritis." Osteoarthritis Cartilage **12 Suppl A**: S31-3.

Martel-Pelletier, J., N. Alaaeddine, et al. (1999). "Cytokines and their role in the pathophysiology of osteoarthritis." Front Biosci **4**: D694-703.

Mauck, R. L., B. A. Byers, et al. (2007). "Regulation of cartilaginous ECM gene transcription by chondrocytes and MSCs in 3D culture in response to dynamic loading." Biomech Model Mechanobiol **6**(1-2): 113-25.

Mauck, R. L., C. T. Hung, et al. (2003). "Modeling of Neutral Solute Transport in a Dynamically Loaded Porous Permeable Gel: Implications for Articular Cartilage Biosynthesis and Tissue Engineering." J Biomech Eng **125**(5): 602-614.

Mauck, R. L., X. Yuan, et al. (2006). "Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture." Osteoarthritis Cartilage **14**(2): 179-89.

Meneghini, R. M., J. L. Pierson, et al. (2007). "The effect of retropatellar fat pad excision on patellar tendon contracture and functional outcomes after total knee arthroplasty." J Arthroplasty **22**(6 Suppl 2): 47-50.

Messner, K. (2001). Healing of articular cartilage injuries. Surgery of the knee. R. H. Lampert. Philadelphia, Churchill Livingstone. **1:** 327-340.

Meyer, E. G., C. T. Buckley, et al. (2011). "The effect of cyclic hydrostatic pressure on the functional development of cartilaginous tissues engineered using bone marrow derived mesenchymal stem cells." J Mech Behav Biomed Mater: DOI: 10.1016/j.jmbbm.2011.04.012.

Meyer, U. and H. P. Wiesmann (2006). Bone and Cartilage Engineering. Bone and Cartilage. G. Schröder. Heidelberg, Springer**:** 7-43.

Minas, T., A. H. Gomoll, et al. (2009). "Increased failure rate of autologous chondrocyte implantation after previous treatment with marrow stimulation techniques." Am J Sports Med **37**(5): 902-8.

Miura, Y., J. Parvizi, et al. (2002). "Brief exposure to high-dose transforming growth factor-beta1 enhances periosteal chondrogenesis in vitro: a preliminary report." J Bone Joint Surg Am **84-A**(5): 793-9.

Miyamoto, C., T. Matsumoto, et al. (2007). "Osteogenic protein-1 with transforming growth factor-beta1: potent inducer of chondrogenesis of synovial mesenchymal stem cells in vitro." J Orthop Sci **12**(6): 555-61.

Miyanishi, K., M. C. Trindade, et al. (2006). "Dose- and time-dependent effects of cyclic hydrostatic pressure on transforming growth factor-beta3-induced chondrogenesis by adult human mesenchymal stem cells in vitro." Tissue Eng **12**(8): 2253-62.

Miyanishi, K., M. C. Trindade, et al. (2006). "Effects of hydrostatic pressure and transforming growth factor-beta 3 on adult human mesenchymal stem cell chondrogenesis in vitro." Tissue Eng **12**(6): 1419-28.

Miyazaki, Y., T. Tsukazaki, et al. (2000). "Dexamethasone inhibition of TGF beta-induced cell growth and type II collagen mRNA expression through ERK-integrated AP-1 activity in cultured rat articular chondrocytes." Osteoarthritis Cartilage **8**(5): 378-85.

Mobasheri, A., C. Csaki, et al. (2009). "Mesenchymal stem cells in connective tissue engineering and regenerative medicine: applications in cartilage repair and osteoarthritis therapy." Histol Histopathol **24**(3): 347-66.

Mochizuki, T., T. Muneta, et al. (2006). "Higher chondrogenic potential of fibrous synovium- and adipose synovium-derived cells compared with subcutaneous fat-derived cells: distinguishing properties of mesenchymal stem cells in humans." Arthritis Rheum **54**(3): 843-53.

Moretti, M., D. Wendt, et al. (2005). "Structural characterization and reliable biomechanical assessment of integrative cartilage repair." J Biomech **38**(9): 1846-1854.

