2013-09-24

# The response of human synovial progenitor cells in a tissue-engineered construct to mechanical loading

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# The response of human synovial progenitor cells in a tissue-engineered construct to mechanical loading

by

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A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF MASTERS OF SCIENCE

BIOMEDICAL ENGINEERING GRADUATE PROGRAM

CALGARY, ALBERTA
SEPTEMBER, 2013

# Abstract

**Background** - Existing therapies for joint injury and osteoarthritis generally fail to restore the biomechanical equilibrium that is critical to joint homeostasis. The potential of cartilage tissue engineering using autologous stem cells is a promising field, but much remains to be understood. In particular, mechanical loading as a means of directing stem cell behaviour is an area of great interest.

**Methods** - Exogenous scaffold-free tissue-engineered constructs were generated from human synovial membrane and/or fluid-derived mesenchymal progenitor cells from five different donors. Cell monolayers were treated with either ascorbic acid-supplemented (AA) media or chondrogenic differentiation media, causing matrix deposition that could be aggregated into constructs. The aggregated constructs were then subjected to confined compressive loading.

**Results** - Constructs were successfully generated using both methods. Gene expression markers of chondrogenic differentiation were substantially different from controls, but varied dramatically between constructs and donors. The generated constructs exhibited a complex, heterogeneous structure under histological analysis that stained positively for cartilage markers regardless of loading.

**Conclusions** - The findings of this study likely indicate that the constructs retained their chondrogenic potential following construct generation and highlight the utility of mechanical loading in directing differentiation in tissue-engineered constructs. They also emphasize some of the challenges presented by the variability in donor tissues, and may serve as a useful platform for future studies investigating the generation of autologous tissue-engineered cartilage.

# Acknowledgements

I would like to express my deep gratitude to my supervisors, Dr. David Hart and Dr. Nigel Shrive for their guidance and expertise throughout my program.

I would also like to thank the members of my committee, Dr. Roman Krawetz, Dr. Neil Duncan, and Dr. Wendy Hutchins for their assistance and reading my thesis. In particular, I would like to thank Dr. Krawetz for his mentorship throughout my program.

I'm also very thankful to my lab mates, past and present, for all the assistance, knowledge, and entertainment they provided throughout my program: Olesja Hazenbiller, Poh Soo Lee, Joyce Mak, Katie O'Brien, Akash Fichadya,and Jon Seto. I would like to thank Olesja and Poh in particular for their frequent technical assistance and illuminating conversation.

Finally, I would like to thank my friends and family for their support throughout my program, and Lisa for her seemingly endless patience. I would also like to thank Mike and Kiran for taking the time to help proofread my thesis.

This project was supported by the Alberta Osteoarthritis Team, funded by AIHS; the University of Calgary CREATE Program, funded by NSERC; and the Queen Elizabeth II Graduate Scholarship.

# List of Presentations

Various findings from this study were presented at:

2011 OARSI World Congress on Osteoarthritis, *San Diego, USA* (September 2011)

Podium Presentation- "Normal and osteoarthritic synovial stem cell-derived tissue-engineered constructs respond to mechanical stimulus following chondrogenic differentiation"

**Geoff Buckley-Herd**, Roman Krawetz, Nigel Shrive, David Hart

12th Annual Alberta Biomedical Engineering Conference, *Banff, Canada* (October 2011)

Poster Presentation- "Normal and osteoarthritic synovial stem cell-derived tissue-engineered constructs respond to mechanical stimulus following chondrogenic differentiation"

**Geoff Buckley-Herd**, Roman Krawetz, Nigel Shrive, David Hart

2011 Canadian Arthritis Network Annual Scientific Conference, *Quebec City, Canada* (September 2011)

Poster Presentation- "Mechanical loading for the enhancement of synovial mesenchymal stem cell scaffold-free tissue-engineered constructs for cartilage repair"

**Geoff Buckley-Herd**, Roman Krawetz, Nigel Shrive, David Hart

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# List of Symbols, Abbreviations and Nomenclature

| Symbol | Definition |
|---|---|
| AA | Ascorbic acid |
| BMP-2 | Bone morphogenetic protein-2 |
| Col1 | Type-I collagen |
| Col2 | Type-II collagen |
| Col10 | Type-X collagen |
| $C_t$ value | Cycle threshold value |
| Dex | Dexamethasone |
| ECM | Extracellular matrix |
| GAG | Glycosaminoglycan |
| L+/L- | Loaded/non-loaded |
| MPC | Mesenchymal progenitor cell |
| MSC | Mesenchymal stem cell |
| rt-qPCR | Real-time quantitative polymerase chain reaction |
| SF | Synovial fluid |
| SM | Synovial membrane |
| TEC | Tissue-engineered construct |
| TGF-β | Transforming growth factor-β |

# Chapter 1

# Introduction

## 1.1 Background

Joint injury and osteoarthritis represent a substantial and growing burden on the Canadian healthcare system, and healthcare systems around the world. In 2007-2008, approximately 16% of Canadians aged 15 or older reported a form of arthritis. This figure is expected to rise to 20%, or 7 million people, by 2031 (O'Donnell *et al.*, 2010). Osteoarthritis is the most prevalent type of arthritis, affecting approximately 10% of Canadian adults. Future rates of osteoarthritis may also be affected by the increasing rates of obesity over the last several years, as obesity has been positively correlated with the development of osteoarthritis (Felson *et al.*, 1988). The direct and indirect costs of all types of arthritis in Canada in 2000 (adjusted for inflation in 2008) was $6.4 billion (O'Donnell *et al.*, 2010). However, these costs are expected to rapidly increase, with the economic burden of osteoarthritis alone estimated at $27.5 billion in 2010 (Bombardier *et al.*, 2011). Therefore, strategies for treating osteoarthritis and mitigating the resulting disability and reduction in quality of life could substantially reduce healthcare expenditures.

Most conventional methods of treating articular cartilage defects resulting from joint injury or following the onset of osteoarthritis focus on reducing pain in the joint and/or repair of the joint surface in order to restore joint function and mobility. Many surgical interventions involve recruiting bone marrow progenitor cells from the subchondral marrow below the joint cartilage. The stem cells migrate into the lesion and repair it with the deposition of fibrocartilage. Although often successful in reducing pain and restoring joint function, the fibrocartilage produced by this method possesses different mechanical properties from articular cartilage and degrades over time, rendering these treatments temporary. Other inter-

ventions involve transplanting healthy cartilage tissue. Mosaicplasty involves transplanting circular plugs of cartilage from non-articulating parts of the joint into the lesion. The gaps left at the donor site and around the transplanted plugs are filled in with fibrocartilage by the subchondral bone progenitors. However, chondrocyte death at the margin of the plugs can result in graft failure, and the risk of donor site morbidity is a possible disadvantage of this approach. Autologous chondrocyte implantation is another approach that involves the *in vitro* expansion of chondrocytes surgically obtained from non-load-bearing cartilage in the joint, such as the intercondylar notch. The chondrocytes are then reimplanted at the lesion site in a separate surgery. Despite varying degrees of success, there are a number of disadvantages associated with this intervention. Donor site morbidity is a risk, and requiring multiple surgeries is another drawback. As well, the *in vitro* expansion of chondrocytes can also result in some loss of their chondrogenic function due to dedifferentiation (Darling and Athanasiou, 2005), which can impact their performance post-implantation. Partial- or whole-joint grafts from cadaveric donors have been successful, but as with many allogeneic transplant strategies the shortage of donors is an ongoing issue and major obstacle.

Due to the limitations surrounding the current strategies for the treatment of joint injury and osteoarthritis, alternate therapies should be investigated.

## 1.2 Articular Cartilage

Articular cartilage is the thin layer of connective tissue found lining the ends of bones in articulating joints. It is typically 1-2 mm thick in the human knee, and serves to provide low-friction joint movement and a limited amount of shock absorption. Cartilage is avascular and aneural, and believed to possess limited capacity for repair.

Articular cartilage is generally modelled as a biphasic tissue comprising a fluid and a solid phase. The fluid phase makes up approximately 60-85% of the tissue wet weight and contains water and dissolved electrolytes. The solid phase comprises all of the cells, extracellular

matrix (ECM) proteins, and other substances within the tissue. The most abundant protein in articular cartilage is collagen, making up 10-30% of the wet weight of the tissue. The collagen in articular cartilage contributes to the tissue's tensile properties. While collagen is ubiquitous throughout the body, articular cartilage is unique in that type-II collagen is by far the most common isoform. It accounts for approximately 90-95% of the collagen present in articular cartilage, with the remainder comprising types VI, IX, X, and XI in small amounts.

The other main type of protein in the cartilage matrix are proteoglycans, making up 3-10% of the tissue wet weight. These are heavily glycosylated proteins in which a large number of glycosaminoglycans (GAGs) are bound to a protein core. In articular cartilage the most abundant GAGs are chondroitin sulfate and keratin sulfate. Aggrecan is the most common type of proteoglycan found in articular cartilage. Aggrecan proteins form aggregates along with hyaluronan, another type of GAG, resulting in a large, negatively charged mass. The high concentrations of aggrecan in cartilage attract water molecules, resulting in a swelling pressure that gives articular cartilage its impressive compressive properties. These forces are offset by the collagen network that exists to support the tissue structure. The function of cartilage can be thought of in terms of a dynamic balance between two opposing forces: the swelling pressure exerted by the proteoglycan activity and the constraining forces imposed by the collagen. The proper maintenance of this activity is believed to be critical for articular cartilage homeostasis and mechanical stability of a joint.

Articular cartilage is maintained by a resident population of cells called chondrocytes. Located individually or in smalls groups within cavities known as lacunae, these cells are responsible for the production of the proteins that make up articular cartilage. They constitute approximately 10% of the wet weight of articular cartilage and are distributed throughout the tissue.

## 1.3 Cartilage Structure and Function

The composition and structure of articular cartilage varies with depth. The uppermost region, the superficial zone, consists of the first 10-20% of the tissue thickness and includes the joint surface. It contains the largest proportion of collagen compared to the rest of the cartilage, and the collagen fibres are densely packed and oriented parallel to the cartilage surface. There is relatively little proteoglycan in this layer. The chondrocytes in this layer are elongated along the cartilage surface and are distinct because they express the protein lubricin, a lubricating proteoglycan that is believed to be critical for healthy joint function, which is not expressed by chondrocytes found in the deeper layers of cartilage (Schumacher *et al.*, 1994).

The middle zone constitutes the next 40-60% of cartilage thickness. The chondrocytes in this zone are more spherical than those of the superficial layer. The collagen fibres are aligned in a less orderly fashion, and the layer contains more proteoglycan than the superficial layer. Consequently, this region has a higher compressive modulus compared to the superficial layer.

The deep layer encompasses the remaining 30% of cartilage thickness. The collagen fibres are oriented perpendicular to the articular surface and help to anchor the cartilage to the underlying subchondral bone. This layer also contains the most proteoglycans in the cartilage. It is the stiffest layer in the cartilage and has the lowest concentration of fluid. The deep layer is separated from a transitional layer of mineralized cartilage and the subchondral bone by a boundary known as the tide mark.

Articular cartilage is an important tissue with unique biomechanical properties owing to its composition and biphasic structure. Its mechanical properties vary with position (heterogeneity), direction (anisotropy), as well as time (viscoelasticity). The network of proteins in the cartilage make for a relatively impermeable structure that, coupled with the hydrophilic aggrecan proteins, attract water molecules and severely limits their mobility.

The resulting interstitial fluid pressure is responsible for the majority of load transmission in articular cartilage and also gives it its unique viscoelastic properties (Soltz and Ateshian, 2000). The loss and decline in composition of proteoglycan, resulting in a more permeable, less pressurized tissue, is closely related to the impaired function of articular cartilage in the development of osteoarthritis (Akizuki *et al.*, 1987; Setton *et al.*, 1994).

Any therapy directed at the treatment of joint injury is therefore necessarily concerned with the restoration of a biomechanical environment conducive to sustained healthy joint function.

## 1.4 Cartilage Tissue Engineering

Articular cartilage possesses a minimal capacity for healing and repair. Its limited supply of nutrients and oxygen is delivered via diffusion through the synovial fluid. In other tissues, repair is typically supported by recruited progenitor cells and the local vasculature. There is a marked increase in the concentration of cells in the synovial fluid coinciding with the development of osteoarthritis (Morito *et al.*, 2008; Jones *et al.*, 2008; Sekiya *et al.*, 2012), but their contribution to cartilage repair, if any, is not fully understood.