Morigi, M., B. Imberti, et al. (2004). "Mesenchymal stem cells are renotropic, helping to repair the kidney and improve function in acute renal failure." J Am Soc Nephrol **15**(7): 1794-804.

Moseley, J. B., K. O'Malley, et al. (2002). "A controlled trial of arthroscopic surgery for osteoarthritis of the knee." N Engl J Med **347**(2): 81-8.

Mouw, J. K., J. T. Connelly, et al. (2007). "Dynamic compression regulates the expression and synthesis of chondrocyte-specific matrix molecules in bone marrow stromal cells." Stem Cells **25**(3): 655-663.

Mow, V. C. and X. E. Guo (2002). "Mechano-electrochemical properties of articular cartilage: their inhomogeneities and anisotropies." Annu Rev Biomed Eng **4**: 175-209.

Mow, V. C. and R. Huiskes (2005). Basic Orthopaedic Biomechanics and Mechano-biology. W. W. Lippincott. Philadelphia.

Mow, V. C. and C. T. Hung (2001). Biomechanics of articular cartilage. Basic biomechanichs of the musculoskeletal muscle. M. Nordin and V. H. Frankel. Philadelphia, Lippincot Williams & Wilkins**:** 60-101.

Mow, V. C., S. C. Kuei, et al. (1980). "Biphasic creep and stress relaxation of articular cartilage in compression? Theory and experiments." J Biomech Eng **102**(1): 73-84.

Mow, V. C., C. S. Proctor, et al. (1989). Biomechanics of articular cartilage. Basic biomechanichs of the musculoskeletal muscle. M. Nordin and V. H. Frankel. Philadelphia, Lea & Febiger**:** 31-57.

Mueller, M. B. and R. S. Tuan (2008). "Functional characterization of hypertrophy in chondrogenesis of human mesenchymal stem cells." Arthritis Rheum **58**(5): 1377-88.

Murphy, J. M., D. J. Fink, et al. (2003). "Stem cell therapy in a caprine model of osteoarthritis." Arthritis Rheum **48**(12): 3464-74.

Nagase, T., T. Muneta, et al. (2008). "Analysis of the chondrogenic potential of human synovial stem cells according to harvest site and culture parameters in knees with medial compartment osteoarthritis." Arthritis Rheum **58**(5): 1389-98.

Nakano, T., Y. W. Wang, et al. (2004). "Chemical composition of the infrapatellar fat pad of swine." J Anat **204**(4): 301-6.

Ng, K. W., R. L. Mauck, et al. (2006). "Dynamic deformational loading results in selective application of mechanical stimulation in a layered, tissue-engineered cartilage construct." Biorheology **43**(3-4): 497-507.

Ni, Y. F., X. F. Li, et al. (2008). "In vivo chondrogenesis by co-culture of rabbit bone marrow-derived mesenchymal stem cells and chondrocytes." J Clin Rehab Tissue Eng Res **12**(16): 3185-3188.

Nishimura, K., L. A. Solchaga, et al. (1999). "Chondroprogenitor cells of synovial tissue." Arthritis Rheum **42**(12): 2631-2637.

Nowlan, N. C., C. Bourdon, et al. (2010). "Developing bones are differentially affected by compromised skeletal muscle formation." Bone **46**(5): 1275-85.

O'Driscoll, S. W., F. W. Keeley, et al. (1988). "Durability of regenerated articular cartilage produced by free autogenous periosteal grafts in major full-thickness defects in joint surfaces under the influence of continuous passive motion. A follow-up report at one year." J Bone Joint Surg Am **70**(4): 595-606.

O'Driscoll, S. W. and D. B. F. Saris (2007). Articular Cartilage Repair. Orthopaedic Basic Science. Foundations of clinical practice. T. A. Einhorn, R. J. O'Keefe and J. A. Buckwalter. Rosemont. IL, AAOS.

Obradovic, B., I. Martin, et al. (2001). "Integration of engineered cartilage." J Orthop Res **19**(6): 1089-97.

Ogawa, R., S. Mizuno, et al. (2009). "The effect of hydrostatic pressure on three-dimensional chondroinduction of human adipose-derived stem cells." Tissue Eng Part A **15**(10): 2937-45.

Ogilvie-Harris, D. J. and J. Giddens (1994). "Hoffa's disease: arthroscopic resection of the infrapatellar fat pad." Arthroscopy **10**(2): 184-7.

Oliviero, F., R. Ramonda, et al. (2010). "New horizons in osteoarthritis." Swiss Med Wkly **140**: w13098.

Palmoski, M. J., R. A. Colyer, et al. (1980). "Joint motion in the absence of normal loading does not maintain normal articular cartilage." Arthritis Rheum **23**(3): 325-34.

Park, J. S., J. S. Chu, et al. (2011). "The effect of matrix stiffness on the differentiation of mesenchymal stem cells in response to TGF-beta." Biomaterials **32**(16): 3921-30.

Park, Y., M. Sugimoto, et al. (2005). "BMP-2 induces the expression of chondrocyte-specific genes in bovine synovium-derived progenitor cells cultured in three-dimensional alginate hydrogel." Osteoarthritis Cartilage **13**(6): 527-36.

Parkkinen, J. J., J. Ikonen, et al. (1993). "Effects of cyclic hydrostatic pressure on proteoglycan synthesis in cultured chondrocytes and articular cartilage explants." Arch Biochem Biophys **300**(1): 458-65.

Pascual, M. (2011). The chondrocelect experience: durable and effective treatments for cartilage damage in the knee. 5th European Chapter of the Tissue Engineering and Regenerative Medicine International Society. C. g. TiGenix. Granada, Spain.

Pedrozo, H. A., Z. Schwartz, et al. (1998). "Growth plate chondrocytes store latent transforming growth factor (TGF)- ?1 in their matrix through latent TGF-?1 binding protein-1." J Cell Physiol **177**(2): 343-354.

Pei, M., F. He, et al. (2009). "Repair of full-thickness femoral condyle cartilage defects using allogeneic synovial cell-engineered tissue constructs." Osteoarthritis Cartilage **17**(6): 714-22.

Pei, M., F. He, et al. (2008). "Engineering of functional cartilage tissue using stem cells from synovial lining: a preliminary study." Clin Orthop Relat Res **466**(8): 1880-9.

Pei, M., F. He, et al. (2008). "Synovium-derived stem cell-based chondrogenesis." Differentiation **76**(10): 1044-56.

Pelttari, K., E. Steck, et al. (2008). "The use of mesenchymal stem cells for chondrogenesis." Injury **39 Suppl 1**: S58-65.

Pelttari, K., A. Winter, et al. (2006). "Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice." Arthritis Rheum **54**(10): 3254-66.

Pelttari, K., A. Wixmerten, et al. (2009). "Do we really need cartilage tissue engineering?" Swiss Med Wkly **139**(41-42): 602-9.

Peng, L., Z. Jia, et al. (2008). "Comparative analysis of mesenchymal stem cells from bone marrow, cartilage, and adipose tissue." Stem Cells Dev **17**(4): 761-73.

Peretti, G. M., L. J. Bonassar, et al. (1999). "Biomechanical analysis of a chondrocyte-based repair model of articular cartilage." Tissue Eng **5**(4): 317-326.

Peterson, L. (2001). International experience with autologous chondrocyte implantation. Surgery of the knee. R. H. Lampert. Philadelphia, Churchill Livingstone. **1**.

Peterson, L., T. Minas, et al. (2000). "Two-to 9-year outcome after autologous chondrocyte transplantation of the knee." Clin Orthop Relat Res(374): 212-234.

Pittenger, M. F., A. M. Mackay, et al. (1999). "Multilineage potential of adult human mesenchymal stem cells." Science **284**(5411): 143-7.

Polacek, M., J. A. Bruun, et al. (2010). "The secretory profiles of cultured human articular chondrocytes and mesenchymal stem cells: implications for autologous cell transplantation strategies." Cell Transplant.