Tissue engineering is therefore one of the most promising fields in terms of developing strategies to treat cartilage defects in order to restore the joint surface and recapitulate the biomechanical environment. Conventionally, tissue engineering is seen as the designed combination of cells, scaffold, and signals: a scaffold laden with appropriately chosen cells whose behaviour is directed by carefully administered signals in order to restore some sort of biological function. This definition is broad and encompasses a vast range of biomedical research and technologies, and this review will focus on developments closely related to the tissue engineering of articular cartilage.

### 1.4.1 Cells

A common starting point in tissue-engineering is to attempt to utilize the native cells of a target tissue. Many early studies investigating tissue-engineered articular cartilage used the chondrocytes within the native as they are known to be responsible for the production and maintenance of the cartilage matrix. These studies experimented with chondrocytes seeded in a variety of scaffold materials and yielded promising results as the cells expressed many of the proteins present in native articular cartilage, and were also capable of modifying the mechanical properties of the scaffolds (Ma *et al.*, 1995; Buschmann *et al.*, 1995). However, the use of chondrocytes in cartilage tissue engineering has a number of disadvantages. As a terminally differentiated cell, they possess limited capacity for proliferation in culture. Moreover, there is also the issue of dedifferentiation identified earlier (Darling and Athanasiou, 2005). Tissue engineering typically requires substantial expansion of cells in order to generate a sufficient number of cells, and therefore chondrocytes are not likely to be a successful candidate. As well, if autologous cells are being considered there is the risk of morbidity at the donor site.

In order to overcome these limitations, there is a great deal of interest in the use of stem cells. These types of cells hold a great deal of promise for tissue engineering because they are able to differentiate along a variety of different lineages. As well, their property of self-renewal makes them an appealing cell source for tissue engineering because, theoretically, vast quantities of cells can be produced without compromising their ability to differentiate.

Pluripotent stem cells generally refer either to embryonic stem cells (ESCs) derived from the inner cell mass of a blastocyst, or induced pluripotent stem cells (iPSCs), which are adult cells that have been reprogrammed using specific factors to induce controlled dedifferentiation toward a pluripotent state. Pluripotent stem cells exhibit the best capacity for expansion among types of stem cells, however they have a number of possible risks and disadvantages. Since they are pluripotent, they are at risk of forming tumours comprising undesired tissue

and cell types *in vivo*, necessitating strict control of their differentiation. To date, ESCs have not been successfully used to generate stable, mature articular chondrocytes (Oldershaw, 2012), necessitating further research and optimization before they can be used in any clinical setting. These practical concerns, combined with the potential ethical issues related to the use of ESCs, do not make pluripotent stem cells an ideal choice for the use in cartilage tissue engineering.

There is a significant amount of interest in the use of adult multipotent stem cells for cartilage tissue engineering. In particular, mesenchymal stem cells (MSCs) are a type of adult stem cell found in a number of tissues including bone marrow, adipose tissue, and synovial tissue. A number of terms are used interchangeably for MSCs, and they are also referred to as mesenchymal progenitor cells (MPCs) in some of the literature. For the purposes of this thesis, MPCs will be used to describe the melange of cells in adult tissues that likely contain MSCs, but may contain other cell types as well.

Related multipotent cells can be obtained from a variety of mesenchymal tissues. There is evidence to suggest that MPCs from certain tissue sources may be more suitable for differentiation along certain lineages than others. MPCs have been recently identified in the synovial membrane (SM), and may possess a uniquely enhanced capacity for chondrogenic differentiation compared to MPCs from other tissues (Sakaguchi *et al.*, 2005). MPCs have also been isolated from the synovial fluid (SF) that are thought to originate in the synovium (Jones *et al.*, 2008) and may share a similar propensity toward chondrogenic differentiation. Synovial-derived MPCs have therefore been identified as a suitable candidate for cartilage tissue engineering (Jones and Pei, 2012).

### 1.4.2 Scaffolds

In general, the scaffold in a tissue-engineered construct serves two main purposes. First, it supports the growth of the cells seeded on it. Secondly, depending on the tissue, it may be specifically designed to support the function of that tissue. For instance, the scaffold in a typ-

ical tissue-engineered bone construct is designed to have mechanical properties comparable to the native bone around it. Similar biomechanical considerations exist for cartilage tissue engineering. Restoring biomechanical equilibrium would be the main goal of tissue-engineered articular cartilage, and therefore a successful scaffold for articular cartilage tissue engineering will likely attempt to approximate similar mechanical properties such as compressive modulus, viscoelasticity, friction, etc. Other important considerations include biodegradability and integration of the scaffold material with the surrounding cartilage following implantation (Almarza and Athanasiou, 2004; Little *et al.*, 2011).

Numerous different materials are used as scaffolds for tissue-engineered articular cartilage. These can be broadly categorized into natural and synthetic biomaterials. Natural biomaterials encompass a number of diverse substances. Carbohydrate-based hydrogels such as agarose, alginate and chitosan are popular as they are generally biocompatible and are easily formed. Also, these materials have the advantage of allowing cells to retain a spherical morphology similar to chondrocytes *in vivo*. There are also a variety of protein-based natural scaffolds including collagen and fibrin. Collagen has been studied extensively because of its ubiquity. However, the collagen gels produced by existing methods do not replicate the mechanical properties of native articular cartilage. This is often attributed to the lack of organisation of the collagen fibres. Without reproducing the complex network of collagen found within native cartilage, collagen alone is not a suitable scaffold material. As well, one of the main drawbacks of natural scaffold biomaterials is that they are derived from organic sources, thus there is a risk of transmission of infectious agents as they generally cannot be sterilized by conventional means.

Most synthetic scaffold materials form polymeric hydrogels, such as polylactic acid and polyglycolic acid. Co-polymers such as polylactic-*co*-glycolic acid are also used. One of the benefits of these synthetic biomaterials is that many already have demonstrated biomedical applications, and it is consequently easier and quicker to implement them clinically. As

well, the mechanical properties of synthetic biomaterials can be fine-tuned to a certain extent, giving investigators more control as opposed to natural scaffolds. However, these also generally do not possess mechanical properties comparable to native cartilage.

A recently developed approach attempted to bypass the use of an exogenous scaffold by utilizing the matrix deposited by a cell monolayer. Specifically, porcine synovial MPCs were treated with ascorbic acid (AA) over several weeks in monolayer culture (Ando *et al.*, 2007). The cells formed monolayers of matrix proteins that, once detached, aggregated into a three-dimensional form. The resulting constructs were rich in type-I collagen and were also shown to retain their chondrogenic potential. Subsequent tests of the undifferentiated constructs in a porcine defect model yielded promising results in terms of construct integration at the defect site, cartilage-like repair tissue, and mechanical properties. However, the investigators of this study noted that some direction of cell differentiation, such as pre-differentiation with biochemical factors, may be necessary due to the presence of some fibrous tissue at the repair site. Constructs were later successfully generated from human synovial MPCs with the same method, which also possessed similar properties to those used in the porcine study (Ando *et al.*, 2008). While generating constructs using this approach is less controlled than an approach utilizing an exogenous scaffold material, it circumvents many of the major issues of most scaffold materials, such as potential byproduct cytotoxicity resulting from scaffold biodegradation, or the transmission of infectious agents.

### 1.4.3 Signals

In the context of cartilage tissue engineering, signals generally refer to the set of stimuli used to direct cells in the production and maintenance of cartilage matrix proteins. With MPCs this also implies the differentiation of the cells to a stable and mature chondrocyte phenotype. Conventionally this is achieved using biochemical factors. One of the main factors is transforming growth factor-β (TGF-β). A TGF-β protein binds to a TGF-β surface receptor, and the resulting signal is transmitted to the nucleus via the phosphorylation of

a SMAD protein (Miyazono, 2000) as part of the TGF-β signalling pathway. Once in the nucleus, it acts as a transcription factor for various chondrogenic genes. Dexamethasone is a glucocorticoid commonly used in differentiation media as it is known to act synergistically with TGF-β in promoting chondrogenic differentiation (Yoo *et al.*, 1998). Bone morphogenetic proteins (BMPs) represent another group of growth factors that also act through the TGF-β signalling pathway. In particular, BMP-2 is thought to have a greater chondrogenic effect compared to other BMPs (Sekiya *et al.*, 2005). Finally, ascorbic acid (AA) is another common biochemical in chondrogenic differentiation media due to its important role in the production of collagen and positive effect on MSC proliferation (Na *et al.*, 2007).

## 1.5 Mechanical Loading for Cartilage Tissue Engineering

A growing area of research is focused on the use of mechanical loading as a means of directing MPC differentiation, given the mechanical environment that joint tissues are exposed to on a regular basis. Typical physiological loads on joint cartilage range from 0-20 MPa at 0-1 Hz (Quinn *et al.*, 1999). Chondrocytes are known to be responsive to mechanical loading, which can induce changes in their production of matrix proteins (Buschmann *et al.*, 1995). The role of mechanical loading is well-known in other tissues such as bone, where the absence of mechanical loading (e.g. microgravity) can lead to losses in bone density and strength (Vico *et al.*, 1998).

Mechanical signals are detected and translated into biochemical signals via a process known as mechanotransduction. A variety of mechanosensitive surface receptors respond to different types of loading and transmit information along one of a number of signalling pathways into the cell's interior. Integrins are a group of transmembrane proteins that attach to extracellular matrix proteins such as collagen. Changes in the external mechanical environment are transferred via integrins into the cell. Other surface receptors include stretch-activated channels and G-proteins which are also able to detect changes in the me-

chanical environment outside of the cell. Li *et al.* (2010a) reported that mechanical signals modulate chondrogenesis through the TGF-β signalling pathway, however mechanotransduction and its implications for cartilage tissue engineering remain to be fully understood, and much research in the field is still ongoing.

The most commonly used form of mechanical loading for cartilage tissue engineering is compression (Delaine-Smith and Reilly, 2011). Compressive forces can be applied in a variety of ways, but typically rely on either hydrostatic pressure or the direct application of force, *i.e.* via a loading platen (Brown, 2000). Generally, hydrostatic pressure is used to load cells alone, while direct loading is used to compress three-dimensional specimens, such as cell-seeded hydrogels.

Despite the growing body of research the relationship between mechanical loading and MPCs remains unclear. One important aspect appears to be the timing of the loading. A number of studies have investigated bone marrow MPCs in agarose constructs under compressive load, and found that the timing of the loading relative to the chondrogenic differentiation of the cells may be an important factor in promoting MPC chondrogenesis through mechanical loading (Mouw *et al.*, 2007; Huang *et al.*, 2010). This has also been reflected in studies utilizing porcine MPCs, where Thorpe *et al.* (2010) reported similar results indicating that applying dynamic compression without prior chondrogenic differentiation inhibited chondrogenesis. One possible reason for this is the need to establish a functional pericellular matrix in order for cells to be able to detect mechanical stimuli (Haugh *et al.*, 2011).

Studies have suggested that treatment with biochemical factors may be more effective than driving differentiation with mechanical loading (Thorpe *et al.*, 2010). This was reflected by another study that used equine MPCs in agarose gels, which reported greater chondrogenesis in unloaded constructs treated with TGF-β3 than loaded and treated constructs, compared to unloaded constructs in control media (Kisiday *et al.*, 2009). Even if mechanical loading is not the most potent means of promoting chondrogenic differentiation, it may serve

as an effective complement to other protocols. Some studies have demonstrated a possible stabilizing effect of mechanical loading in order to suppress undesired cell behaviour, such as MPC hypertrophy and subsequent matrix mineralization (Bian *et al.*, 2012). The role of mechanical loading in maintaining tissue homeostasis has been suggested in other load-bearing tissues such as the meniscus (Natsu-Ume *et al.*, 2005).

Other studies have attempted to combine modes of loading in order to provide more complex signals to cells in order to drive chondrogenic differentiation. One study utilizing human bone marrow MPCs in fibrin-polyurethane scaffolds used a combination of compression and shear loading (Schätti *et al.*, 2011). They concluded that the use of combined compression and shear loading may be able to induce chondrogenesis in the absence of TGF-β. However, the investigators of that study utilized fibroblast growth factor-2 (FGF-2) and AA at different times during their experiments. These are utilized in some differentiation media (Vater *et al.*, 2011) and may have contributed to chondrogenesis and matrix formation in the constructs. Therefore, it can't be said whether the combined loading is capable of driving chondrogenic differentiation without biochemical factors.