Poole, A. R., T. Kojima, et al. (2001). "Composition and structure of articular cartilage: a template for tissue repair." Clin Orthop Relat Res(391 Suppl): S26-33.

Pretzel, D., S. Linss, et al. (2011). "Relative percentage and zonal distribution of mesenchymal progenitor cells in human osteoarthritic and normal cartilage." Arthritis Res Ther **13**(2): R64.

Radin, E. L. (2001). Structure and function of joints. Arthritis and allied conditions. A textbook of rheumatology. W. J. Koopman. Philadelphia, Lippincott Williams and Wilkins. **1:** 157-173.

Rho, G. J., B. M. Kumar, et al. (2009). "Porcine mesenchymal stem cells--current technological status and future perspective." Front Biosci **14**: 3942-61.

Richardson, S. M., R. V. Walker, et al. (2006). "Intervertebral disc cell-mediated mesenchymal stem cell differentiation." Stem Cells **24**(3): 707-716.

Richter, W. (2009). "Mesenchymal stem cells and cartilage in situ regeneration." J Intern Med **266**(4): 390-405.

Riggs, C. M. (2006). "Osteochondral injury and joint disease in the athletic horse." Equine Vet Ed **18**(2): 100-112.

Ringe, J., C. Kaps, et al. (2002). "Porcine mesenchymal stem cells. Induction of distinct mesenchymal cell lineages." Cell Tissue Res **307**(3): 321-7.

Roddy, K. A., P. J. Prendergast, et al. (2011). "Mechanical influences on morphogenesis of the knee joint revealed through morphological, molecular and computational analysis of immobilised embryos." PLoS One **6**(2): e17526.

Ruano-Ravina, A. and M. Jato Diaz (2006). "Autologous chondrocyte implantation: a systematic review." Osteoarthritis Cartilage **14**(1): 47-51.

Ruoslahti, E. and Y. Yamaguchi (1991). "Proteoglycans as modulators of growth factor activities." Cell **64**(5): 867-869.

Sakaguchi, Y., I. Sekiya, et al. (2005). "Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source." Arthritis Rheum **52**(8): 2521-9.

Sakao, K., K. A. Takahashi, et al. (2008). "Induction of chondrogenic phenotype in synovium-derived progenitor cells by intermittent hydrostatic pressure." Osteoarthritis Cartilage **16**(7): 805-14.

Sakimura, K., T. Matsumoto, et al. (2006). "Effects of insulin-like growth factor I on transforming growth factor beta1 induced chondrogenesis of synovium-derived

mesenchymal stem cells cultured in a polyglycolic acid scaffold." Cells Tissues Organs **183**(2): 55-61.

Salisbury Palomares, K. T., L. C. Gerstenfeld, et al. (2010). "Transcriptional profiling and biochemical analysis of mechanically induced cartilaginous tissues in a rat model." Arthritis Rheum **62**(4): 1108-18.

Sampat, S. R., G. D. O'Connell, et al. (2011). "Growth Factor Priming of Synovium-Derived Stem Cells for Cartilage Tissue Engineering." Tissue Eng Part A.

Schaffler, A. and C. Buchler (2007). "Concise review: adipose tissue-derived stromal cells--basic and clinical implications for novel cell-based therapies." Stem Cells **25**(4): 818-27.

Schneiderman, R., E. Snir, et al. (1995). "Insulin-like growth factor-I and its complexes in normal human articular cartilage: Studies of partition and diffusion." Arch of Biochem and Biophys **324**(1): 159-172.

Schumann, D., R. Kujat, et al. (2006). "Mechanobiological conditioning of stem cells for cartilage tissue engineering." Biomed Mater Eng **16**(4 Suppl): S37-52.

Scotti, C., B. Tonnarelli, et al. (2010). "Recapitulation of endochondral bone formation using human adult mesenchymal stem cells as a paradigm for developmental engineering." Proc Natl Acad Sci U S A **107**(16): 7251-6.

Segawa, Y., T. Muneta, et al. (2009). "Mesenchymal stem cells derived from synovium, meniscus, anterior cruciate ligament, and articular chondrocytes share similar gene expression profiles." J Orthop Res **27**(4): 435-441.