There is a great deal of complexity associated with the different variables involved in a mechanical loading protocol. Loading magnitude, frequency and duration make up just some of the possible parameters of a mechanical loading protocol, and it can be made more complicated with the use of repeated intermittent applications of loading. It has been reported that careful selection of these parameters may be critical in optimizing chondrogenesis in cartilage tissue engineering (Li *et al.*, 2010b).

The potential of mechanical loading in cartilage tissue engineering has yet to be fully elucidated, but it does have demonstrated potential in driving chondrogenic differentiation in tissue-engineered constructs. As the underlying mechanisms of mechanotransduction become understood and more is known about the optimization of mechanical loading protocols, it is quite possible that physical stimuli may become an indispensable component in successful

cartilage tissue engineering.

## 1.6 Specific Aims and Hypothesis

Therefore, the specific aims of the present study were:

1. To investigate the differences between scaffold-free tissue-engineered constructs generated using chondrogenic differentiation media and constructs generated with AA-supplemented media.

2. To investigate the response of both of these types of constructs to compressive mechanical loading.

In the present study, it was hypothesized that the generation of exogenous scaffold-free tissue-engineered constructs by treatment with AA-supplemented media or chondrogenic differentiation media would ensure the presence of a sufficiently functional extracellular matrix such that subsequent compressive mechanical loading would promote chondrogenic differentiation in the constructs based on the expression of certain chondrogenic markers.

# Chapter 2

# Materials and Methods

## 2.1 Synovial Mesenchymal Progenitor Cell Derivation

All synovial mesenchymal progenitor cells (MPCs) used in this study were derived from primary knee synovial tissue from cadaveric donors with no clinical indications of osteoarthritis or joint injury. Specifically, MPCs were derived from both synovial membrane (SM) and synovial fluid (SF) for use in experiments. For SM MPCs, synovial membrane biopsies of approximately 1 mm diameter were obtained from the tissue and cultured in a six-well culture dish with MPC culture media. For MPCs derived from SF, a small amount (<1 mL) of the harvested SF was plated directly on a 9.5 $cm^2$ culture dish and then supplied with MPC culture media. Both SM and SF tissue biopsies were cultured for 10-14 days, during which substantial cell outgrowth and expansion was observed. The tissue biopsies were then appropriately disposed of and the cells were cultured normally. Information corresponding to the donor tissue is summarized in Table 2.1.

Table 2.1: List of Tissue Donors

| **Donor ID** | **Age** | **Sex** | **Cell Source** |
|---|---|---|---|
| 1 | 75 | M | SM and SF |
| 2 | 60 | M | SM |
| 3 | 33 | F | chondrocytes |
| 4 | 49 | M | SF |
| 5 | 75 | M | SM |
| 6 | 49 | F | SM and SF |

## 2.2 Cell Culture

### 2.2.1 Culture Media

Cells from above were expanded in MPC culture media consisting of DMEM/F12 media (Gibco, Life Technologies, Rockville, MD, USA, #11330032) supplemented with 10% FBS (Gibco, #12483020) , 1× antibiotic-antimycotic (Gibco, #15240062), 1× minimum-essential media non-essential amino acids (MEM-NEAA) (Gibco, #11140050), and 0.18% 2-mercaptoethanol (Gibco, #21985023). Cells were cultured at 37°C with 5% $CO_2$ and 95% humidity at all times. After two passages, cells were enriched for synovial progenitors via magnetic purification before additional expansion in MPC media (4-6 passages) for experiments.

Experiments involved treating cells with either chondrogenic differentiation media or ascorbic acid-supplemented media. Chondrogenic differentiation media consisted of MPC culture media further supplemented with 500 ng/mL BMP-2 (Biozentrum, Universität Würzburg, Würzburg, Germany), 10 ng/mL TGF-β3 (PeproTech, Rocky Hill, NJ, USA, #10036E), 50 mg/mL ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA, #A4403), $10^{-8}$M dexamethasone (Sigma-Aldrich, #D2915), 40 µg/mL proline as 2.48% v/v MEM-NEAA, 100 µg/mL sodium pyruvate (Sigma-Aldrich, #S8636), and 1× insulin-transferrin-selenium (Lonza, Allendale, NJ, USA, #178382). AA media consisted of MPC culture media supplemented with 50 mg/mL ascorbic acid.

### 2.2.2 Magnetic Cell Purification

The MPCs used in this study were first depleted for various somatic cell types using a commercially available kit and subsequently enriched for $CD90^+$ cells. Human synovial progenitors that express CD90 have been shown to possess an enhanced capacity for chondrogenesis without a micro-mass step (Krawetz *et al.*, 2012). Briefly, cells were washed with 1× PBS and trypsinized using 0.25% trypsin-EDTA (Gibco, #25200072) to produce a single-cell sus-

pension. Cells were then pelleted and resuspended in 1× MAG buffer (BD Biosciences, Mississauga, ON, Canada, #552362). Cells were then labelled using a biotinylated antibody solution for human lineage depletion (BD Biosciences, #519005225), which were then coupled with streptavidin-bound magnetic particles (BD Biosciences, #519003746). Labelled and unlabelled cells were then separated using a cell separation magnet (BD Biosciences, #552311). The unlabelled fraction (i.e. the progenitor cell-rich fraction) was then labelled with a biotinylated CD90 antibody (BD Biosciences, #555594) and bound to streptavidin-bound magnetic particles. The magnetic separation yielded an enriched CD90+ population of progenitor cells which were used for all experiments in this study.

## 2.3 Generation of Tissue-Engineered Constructs

For experiments, cells were plated in six-well culture dishes at $2.0 \times 10^5$ cells/well ($20.8 \times 10^4$ cells/cm$^2$) in MPC culture media and left overnight to allow cell adhesion. Cells were then treated with either chondrogenic differentiation media or AA media for 14-21 days to generate chondrogenic or AA constructs, respectively. Differentiation and AA media were prepared fresh as needed for experiments. Within 7 days a visible layer of extracellular matrix had formed in both sets of cultures. Between days 10-21 some of the cell monolayers were observed to undergo auto-aggregation, in which they spontaneously detached and contracted into a three-dimensional construct form. Monolayers that did not auto-aggregate by day 18 were gently detached manually using a cell scraper. During the remaining days of differentiation, the constructs underwent substantial and visible three-dimensional aggregation to form tissue-engineered constructs (TECs). Constructs were subsequently referred to as AA or chondrogenic constructs based on their respective method of generation.

The number of constructs that could be generated from each cell source was primarily limited by the number of available cells. For most experiments there were sufficient cells for 8 constructs, divided equally into chondrogenic and AA construct groups.

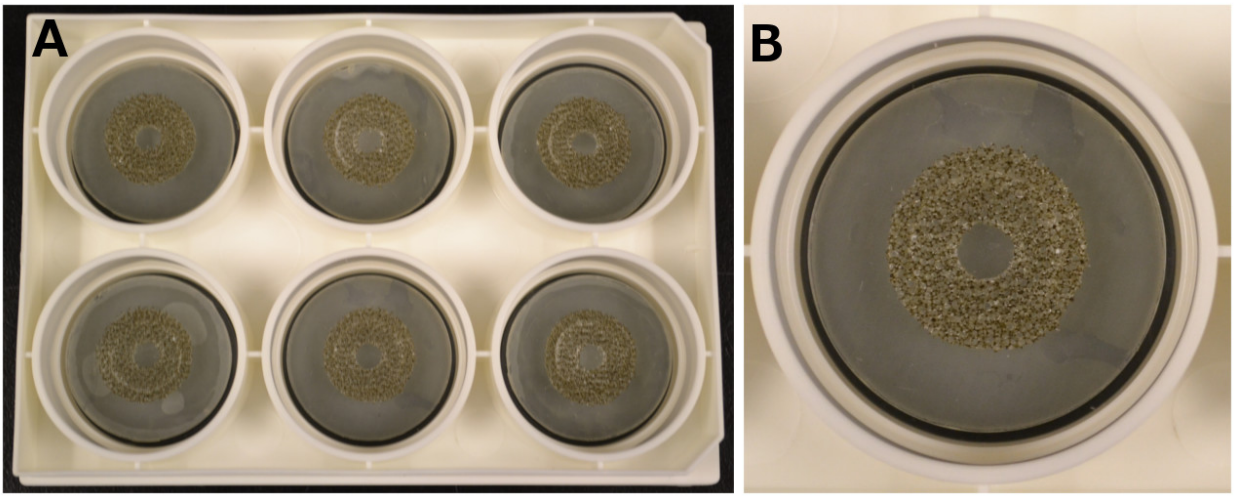


Figure 2.1: A standard Flexcell Biopress plate (A) with detail of an individual well and platen (B)

## 2.4 Mechanical Loading of Constructs

### 2.4.1 Initial Tests

Early tests used the Flexcell Biopress system to subject TECs to mechanical loading. The Biopress system is designed to administer unconfined compression. It makes use of special compression plates (Flexcell International, Hillsborough, NC, USA, #BF-3000C), which resemble a standard six-well culture dish with a silastic membrane in place of the well bottoms (Fig. 2.1A). A stiff plastic platen is adhered to the membrane, with a foam ring centred on the platen that helps position a specimen during loading (Fig. 2.1B). When set up in the Flexcell FX-4000 Compression system (Flexcell), compressed air applied against the silastic membrane forces the stiff disc against the well lid, compressing the specimen. Early test results indicated that this system was not suited to compressing the TECs, as the constructs tended to become entangled in the foam rings. This resulted in substantial construct fragmentation and degradation, and made them exceptionally difficult to remove after loading.

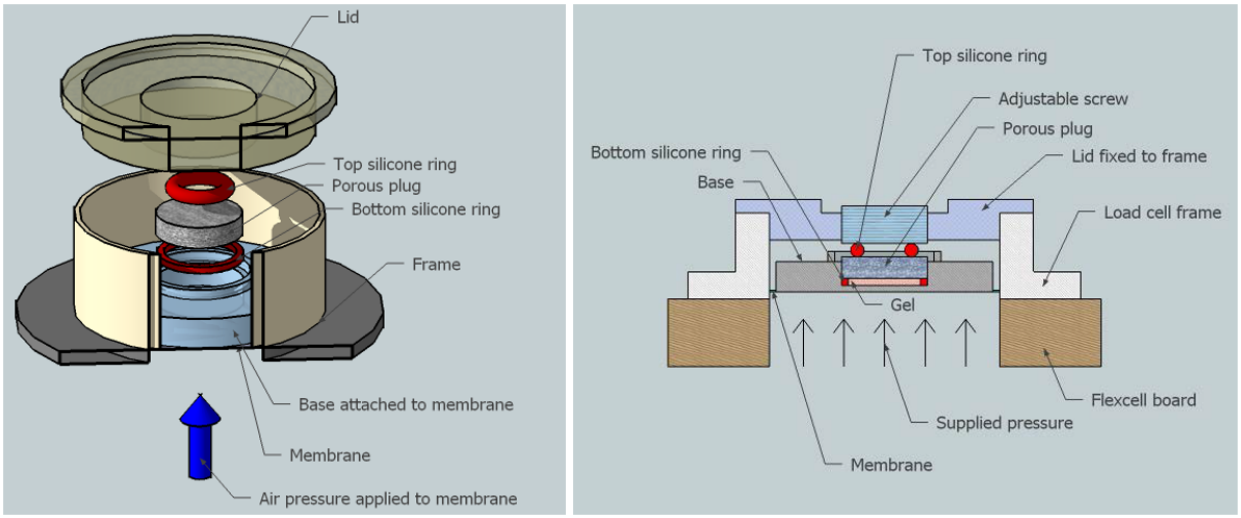


Figure 2.2: Overview of the modified Flexcell system, showing an exploded assembly (left) and a cross-section of the assembled system (right). Adapted from Hazenbiller (2012) with kind permission from Olesja Hazenbiller (see Appendix B).

### 2.4.2 Modified Compression

The TECs were then subjected to compressive mechanical loading using a modified Flexcell system developed by Hazenbiller (2012) for the confined compression of cell-laden collagen gels. The modified loading system is shown schematically in Fig. 2.2. Briefly, the modification involved replacing the original platen and foam ring component with a custom-designed platen adhered to the silastic membrane of a standard Flexcell six-well plate. The platen contains a well that is fitted with a square cross-section silicone O-ring, a porous stainless steel plug, and a circular cross-section silicone O-ring. The gel resides in the void formed between the porous plug, the square O-ring, and the bottom of the platen well. During loading, the loading space is supported partly through the action of the square O-ring. As in the unmodified system, positive air pressure is then used to produce mechanical loading by compressing the circular O-ring and porous plug series, resulting in a controlled strain in the gel. The main difference in the modified system is a transition from unconfined to confined compression.