Sekiya, I., B. L. Larson, et al. (2005). "Comparison of effect of BMP-2, -4, and -6 on in vitro cartilage formation of human adult stem cells from bone marrow stroma." Cell Tissue Res **320**(2): 269-76.

Selmi, T. A. S., P. Verdonk, et al. (2007). "Autologous chondrocyte transplantation in combination with an alginate-agarose based hydrogel (Cartipatch)." Techniques in Knee Surgery **6**: 253-258.

Selmi, T. A. S., P. Verdonk, et al. (2008). "Autologous chondrocyte implantation in a novel alginate-agarose hydrogel: Outcome at two years." J Bone Joint Surg - Series B **90**(5): 597-604.

Shake, J. G., P. J. Gruber, et al. (2002). "Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects." Ann Thorac Surg **73**(6): 1919-25; discussion 1926.

Shintani, N. and E. B. Hunziker (2007). "Chondrogenic differentiation of bovine synovium: bone morphogenetic proteins 2 and 7 and transforming growth factor beta1 induce the formation of different types of cartilaginous tissue." Arthritis Rheum **56**(6): 1869-79.

Shintani, N. and E. B. Hunziker (2011). The variable effects of dexamethasone on the chondrogenic differentiation of mesenchymal stem cells. Orthopaedic Research Society. Long Beach, California, USA.

Shirasawa, S., I. Sekiya, et al. (2006). "In vitro chondrogenesis of human synovium-derived mesenchymal stem cells: optimal condition and comparison with bone marrow-derived cells." J Cell Biochem **97**(1): 84-97.

Silverman, R. P., L. Bonasser, et al. (2000). "Adhesion of tissue-engineered cartilage to native cartilage." Plast Reconstr Surg **105**(4): 1393-1398.

Smith, R. L., S. F. Rusk, et al. (1996). "In vitro stimulation of articular chondrocyte mRNA and extracellular matrix synthesis by hydrostatic pressure." J Orthop Res **14**(1): 53-60.

Solchaga, L. A., K. Penick, et al. (2005). "FGF-2 enhances the mitotic and chondrogenic potentials of human adult bone marrow-derived mesenchymal stem cells." J Cell Physiol **203**(2): 398-409.

Steadman, J. R., B. S. Miller, et al. (2003). "The microfracture technique in the treatment of full-thickness chondral lesions of the knee in National Football League players." J Knee Surg **16**(2): 83-6.

Steadman, J. R., W. G. Rodkey, et al. (1999). "[The microfracture technic in the management of complete cartilage defects in the knee joint]." Orthopade **28**(1): 26-32.

Steck, E., J. Fischer, et al. (2009). "Mesenchymal stem cell differentiation in an experimental cartilage defect: restriction of hypertrophy to bone-close neocartilage." Stem Cells Dev **18**(7): 969-78.

Stewart, A. A., C. R. Byron, et al. (2007). "Effect of fibroblast growth factor-2 on equine mesenchymal stem cell monolayer expansion and chondrogenesis." Am J Vet Res **68**(9): 941-5.

Stewart, M. C., K. M. Saunders, et al. (2000). "Phenotypic stability of articular chondrocytes in vitro: the effects of culture models, bone morphogenetic protein 2, and serum supplementation." J Bone Miner Res **15**(1): 166-74.

Stoddart, M. J., S. Grad, et al. (2009). "Cells and biomaterials in cartilage tissue engineering." Regen Med **4**(1): 81-98.

Tallheden, T., C. Bengtsson, et al. (2005). "Proliferation and differentiation potential of chondrocytes from osteoarthritic patients." Arthritis Res Ther **7**(3): R560-568.

Tam, H. K., A. Srivastava, et al. (2007). "In vitro model of full-thickness cartilage defect healing." J Orthop Res **25**(9): 1136-1144.

Tang, Q. O., K. Shakib, et al. (2009). "TGF-beta3: A potential biological therapy for enhancing chondrogenesis." Expert Opin Biol Ther **9**(6): 689-701.