The constructs were cast in 0.5% m/v agarose gels (Invitrogen, Life Technologies, #15510027)

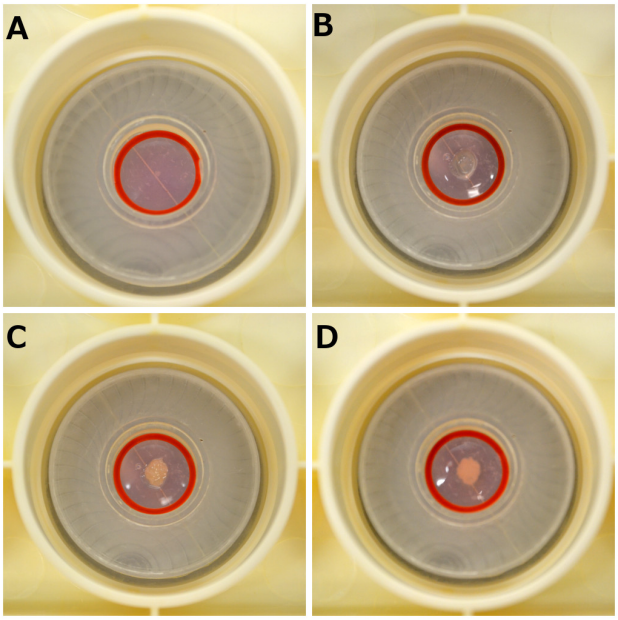


Figure 2.3: Agarose gels are formed in the platen wells (A), where a cavity is made with a pipette tip (B). The construct is positioned (C) and then capped with a thin film of agarose (D).

using MPC media in the loading space of the platen well. This was done to improve specimen consistency for loading, since the aggregated TECs varied in size and shape. First, 0.5% agarose gels were formed in the well with the square O-ring in place (Fig. 2.3A). Small cavities were formed in the centre of the gels using 200 µL pipette tips (Fig. 2.3B). The size of the cavities were approximately matched with the size of the corresponding TECs. The TECs were then placed in the the gel cavities (Fig. 2.3C), followed by a thin film of agarose (Fig. 2.3D). Lastly, 100µL MPC media was then pipetted on top of the gels before placing the porous plug in order to eliminate air pockets. The embedded TECs were then left overnight at 37°C, 5% $CO_2$ and 95% humidity.

For loading, the TEC-loaded Flexcell plates were fully assembled (i.e. the circular O-rings and well lids were positioned) and set up in the Flexcell system in a separate incubator at 37°C with 5% $CO_2$ and 95% humidity. The constructs were then loaded at approximately 5% compressive strain, based on characterization tests performed on the system using collagen gels (Hazenbiller, 2012). It was assumed that the strains in the agarose gels would

closely mirror those measured in the characterization tests with collagen gels and would be substantially more compliant than the square O-ring. The loading was applied cyclically at 1 Hz for 4 hours. Unloaded controls consisted of fully assembled plates with embedded TECs incubated alongside the loaded constructs during loading. The total number of constructs were evenly divided between loaded and unloaded groups, giving 2 constructs per treatment group (i.e. 2 constructs each generated from chondrogenic media or AA media, and unloaded or loaded). Following loading, the TECs were incubated at 37°C for an additional 24 hours before harvesting for downstream analysis. Contamination has been identified as a possible issue with the modified system, since some components can't be autoclaved (Hazenbiller, 2012). However, no instances of contamination occurred during these experiments.

## 2.5 Gene Expression

### 2.5.1 Total RNA Extraction

After mechanical loading, one TEC from each treatment group was placed in a 1.5 mL microfuge tube with 1 mL Trizol (Ambion, Life Technologies, #15596018) and stored at -80°C. For RNA extraction, samples were removed from -80°C and allowed to thaw on ice. The samples were then homogenized by repeated drawing through 18-gauge and 21-gauge needles until the constructs were fragmented. Glycogen (Ambion, #AM9510) was then added at 0.25 µg/µL and left to incubate at room temperature for 5 minutes. Phase separation was performed by adding 200 µL chloroform to the samples and vortexing until mixed. The samples were then incubated at room temperature for 10 minutes, and then centrifuged at 12 000g and 4°C for 15 minutes. The resulting RNA-containing aqueous top phase was transferred to a new 1.5 mL microfuge tube. The RNA in solution was then precipitated with 500 µL isopropanol, and the samples were left to incubate at room temperature for 10 minutes. The precipitated RNA was pelleted through centrifugation at 12000g and 4°C for 10 minutes, and the supernatant was discarded. The pellet was then washed with 1 mL

75% ethanol and centrifuged again at 12 000g for 5 minutes, and the wash was discarded. The tubes were then inverted and allowed to air dry for 10 minutes. Finally, the RNA was resuspended by adding 22 µL nuclease-free ultrapure water. The tubes of RNA were put on ice for immediate RNA quantification, or otherwise stored at -80°C.

### 2.5.2 RNA Quantification

The isolated total RNA was quantified using a NanoVue spectrophotometer (GE Healthcare Life Sciences, Pittsburgh, PA, USA). For a reference, the same nuclease-free ultrapure water used in RNA resuspension was used. After calibrating the system with two 2 µL reference measurements, the sample RNA concentrations were measured using 2 µL total RNA.

### 2.5.3 cDNA Synthesis

Complementary DNA (cDNA) synthesis was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, #4368813), based on the supplied manufacturer directions. Briefly, the component reagents were thawed on ice and a 2× cDNA master mix was prepared according to manufacturer's instructions (Table 2.2). Reaction volumes were adjusted as necessary for volume requirements, but for convenience a 30µL reaction volume is assumed here. The amount of total RNA used for a cDNA synthesis reaction varied depending on the total amount of RNA available from an experiment, but typically varied within 0.25-0.5 µg total RNA for a single cDNA synthesis reaction. The calculated volume of total RNA was transferred to a 0.2 mL microfuge tube along with an appropriate volume of nuclease-free ultrapure water to bring the total RNA volume to 15µL. The RNA was then combined with 15µL of the 2× cDNA master mix and mixed with brief pipetting. The mixed tubes were then placed in a C1000 Thermocycler (Bio-Rad, Hercules, CA, USA) and treated with a standard thermal protocol (Table 2.3). The resulting synthesized cDNA was then immediately used for quantitative polymerase chain reaction.

Table 2.2: cDNA reverse transcription master mix composition

| **Reagent** | **Volume (µL)** |
|---|---|
| 10× RT buffer | 3.0 |
| 10× Random primers | 3.0 |
| 25× dNTP mix | 1.2 |
| Reverse transcriptase | 1.5 |
| Nuclease-free water | 6.3 |
| **Total** | **15.0** |

Table 2.3: cDNA reverse transcription thermal protocol

| **Step** | **Temperature (°C)** | **Time (minutes)** |
|---|---|---|
| 1 | 25 | 10 |
| 2 | 37 | 120 |
| 3 | 85 | 5 |
| 4 | 4 | indef. |

### 2.5.4 Quantitative Polymerase Chain Reaction

Quantitative polymerase chain reaction (kqPCR) analysis to investigate gene expression was performed using the TaqMan platform, with TaqMan Gene Expression Master Mix (Applied Biosystems, #4369016) and a variety of primer-probes (all from Applied Biosystems). The probes used in this study are summarized in Table 2.4. For all analysis in the present study, kqPCR was performed using three replicates per sample per gene. Probe master mixes for each gene were prepared by combining appropriate volumes of 2× TAQ master mix, primer-probe, and nuclease-free ultrapure water in separate 1.5 mL microfuge tubes. The specific volumes for a single 10µL kqPCR reaction are given in Table 2.5. A 384-well PCR plate (Applied Biosystems) was prepared using a BioMek 3000, an automated laboratory pipetting workstation (Beckman Coulter, Indianapolis, IN, USA, #986120). The pipetting protocol was programmed in two main phases. In the first phase, 9 µL volumes of the prepared primer-probe master mixes were transferred into the reaction plate. Simultaneously, the synthesized cDNA was transferred from the microfuge tubes to a 96-well PCR plate because the automated workstation was not equipped to handle PCR microfuge tubes. The second phase involved transferring 1 µL volumes of cDNA from the 96-well plate to the

appropriate wells of the reaction plate. Each combination of primer-probe and sample cDNA was conducted using three technical replicates (i.e. triplicate wells on the PCR plate). Finally, negative controls were added by transferring 10µL of each primer master mix into separate wells of the reaction plate. The reaction plate was then sealed with PCR adhesive film (VWR, Radnor, PA, USA, #60941078). The plate was then briefly mixed using a microplate mixer and centrifuged. The reaction plate was then amplified and analyzed using a 7900HT Fast Real-Time PCR system (Applied Biosystems).

Table 2.4: Summary of kqPCR Primer-probes

| **Gene** | **ABI Product no.** | **Description** |
|---|---|---|
| 18s | Hs03928985_g1 | Endogenous control, ribosomal RNA |
| Col1 | Hs00164004_m1 | Type-I collagen |
| Col2 | Hs00264051_m1 | Type-II collagen |
| Col10 | Hs00166657_m1 | Type-X collagen |
| SOX9 | Hs01001343_g1 | Chondrogenic transcription factor |
| Aggrecan | Hs00153936_m1 | Cartilage proteoglycan |
| Runx2 | Hs00231692_m1 | Osteogenic transcription factor |
| Adiponectin | Hs00605917_m1 | Adipogenic marker |

Table 2.5: Composition per 10 µL kqPCR reaction

| **Reagent** | **Volume (µL)** |
|---|---|
| TAQ Master Mix | 5.0 |
| Primer-probe | 0.5 |
| Nuclease-free water | 3.5 |
| cDNA | 1.0 |
| **Total** | **10.0** |

### 2.5.5 kqPCR Data Analysis

Analysis of gene expression data was performed using the relative quantification by comparative $C_t$ method, also known as the $\Delta\Delta C_t$ method. Raw $C_t$ data values were obtained using RQ Manager v1.2.1, the proprietary software supplied with the 7900HT system. The software automatically determines baseline and threshold values in order to calculate $C_t$ values from the produced amplification curves. These values were manually verified in several

experiments to determine that the software was working properly.

Calculating relative gene expression values is done through a series of subtractive calculations. The first operation involves normalizing all values belonging to a given sample to the average $C_t$ value of an endogenous control or housekeeping gene (Eq. 2.1). In these experiments, the ribosomal RNA 18s was used for an endogenous control. The resulting $\Delta C_t$ values of a sample can then be normalized to those of a control sample in order to generate $\Delta\Delta C_t$ values (Eq. 2.2). In general, for these experiments, the controls were either the unloaded AA constructs or the unloaded constructs of a particular treatment. Note that whereas $\Delta C_t$ values are calculated within a single sample but across all genes, $\Delta\Delta C_t$ values are calculated within a single gene but across all samples. Finally, the $\Delta\Delta C_t$ values can be converted to a relative quantification (RQ) value (Eq. 2.3).

$$\Delta C_{\text{t, sample}} = C_{\text{t, sample}} - C_{\text{t avg, endogenous control}} \tag{2.1}$$

$$\Delta\Delta C_{\text{t, sample}} = \Delta C_{\text{t, sample}} - \Delta C_{\text{t avg, control}} \tag{2.2}$$

$$RQ_{\text{sample}} = 2^{-\Delta\Delta C_{\text{t, sample}}} \tag{2.3}$$

In cases where genes were not detected, the amplification curves either failed to rise above the threshold value, or in some cases only some of replicates passed the threshold. Besides the automatic thresholding performed by the software, only $C_t$ values under 38 were considered to be "amplified" (Burns and Valdivia, 2007). One of two things was done to minimize the impact of non-amplified wells on the data analysis without introducing false positive data. In cases where only some replicates failed to reach threshold, these wells were assigned a $C_t$ value of 40, the theoretical upper limit of the system. If no wells for a given sample and gene combination reached the threshold and thus had an undetermined $C_t$ value, they were assigned a $\Delta C_t$ value of 40, which is the practical equivalent of non-

detection for the purposes of this analysis. This was done to minimize the potential skewing effect of an undetermined $C_t$ value. Without this alteration, it would be possible to produce a significant difference in RQ values for particular gene even if it wasn't detected in either the target sample nor the calibrator sample, as a result of differences in the $\Delta C_t$ values produced by unequal endogenous control $C_t$ values in the first subtraction calculation.

## 2.6 Histology

### 2.6.1 Construct Fixation and Embedding

One construct from each treatment group was fixed in 250µL of 4% paraformaldehyde overnight at 4°C. Constructs were then washed twice in PBS and transferred to a plastic dish where excess liquid was removed by pipette. Constructs were subsequently embedded in Optimal Cutting Temperature (OCT) compound (TissueTek, Sakura Finetek, Torrance, CA, USA, #4583) in cryosectioning moulds and snap-frozen in a methylbutane bath chilled by dry ice. Embedded constructs were stored at -80°C until cryosectioning.

### 2.6.2 Cryosectioning

Frozen constructs were removed from storage at -80°C and warmed in a -20°C freezer for one hour, and then transferred to a cryostat at -18°C. Sections of 7µm thickness were obtained and collected on SuperFrost Plus micro slides (VWR, #48311703). The sections were allowed to air dry at room temperature for 30 minutes, and then stored at -20°C.

### 2.6.3 Histological Staining

Hematoxylin and Eosin (H&E) Stain

Sections were rehydrated in $dH_2O$ for 3 minutes, followed by staining in hematoxylin for 20 mins. The slides were rinsed in running tap water for 15 minutes, and subsequently dipped 3-4 times each in 1% acid alcohol, $dH_2O$, ammonia water, and again in $dH_2O$. They were then

dipped once quickly in 70% ethanol and then stained in eosin for 8 minutes. The sections were then dehydrated through dips in 70% and 95% ethanol, and 2×3-minute immersions in 100% ethanol. Lastly, they were cleared in 2×3-minute dips in xylene, followed by mounting and coverslipping.

Alcian Blue Stain

Sections were rehydrated in $dH_2O$ for 3 minutes, followed by a 1 minute immersion in 3% glacial acetic acid. Sections were then stained for 2 hours in Alcian blue stain composed of filtered 1% m/v Alcian blue 8GX (Sigma-Aldrich, #A5268) in 3% glacial acetic acid adjusted to a pH of 2.5. The sections were then washed in running tap water for 10 minutes and then dehydrated through sequential 2 minute immersions in 70%, 95%, and 100% ethanol. Finally, the slides were dipped in 50% ethanol/xylene and cleared in xylene for 2 minutes followed by mounting and coverslipping.

Alizarin Red

Sections were rehydrated in $dH_2O$ for 3 minutes and then stained in Alizarin red solution for 3 minutes. The Alizarin red solution was composed of filtered 2% m/v Alizarin red S (Sigma-Aldrich, #A5533) in $dH_2O$, with a pH adjusted to 4.1-4.3 with 5% ammonium hydroxide. Excess dye was removed from the slides and the sections were dipped 20 times in acetone, dipped once in 50% acetone/xylene, and cleared twice for 2 minutes in xylene. Slides were then mounted and coverslipped.

Oil Red O Stain

Sections were rehydrated in $dH_2O$ for 3 minutes. They were then dipped in 60% isopropanol and stained in Oil Red O solution for 15 minutes. The Oil Red O solution consisted of 30 mL of 0.5% m/v Oil Red O in 100% isopropanol mixed with 20 mL $dH_2O$. The slides were then dipped 2-3 times in 60% isopropanol and rinsed gently in $dH_2O$. Lastly, slides were mounted with aqueous mountant and coverslipped.

## 2.7 Statistics

Statistics were performed using toolboxes provided in GraphPad Prism 6 and MATLAB v7.1. For the analysis of gene expression, a series of Wilcoxon signed-rank tests were used to investigate several different comparisons in the data sets: AA versus chondrogenic, and unloaded versus loaded for both AA and chondrogenic constructs. The mean $\Delta\Delta C_t$ values for each set of data were calculated (Yuan *et al.*, 2006), and paired within a sample according to the desired comparison. The null hypothesis that the median difference between paired values was equal to zero was then tested using a two-tailed test, with $\alpha = 0.05$. The $\Delta\Delta C_t$ values were then converted to RQ-values for presentation on a base-10 logarithmic scale in figures.

# Chapter 3

# Results

## 3.1 Initial Tests

Synovial membrane (SM) and synovial fluid (SF) MPCs (Donor 1) were used to produce chondrogenic and AA constructs over a 14-day generation period. They were then subjected to mechanical loading for 4 hours alongside non-loaded controls (L+/L-), and harvested for analysis 16 hours after the end of mechanical loading. This time point was selected because upregulation in chondrogenic markers had been seen at the same time point in murine ESCs loaded with the same device (Hazenbiller, 2012), and was a reasonable starting time point in terms of experimental logistics. All constructs in this experiment were analyzed for the expression of commonly-used chondrogenic markers including SOX9, aggrecan, and type-II collagen (Fig. 3.1). An overview of these findings is provided in Table 3.1.

There were no clear effects of either loading or treatment in the chondrogenic markers, partially as a result of variability in the kqPCR findings. Part of this variability may be attributable to methodological issues. Difficult total RNA extraction coupled with low total RNA yields in these experiments likely rendered them vulnerable to error that would become visible as variation during kqPCR. As well, RNA quality was not assessed and may also have impacted these results. Consequently, the application of statistics generated no meaningful relationships in these particular data.

Perhaps the most outstanding feature from these data is the lack of a clear expression pattern. The expression of SOX9 was variable and not substantially different between the AA and chondrogenic constructs. The expression of aggrecan was weak in general, and type-II collagen expression was almost non-existent except for one or two replicates in certain samples.

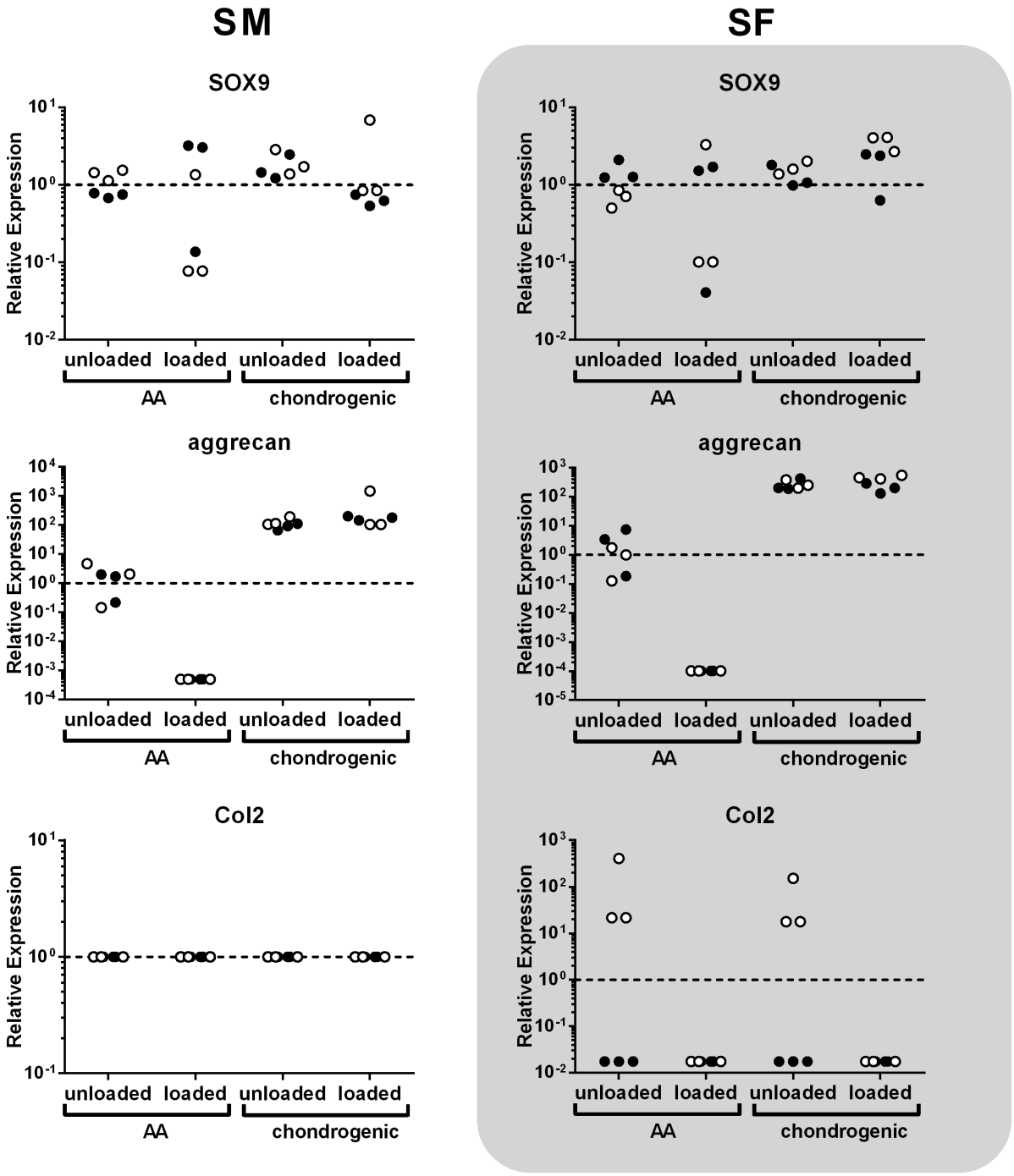


Figure 3.1: Gene expression of chondrogenic markers in constructs generated from SM (left column) and SF (right column) constructs assessed by kqPCR (Donor 1, n = 2 constructs per treatment group). Normalized to unloaded AA constructs. Solid/hollow data points indicate corresponding technical replicates.

Table 3.1: Overview of Donor 1 kqPCR data

| **SOX9** | # | **AA L-** | **AA L+** | **chondrogenic L-** | **chondrogenic L+** |
|---|---|---|---|---|---|
| SM | 1 | pos | mix | pos | pos |
| | 2 | pos | mix | pos | mix |
| SF | 1 | pos | mix | pos | pos |
| | 2 | pos | mix | pos | pos |

| **Aggrecan** | # | **AA L-** | **AA L+** | **chondrogenic L-** | **chondrogenic L+** |
|---|---|---|---|---|---|
| SM | 1 | mix | neg | pos | pos |
| | 2 | mix | neg | pos | mix |
| SF | 1 | mix | neg | pos | pos |
| | 2 | mix | neg | pos | pos |

| **Col2** | # | **AA L-** | **AA L+** | **chondrogenic L-** | **chondrogenic L+** |
|---|---|---|---|---|---|
| SM | 1 | neg | neg | neg | neg |
| | 2 | neg | neg | neg | neg |
| SF | 1 | neg | neg | neg | neg |
| | 2 | mix | neg | mix | neg |

Overview of Donor 1 SM and SF construct kqPCR data, indicating whether genes were positively (pos), partially (mix), or not detected (neg) in a particular sample. Partially detected genes were amplified in some but not all technical replicates.

## 3.2 Loading Response Time Course

In an attempt to improve the construct generation process and elicit more pronounced and consistent construct behaviour, the time in treatment with either AA media or chondrogenic media was extended to 21 days for all subsequent sets of constructs. As well, in order to optimise data collection, the time course of the response of the constructs to mechanical loading was investigated. SM MPC (Donor 2) constructs of both types were subjected to mechanical loading for 4 hours and harvested for analysis at 0, 24, and 48 hours after loading. Two replicate constructs per treatment at each time point were used in this experiment. Gene expression was analyzed for the chondrogenic markers SOX9, aggrecan, and type-II collagen (Fig. 3.2). An overview of these findings is provided in Table 3.2.

The expression of SOX9 was reasonably consistent among the AA constructs. In comparison to the 0-hour post-load time point, there was a substantial upregulation of expression 24

hours later. At the 48-hour time point the expression was highly varied, likely as a result of inconsistent detection of the gene in the samples. The SOX9 expression in the chondrogenic constructs was somewhat similar, though substantially more varied. The constructs exhibited differing levels of SOX9 expression at even the 0-hour time point. There was additional variability at the 24-hour time point, with one construct exhibiting over 10-fold upregulation and the other showing modest downregulation in comparison to the time-zero constructs. The constructs at the 48-hour time point, however, both showed downregulated expression of SOX9 in comparison to the constructs at the first time point.

The gene expression of aggrecan did not exhibit any trends over the time period studied. However, it was not consistently detected in all technical replicates at the 48-hour time point.

Type-II collagen expression was also highly variable. Of the markers investigated in this particular experiment it was detected the least frequently. It was only weakly detected in some constructs, leading to dramatic differences in expression compared to samples with no observable expression. Therefore, little can be said regarding the expression of type-II collagen over the time period investigated.