Tapp, H., E. N. Hanley, et al. (2008). "Adipose-Derived Stem Cells: Characterization and Current Application in Orthopaedic Tissue Repair." Exp Biol Med (Maywood).

Tavian, M., B. Zheng, et al. (2005). "The vascular wall as a source of stem cells." Ann N Y Acad Sci **1044**: 41-50.

Theoret, C. L., S. M. Barber, et al. (1996). "Repair and function of synovium after arthroscopic synovectomy of the dorsal compartment of the equine antebrachiocarpal joint." Vet Surg **25**(2): 142-53.

Thorpe, S. D., C. T. Buckley, et al. (2008). "Dynamic compression can inhibit chondrogenesis of mesenchymal stem cells." Biochem Biophys Res Commun **377**(2): 458-62.

Thorpe, S. D., C. T. Buckley, et al. (2010). "The response of bone marrow-derived mesenchymal stem cells to dynamic compression following TGF-beta3 induced chondrogenic differentiation." Ann Biomed Eng **38**(9): 2896-909.

Todhunter, R. (1996). Anatomy and physiology of synovial joints. Joint disease in the horse. C. W. McIlwraith and G. W. Trotter. Philadelphia, W.B. Saunders**:** 1-28.

Toghraie, F. S., N. Chenari, et al. (2011). "Treatment of osteoarthritis with infrapatellar fat pad derived mesenchymal stem cells in Rabbit." Knee **18**(2): 71-5.

Tognana, E., F. Chen, et al. (2005). "Adjacent tissues (cartilage, bone) affect the functional integration of engineered calf cartilage in vitro." Osteoarthritis Cartilage **13**(2): 129-138.

Torzilli, P. A., T. C. Adams, et al. (1987). "Transient solute diffusion in articular cartilage." J Biomech **20**(2): 203-214.

Torzilli, P. A., J. M. Arduino, et al. (1997). "Effect of proteoglycan removal on solute mobility in articular cartilage." J Biomech **30**(9): 895-902.

Toyoda, T., B. B. Seedhom, et al. (2003). "Upregulation of aggrecan and type II collagen mRNA expression in bovine chondrocytes by the application of hydrostatic pressure." Biorheology **40**(1-3): 79-85.

Toyoda, T., B. B. Seedhom, et al. (2003). "Hydrostatic pressure modulates proteoglycan metabolism in chondrocytes seeded in agarose." Arthritis Rheum **48**(10): 2865-72.

Tsuchiya, K., G. Chen, et al. (2004). "The effect of coculture of chondrocytes with mesenchymal stem cells on their cartilaginous phenotype in vitro." Mater Sci Eng C **24**(3): 391-396.

Tsuji, T., F. J. Hughes, et al. (1990). "Effects of donor age on osteogenic cells of rat bone marrow in vitro." Mech Ageing Dev **51**(2): 121-32.

Vacanti, V., E. Kong, et al. (2005). "Phenotypic changes of adult porcine mesenchymal stem cells induced by prolonged passaging in culture." J Cell Physiol **205**(2): 194-201.

Vahlensieck, M., G. Linneborn, et al. (2002). "Hoffa's recess: incidence, morphology and differential diagnosis of the globular-shaped cleft in the infrapatellar fat pad of the knee on MRI and cadaver dissections." Eur Radiol **12**(1): 90-3.

Vidal, M. A., S. O. Robinson, et al. (2008). "Comparison of chondrogenic potential in equine mesenchymal stromal cells derived from adipose tissue and bone marrow." Vet Surg **37**(8): 713-24.

Vinardell, T., C. T. Buckley, et al. (2010). "Composition-function relations of cartilaginous tissues engineered from chondrocytes and mesenchymal stem cells isolated from bone marrow and infrapatellar fat pad." J Tissue Eng Regen Med: DOI:10.1002/term.

Vinardell, T., S. D. Thorpe, et al. (2009). "Chondrogenesis and integration of mesenchymal stem cells within an in vitro cartilage defect repair model." Ann Biomed Eng **37**(12): 2556-65.