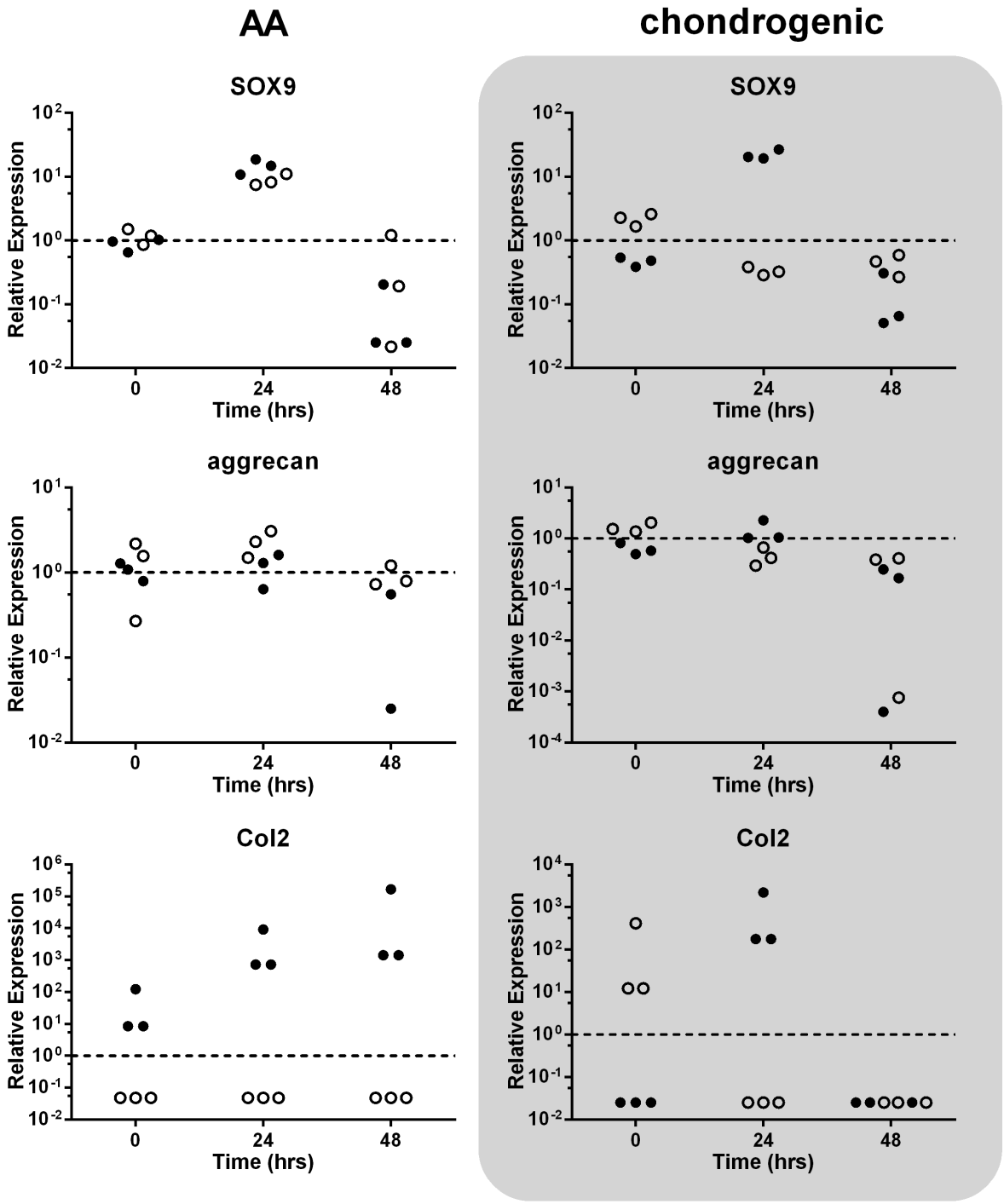


Figure 3.2: Time course of gene expression of AA (left) and chondrogenic (right) SM MPC constructs following mechanical loading assessed by kqPCR (Donor 2, n = 2 constructs per treatment per time point). Normalized to 0-hour constructs. Solid/hollow data points indicate corresponding technical replicates.

Table 3.2: Overview of Donor 2 Time Course kqPCR

| **SOX9** | # | **0h** | **24h** | **48h** |
|---|---|---|---|---|
| AA | 1 | pos | pos | mix |
| | 2 | pos | pos | mix |
| Chondrogenic | 1 | pos | pos | pos |
| | 2 | pos | pos | pos |
| **Aggrecan** | # | **0h** | **24h** | **48h** |
| AA | 1 | pos | pos | mix |
| | 2 | pos | pos | pos |
| Chondrogenic | 1 | pos | pos | mix |
| | 2 | pos | pos | mix |

| **Col2** | # | **0h** | **24h** | **48h** |
|---|---|---|---|---|
| AA | 1 | mix | mix | mix |
| | 2 | neg | neg | neg |
| Chondrogenic | 1 | neg | mix | neg |
| | 2 | mix | neg | neg |

Overview of Donor 2 time course kqPCR data, indicating whether genes were positively (pos), partially (mix), or not detected (neg) in a particular sample. Partially detected genes were amplified in some but not all technical replicates.

## 3.3 Response of Articular Chondrocytes to Mechanical Loading

In order to determine whether the mechanical loading protocol used in this study would elicit a response in human articular chondrocytes, the gene expression of loaded chondrocytes was assessed. Pellets of human articular chondrocytes (Donor 3) at passage 3 ($1.75 \times 10^5$ cells/pellet) were embedded in agarose scaffolds and subjected to mechanical loading for 4 hours. Gene expression was assessed following the 24-hour rest period.

The chondrocytes were then analyzed for expression of chondrogenic markers (Fig. 3.3). An overview of these findings is given in Table 3.3. Due to an extremely limited number of cells available for use in this experiment only one pellet could be generated for each treatment. The loaded chondrocytes did exhibit lower levels of expression of both SOX9 and type-II collagen, while expression of aggrecan appeared to be slightly elevated.

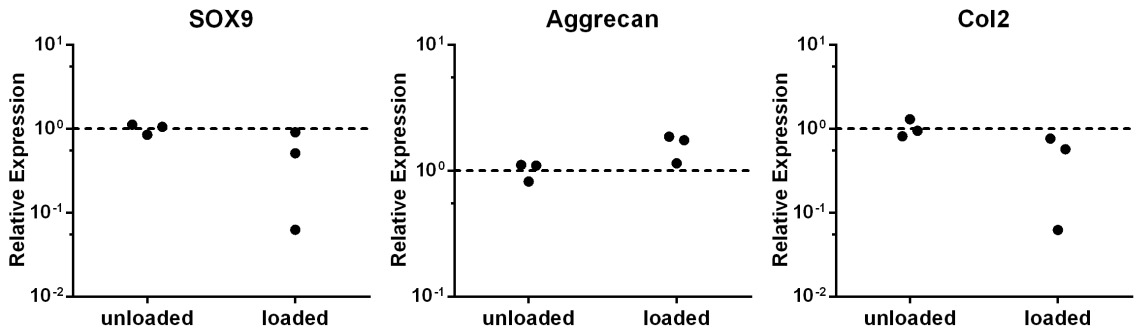


Figure 3.3: Gene expression of articular chondrocytes (Donor 3) following mechanical loading assessed by kqPCR (n = 1). Normalized to unloaded pellet.

Table 3.3: Overview of Donor 3 chondrocyte kqPCR

| Gene | unloaded | loaded |
|---|---|---|
| SOX9 | pos | pos |
| Aggrecan | pos | pos |
| Col2 | pos | mix |

Overview of Donor 3 chondrocyte kqPCR data, indicating whether genes were positively (pos), partially (mix), or not detected (neg) in a particular sample. Partially detected genes were amplified in some but not all technical replicates.

## 3.4 Additional Tests

In order to investigate the response of a larger variety of cells to mechanical loading, and also to assess the protocol modifications made earlier, AA and chondrogenic constructs were generated over 21 days from cells from the left and right synovial fluid of one donor (Donor 4), and two synovial membrane biopsies from the same limb of another donor (Donor 5).

As well, in order to investigate the behaviour and possible differentiation path of the constructs further, the number of genes of interest was expanded to include markers related to osteogenesis and adipogenesis. Specifically, these markers included type-I and type-X collagen, Runx2, and adiponectin.

The gene expression of the synovial fluid constructs is presented in Fig. 3.4. Type-I collagen expression was somewhat varied but largely remained close to control levels. Type-

II collagen was generally not detected except in the Donor 4 L SF chondrogenic constructs, where it was substantially upregulated in comparison to controls. Similarly, type-X collagen expression in one set of constructs generally remained at control levels, where in the other set it was significantly upregulated in the chondrogenic constructs regardless of loading, and in the loaded AA construct. The expression of SOX9 was noticeably downregulated in the chondrogenic constructs, while in the loaded AA constructs it was conflicting but very close to control levels. The expression of aggrecan was highly variable, but generally did not vary substantially from control levels. The expression of Runx2 was similar to that of type-X collagen, in that it was generally absent in one set of constructs and significantly upregulated in the chondrogenic constructs of the other. Moreover, it was the same set of constructs that exhibited this upregulation of both Runx2 and type-X collagen. Lastly, adiponectin was not detected in any of the constructs.

Both sets of Donor 5 SM constructs exhibited apparent lipid globule deposition in constructs treated with chondrogenic differentiation media (Fig. 3.5). The lipid globules became increasingly abundant and evident as construct treatment continued up until mechanical loading. Despite this behaviour, the constructs underwent aggregation into the construct form and were mechanically loaded without difficulty.

Subsequent kqPCR showed dramatically upregulated expression of adiponectin, an adipogenic marker (Fig. 3.6), in comparison to the AA constructs. In fact, it was not detected at all in the AA constructs. This massive increase in expression did not appear to be consistently affected by the application of mechanical loading.

Gene expression in the synovial membrane constructs was otherwise similar to that of the synovial fluid constructs in terms of the variability observed. Type-I collagen expression was somewhat varied, but tended to remain around control levels. Type-II collagen was almost non-existent, except for one chondrogenic construct. Type-X collagen was also largely absent, except in the chondrogenic constructs where it was generally substantially expressed.

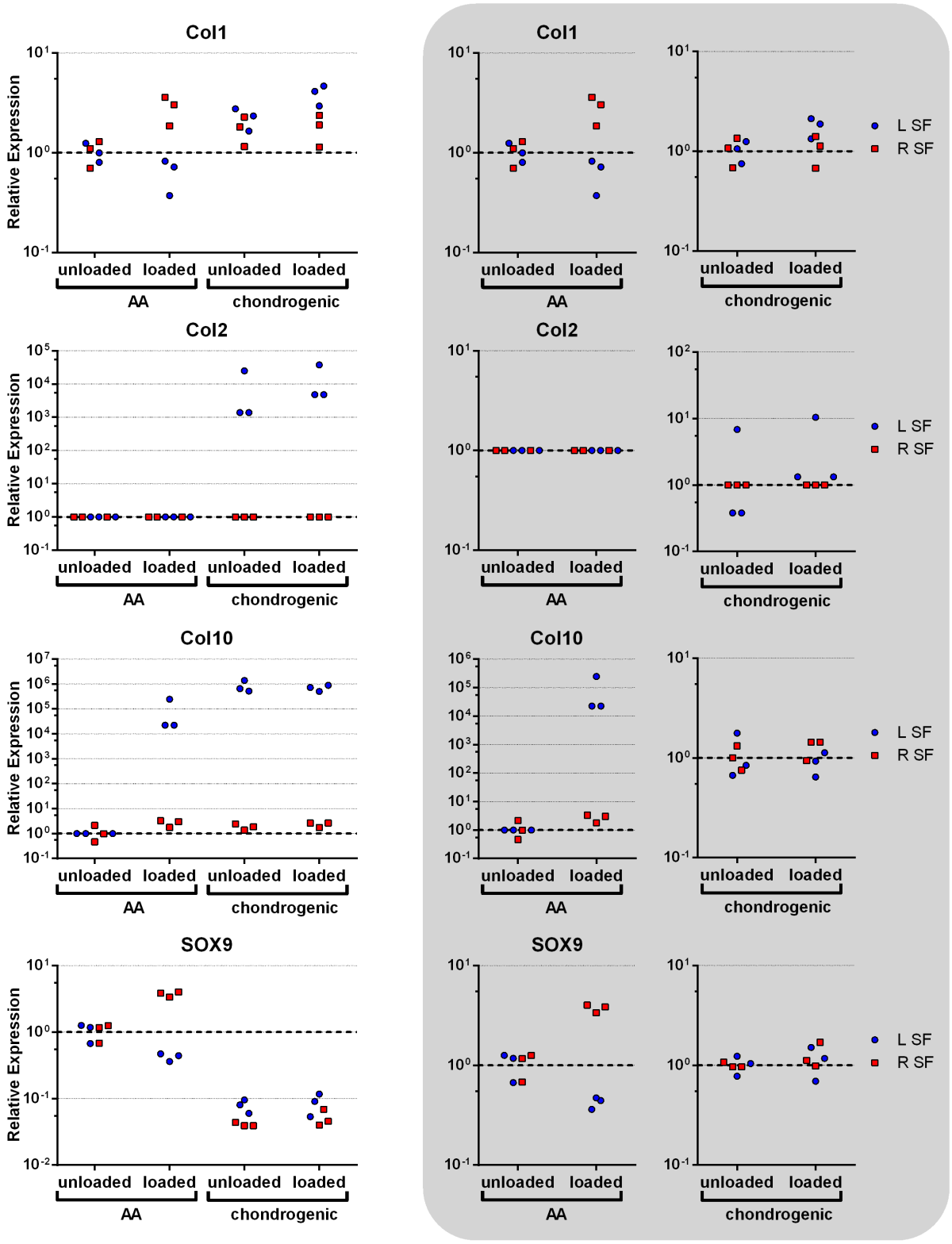


Figure 3.4: Gene expression of SF MPC constructs assessed by kqPCR (Donor 4, n = 2 constructs per treatment group, 1 construct per limb). The data in the left column are normalized to unloaded AA constructs. The data in the middle and right columns (grey box) are normalized to the unloaded AA and chondrogenic constructs, respectively.

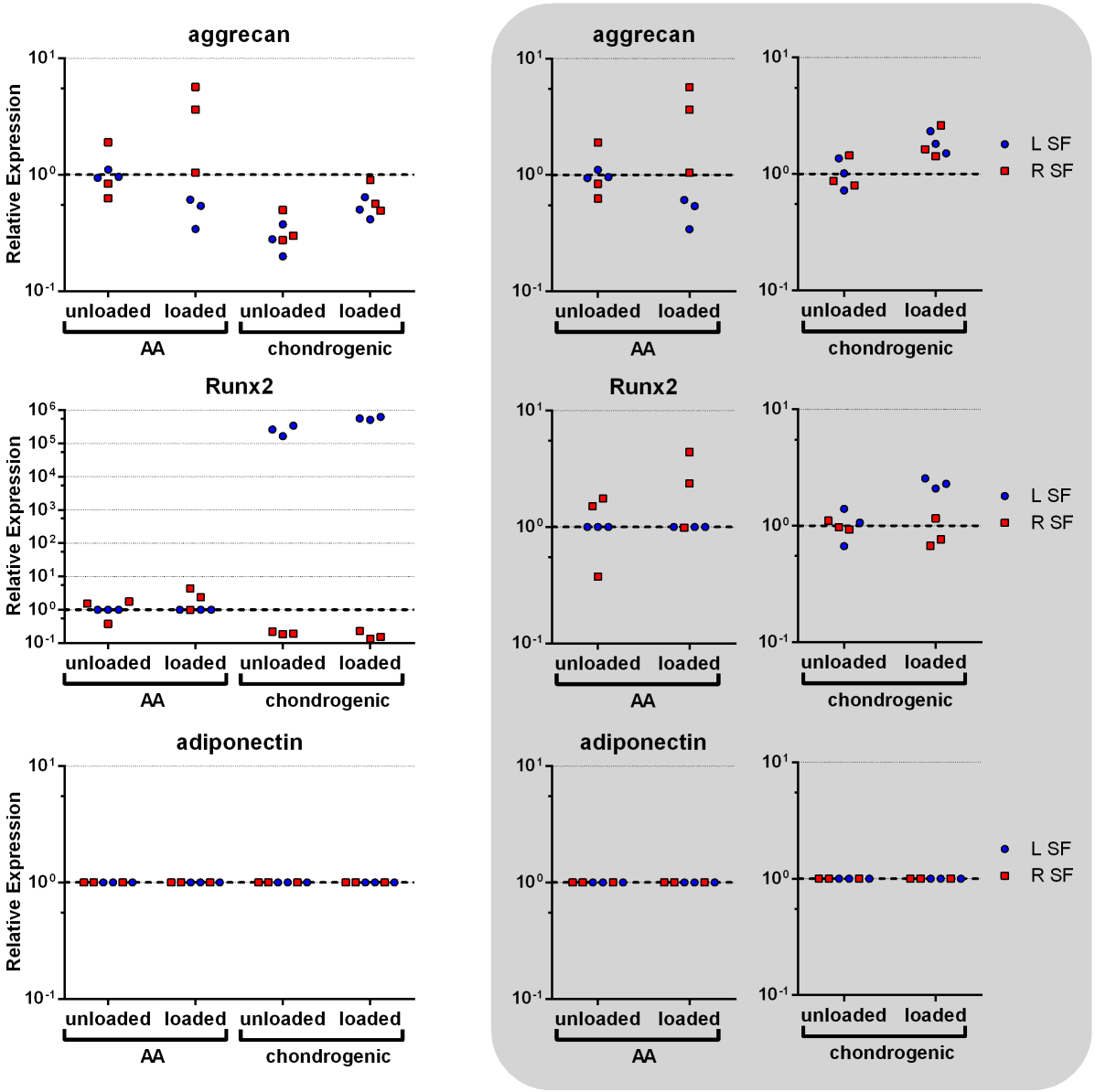


Figure 3.4: Gene expression of Donor 4 SF MPC constructs (cont.).

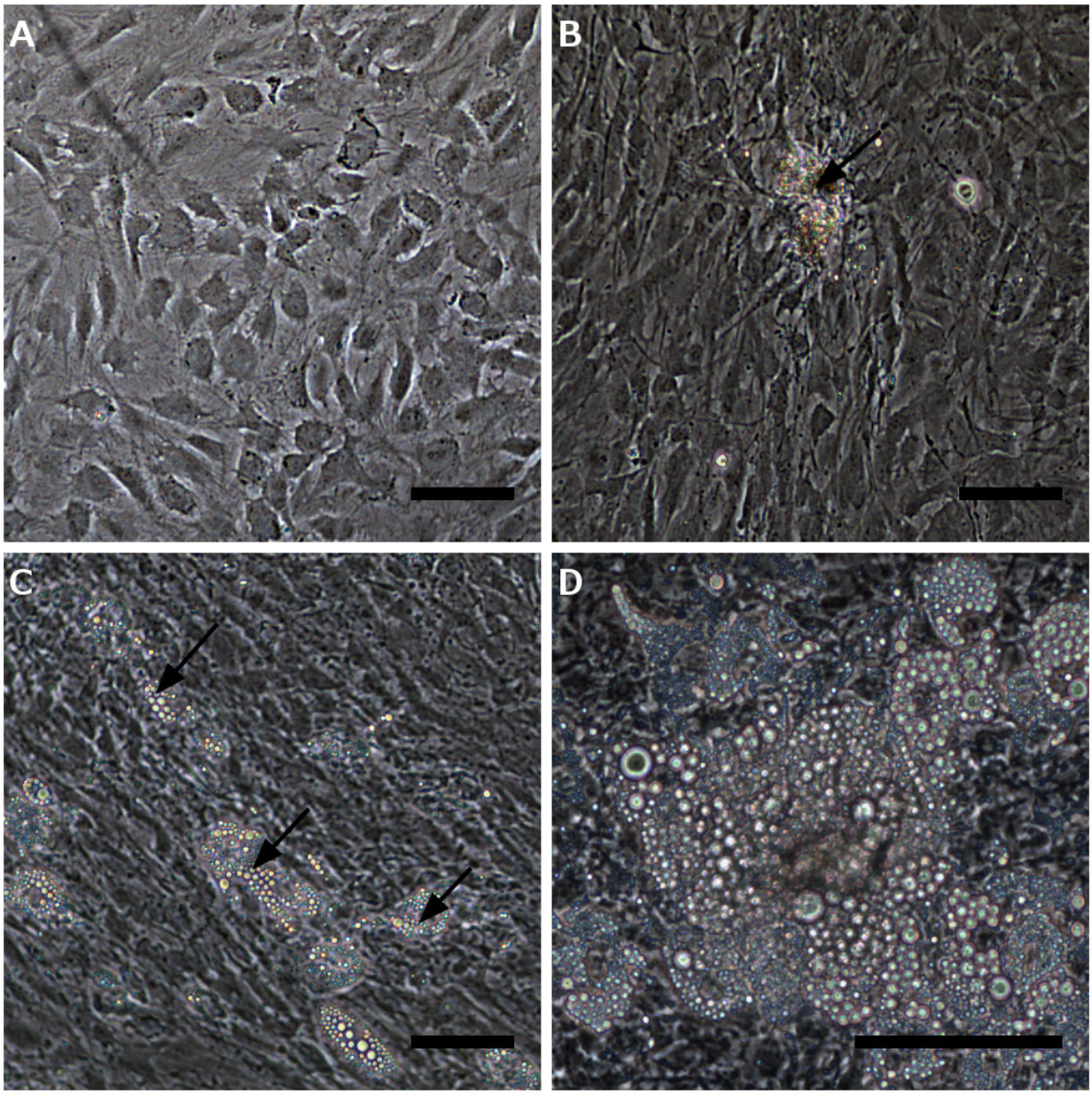


Figure 3.5: Apparent lipid deposition during chondrogenic construct generation using SM MPCs (Donor 5). Lipid globules (arrows) are totally absent at day 0 (A), but are visible at day 7 (B) and day 14 (C, D). Scale bars - 100 µm.

The expression of SOX9 was also variable, as for type-I collagen, and tended to hover around control levels. The expression of aggrecan varied dramatically, but was generally upregulated in all chondrogenic constructs. One loaded AA construct also exhibited upregulated aggrecan, while the other remained at baseline. The expression of Runx2 was generally slightly downregulated in chondrogenic constructs, although no apparent differences were observed as a result of mechanical loading.

To confirm lipid deposition, histological sections were stained with Oil Red O. The chondrogenic constructs exhibited positive staining through the sections, regardless of mechanical loading (Fig. 3.7). No such staining was observed in the AA constructs.

An overview of detected and non-detected (i.e. amplified and non-amplified) genes in Fig. 3.4 and 3.6 is provided in Table 3.4.