Vinatier, C., D. Mrugala, et al. (2009). "Cartilage engineering: a crucial combination of cells, biomaterials and biofactors." Trends Biotechnol **27**(5): 307-14.

Vunjak-Novakovic, G. and S. A. Goldstein (2005). Biomechanical principles of cartilage and bone tissue enginering. Basic orthopaedic biomechanics and mechano-biology. V. C. Mow and R. Huiskes. Philadelphia, Lippincott, Williams and Wilkins.

Wagner, D. R., D. P. Lindsey, et al. (2008). "Hydrostatic pressure enhances chondrogenic differentiation of human bone marrow stromal cells in osteochondrogenic medium." Ann Biomed Eng **36**(5): 813-20.

Wakitani, S., T. Goto, et al. (1994). "Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage." J Bone Joint Surg Am **76**(4): 579-92.

Wakitani, S., K. Imoto, et al. (2002). "Human autologous culture expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in osteoarthritic knees." Osteoarthritis Cartilage **10**(3): 199-206.

Wakitani, S., M. Nawata, et al. (2007). "Repair of articular cartilage defects in the patello-femoral joint with autologous bone marrow mesenchymal cell transplantation: three case reports involving nine defects in five knees." J Tissue Eng Regen Med **1**(1): 74-79.

Wang, N., J. P. Butler, et al. (1993). "Mechanotransduction across the cell surface and through the cytoskeleton." Science **260**(5111): 1124-7.

Wickham, M. Q., G. R. Erickson, et al. (2003). "Multipotent stromal cells derived from the infrapatellar fat pad of the knee." Clin Orthop Relat Res(412): 196-212.

Williams, C. G., T. K. Kim, et al. (2003). "In vitro chondrogenesis of bone marrow-derived mesenchymal stem cells in a photopolymerizing hydrogel." Tissue Eng **9**(4): 679-88.

Winter, A., S. Breit, et al. (2003). "Cartilage-like gene expression in differentiated human stem cell spheroids: a comparison of bone marrow-derived and adipose tissue-derived stromal cells." Arthritis Rheum **48**(2): 418-29.

Wong, M., M. Siegrist, et al. (2003). "Cyclic tensile strain and cyclic hydrostatic pressure differentially regulate expression of hypertrophic markers in primary chondrocytes." Bone **33**(4): 685-93.

Worster, A. A., B. D. Brower-Toland, et al. (2001). "Chondrocytic differentiation of mesenchymal stem cells sequentially exposed to transforming growth factor-beta 1 in monolayer and insulin-like growth factor-I in a three-dimensional matrix." J Orthop Res **19**(4): 738-749.

Wu, M. J., Z. Y. Gu, et al. (2008). "Effects of hydrostatic pressure on cytoskeleton and BMP-2, TGF-beta, SOX-9 production in rat temporomandibular synovial fibroblasts." Osteoarthritis Cartilage **16**(1): 41-7.

Yamaguchi, Y., D. M. Mann, et al. (1990). "Negative regulation of transforming growth factor-? by the proteoglycan decorin." Nature **346**(6281): 281-284.

Yanada, S., M. Ochi, et al. (2006). "Possibility of selection of chondrogenic progenitor cells by telomere length in FGF-2-expanded mesenchymal stromal cells." Cell Prolif **39**(6): 575-84.

Yokoyama, A., I. Sekiya, et al. (2005). "In vitro cartilage formation of composites of synovium-derived mesenchymal stem cells with collagen gel." Cell Tissue Res **322**(2): 289-98.

Yoo, J. U., T. S. Barthel, et al. (1998). "The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells." J Bone Joint Surg - Series A **80**(12): 1745-1757.

Yoshimura, H., T. Muneta, et al. (2007). "Comparison of rat mesenchymal stem cells derived from bone marrow, synovium, periosteum, adipose tissue, and muscle." Cell Tissue Res **327**(3): 449-62.

Zeiter, S., P. Lezuo, et al. (2009). "Effect of TGF beta1, BMP-2 and hydraulic pressure on chondrogenic differentiation of bovine bone marrow mesenchymal stromal cells." Biorheology **46**(1): 45-55.

Zhang, Z., J. M. McCaffery, et al. (2004). "Hyaline cartilage engineered by chondrocytes in pellet culture: histological, immunohistochemical and ultrastructural analysis in comparison with cartilage explants." J Anat **205**(3): 229-37.

Zimmermann, T., E. Kunisch, et al. (2001). "Isolation and characterization of rheumatoid arthritis synovial fibroblasts from primary culture--primary culture cells markedly differ from fourth-passage cells." Arthritis Res **3**(1): 72-6.

Zuk, P. A., M. Zhu, et al. (2002). "Human adipose tissue is a source of multipotent stem cells." Mol Biol Cell **13**(12): 4279-95.

Zuk, P. A., M. Zhu, et al. (2001). "Multilineage cells from human adipose tissue: implications for cell-based therapies." Tissue Eng **7**(2): 211-28.

Appendix A: Publications and conferences

Publications

1. **Vinardell T**, Rolfe R, Buckley C, Meyer E, Murphy P, Kelly D. Hydrostatic pressure and TGF-β3 interact to regulate chondrogenesis of chondrocytes and joint derived mesenchymal stem cells *(Submitted to Eur Cell and Mat).*

2. Haugh M, Meyer E, Thorpe S, **Vinardell T**, Duffy G, Kelly D. Temporal and Spatial Changes in Cartilage-Matrix Specific Gene Expression in Mesenchymal Stem Cells in Response to Dynamic Compression. *Tissue Eng Part A. 2011 Aug 26.*

3. **Vinardell T**, Buckley C, Thorpe S, Kelly D Functional properties of cartilaginous tissues engineered from different tissues sources. *J Tissue Eng Regen Med.* 2010 Dec (29).

4. Buckley C, **Vinardell T**, Kelly D. Oxygen tension differentially regulates the functional properties of cartilaginous tissues engineered from infrapatellar fat pad derived MSCs and articular chondrocytes. *Osteoarthritis and Cartilage* 2010 Oct;18(10):1345-54.

5. Thorpe S, Buckley C, **Vinardell T**, O'Brien F, Campbell V, Kelly D. The Response of Bone Marrow-Derived Mesenchymal Stem Cells to Dynamic Compression Following TGF-beta3 Induced Chondrogenic Differentiation. *Ann Biomed Eng.* 2010 Sep; 38(9):2896-909.

6. Buckley C[1], **Vinardell T**[1], Thorpe S, Haugh M, Jones E, McGonagle D, Kelly D. Functional properties of cartilaginous tissues engineered from infrapatellar fat pad derived mesenchymal progenitor cells. *Journal of Biomechanics* 2010 Mar 22; 43(5):920-6. 1. Both of these authors contributed equally to this study. Kelly D and Vinardell T designed the experiment. Vinardell T and Buckley C performed all experimental work and data analysis. Buckley C prepared the manuscript.

7. **Vinardell T**, Thorpe S, Buckley C, Kelly D. Chondrogenesis of bone marrow mesenchymal stem cells and chondrocytes- an in vitro cartilage defect repair model. *Annals of Biomedical Engineering* 2009 Volume 37, Issue 12 (2009), Page 2556.

8. Thorpe S, Buckley C, **Vinardell T**, O'Brien F, Campbell V, Kelly D. Dynamic compression can inhibit chondrogenesis of mesenchymal stem cells. *Biochemical and Biophysical Research Communications* 2008 Dec 12;377(2):458-62.

Conference presentations

1. **Vinardell T**, Rolfe R, Buckley C, Meyer E, Murphy P, Kelly D. Hydrostatic pressure and TGF-β3 interact to regulate chondrogenesis of chondrocytes and joint derived mesenchymal stem cells. 5th European Chapter of the Tissue Engineering and Regenerative Medicine International Society, Granada, Spain 2011.

2. **Vinardell, T**. Buckley, C. Meyer, E. Kelly, D. Hydrostatic pressure and the cytokine TGF-β3 interact to regulate chondrogenesis of mesenchymal stem cells from synovial