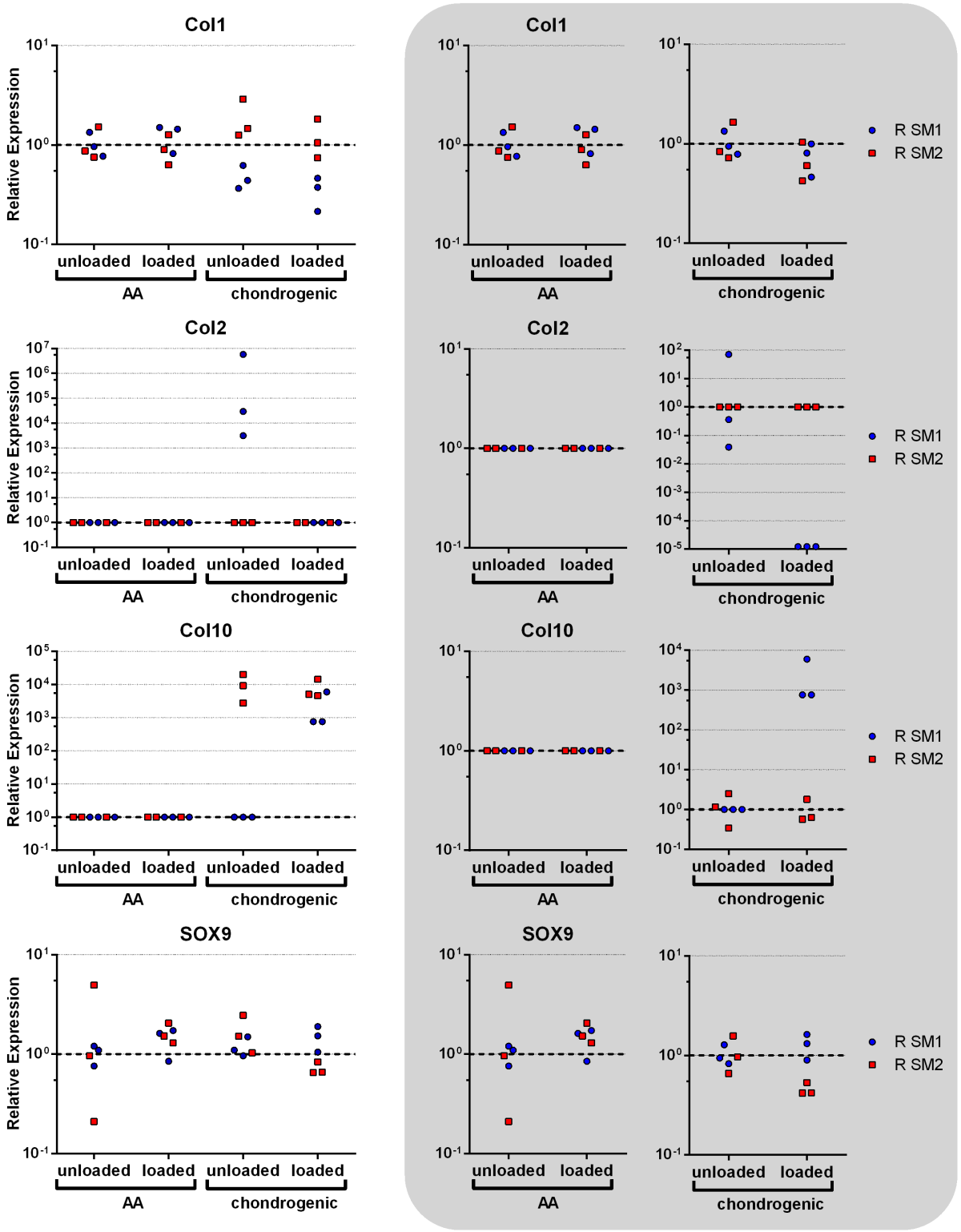


Figure 3.6: Gene expression of SM MPC constructs assessed by kqPCR (Donor 5, n = 2 constructs per treatment group, 1 each from two different SM biopsies). The data in the left column are normalized to unloaded AA constructs.

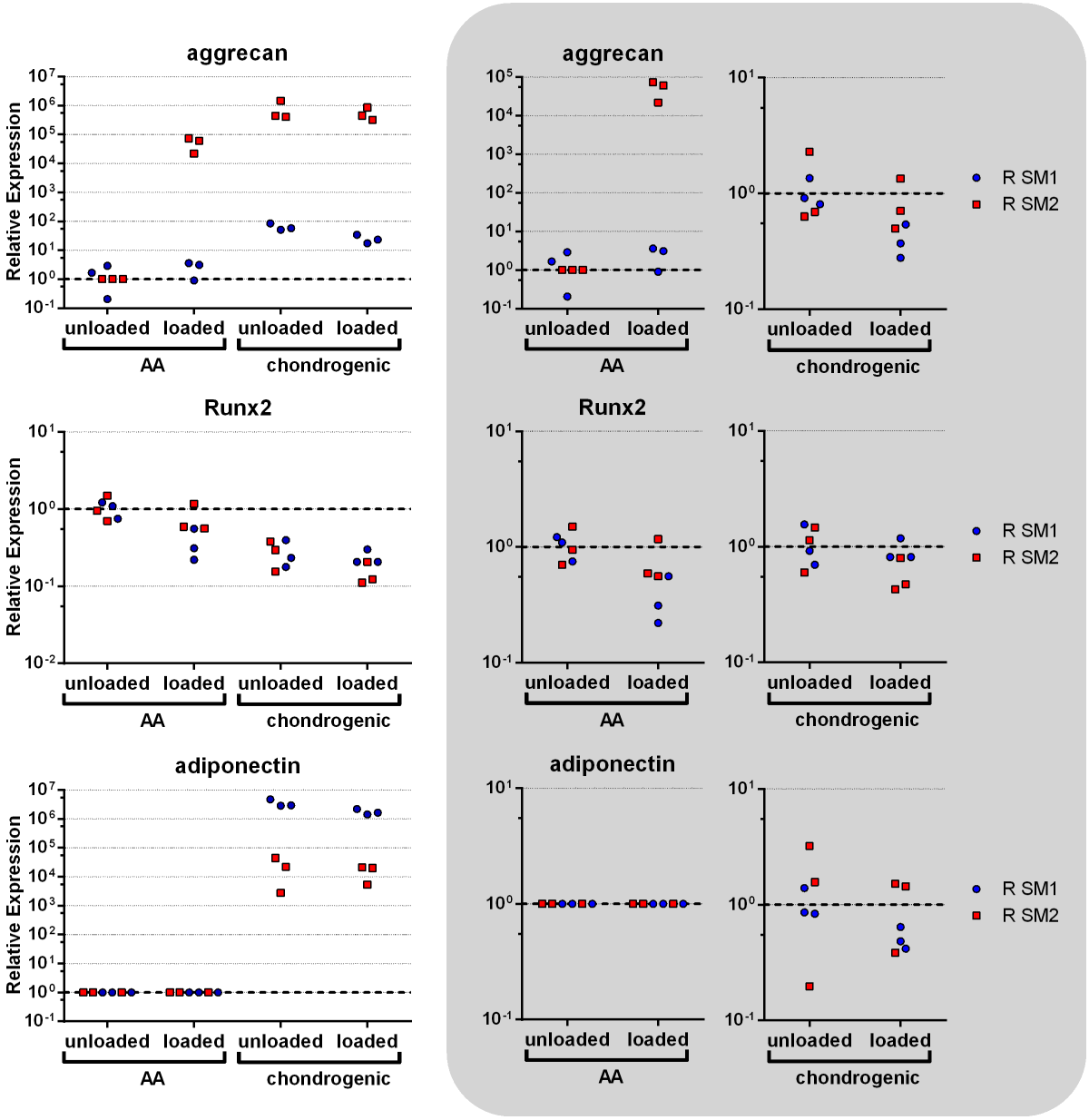


Figure 3.6: Gene expression of Donor 5 SM MPC constructs (cont.)

Table 3.4: Overview of Donor 4 and 5 kqPCR data

| **Col1** | | **AA L-** | **AA L+** | **chondrogenic L-** | **chondrogenic L+** |
|---|---|---|---|---|---|
| Donor 4 | L SF | pos | pos | pos | pos |
| | R SF | pos | pos | pos | pos |
| Donor 5 | SM 1 | pos | pos | pos | pos |
| | SM 2 | pos | pos | pos | pos |

| **Col2** | | **AA L-** | **AA L+** | **chondrogenic L-** | **chondrogenic L+** |
|---|---|---|---|---|---|
| Donor 4 | L SF | neg | neg | mix | mix |
| | R SF | neg | neg | neg | neg |
| Donor 5 | SM 1 | neg | neg | mix | neg |
| | SM 2 | neg | neg | neg | neg |

| **Col10** | | **AA L-** | **AA L+** | **chondrogenic L-** | **chondrogenic L+** |
|---|---|---|---|---|---|
| Donor 4 | L SF | neg | mix | pos | pos |
| | R SF | pos | pos | pos | pos |
| Donor 5 | SM 1 | neg | neg | neg | mix |
| | SM 2 | neg | neg | mix | pos |

| **SOX9** | | **AA L-** | **AA L+** | **chondrogenic L-** | **chondrogenic L+** |
|---|---|---|---|---|---|
| Donor 4 | L SF | pos | pos | pos | pos |
| | R SF | pos | pos | pos | pos |
| Donor 5 | SM 1 | pos | pos | pos | pos |
| | SM 2 | pos | pos | pos | pos |

| **Aggrecan** | | **AA L-** | **AA L+** | **chondrogenic L-** | **chondrogenic L+** |
|---|---|---|---|---|---|
| Donor 4 | L SF | pos | pos | pos | pos |
| | R SF | pos | pos | pos | pos |
| Donor 5 | SM 1 | mix | pos | pos | pos |
| | SM 2 | pos | pos | pos | pos |

| **Runx2** | | **AA L-** | **AA L+** | **chondrogenic L-** | **chondrogenic L+** |
|---|---|---|---|---|---|
| Donor 4 | L SF | neg | neg | pos | pos |
| | R SF | pos | pos | pos | pos |
| Donor 5 | SM 1 | pos | pos | pos | pos |
| | SM 2 | pos | pos | pos | pos |

| **Adiponectin** | | **AA L-** | **AA L+** | **chondrogenic L-** | **chondrogenic L+** |
|---|---|---|---|---|---|
| Donor 4 | L SF | neg | neg | neg | neg |
| | R SF | neg | neg | neg | neg |
| Donor 5 | SM 1 | neg | neg | pos | pos |
| | SM 2 | neg | neg | mix | pos |

Overview of Donor 4 and Donor 5 kqPCR data, indicating whether genes were positively (pos), partially (mix), or not detected (neg) in a particular sample. Partially detected genes were amplified in some but not all technical replicates.

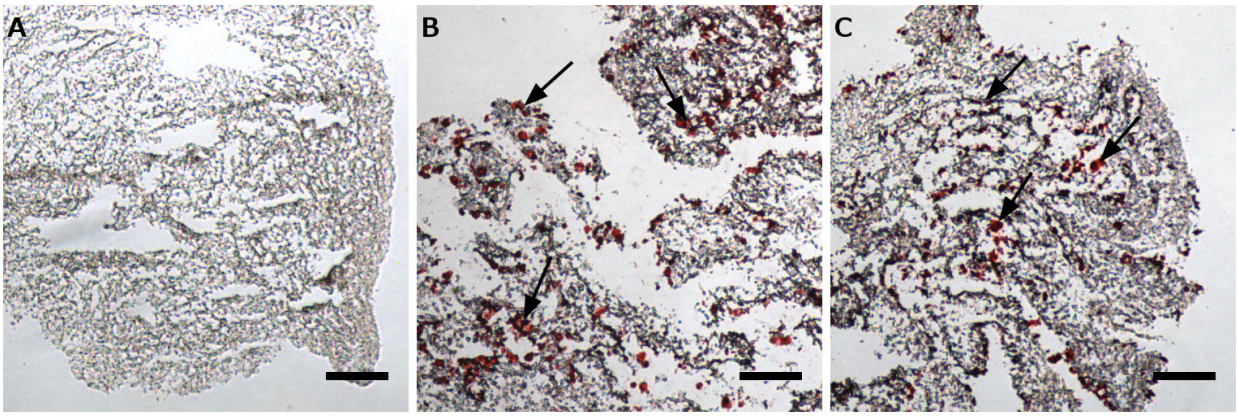


Figure 3.7: Donor 5 SM MPC construct sections stained with Oil Red O for lipid deposition. No staining was observed in AA constructs (A), while both unloaded (B) and loaded (C) chondrogenic constructs exhibited positive staining (arrows). Scale bar 200 µm.

## 3.5 Main Study

### 3.5.1 Construct Generation

To investigate the variability in response to mechanical loading of constructs generated from cells obtained from closely related sources, cells were derived and expanded from SF and three SM biopsies of the left and right limbs of a single donor (Donor 6). Sets of AA and chondrogenic constructs were generated over 21 days using these cells and subjected to mechanical loading. A series of representative images of the morphological changes observed during construct generation are shown in Fig. 3.8. Heterogeneity can be observed in cell morphology throughout the individual monolayers even before treatment with AA or chondrogenic differentiation media. Following the addition of media for construct generation, the cell monolayers undergo dramatic, visible morphological changes. Although the monolayers are seeded at a near-confluent density prior to construct generation, it appears that the cells undergo some amount of proliferation following the application of treatment media, based on the relative appearance of more closely packed cells. This becomes more pronounced at subsequent time points. This could also be the result of an increase in average cell size. At approximately day 7 of treatment it became difficult to distinguish between individual cells

in the chondrogenic sets, likely due to the apparent number of cells as well as changes in cell morphology. The cells appear to elongate in one direction and become significantly more narrow in the other. This elongation also appears to occur in the AA sets, although not to the same extent. Individual cells can still be clearly identified, and the general appearance of the monolayer is 'cleaner'. Interestingly, in both groups the observed elongation appears to occur in a somewhat directed manner. Neighbouring cells orient in a similar direction, giving the appearance of a collective direction.

There was not much observable change in the AA sets from day 7 to day 14. The apparently coordinated elongation observed at day 7 was still present. This behaviour was even more evident in the chondrogenic monolayers. The monolayers appeared to be even more cluttered, although whether this is due to increased cell density from proliferation, matrix deposition, or some other mechanism is unknown. Regardless, there was a very visible directionality in the orientation of the cells.

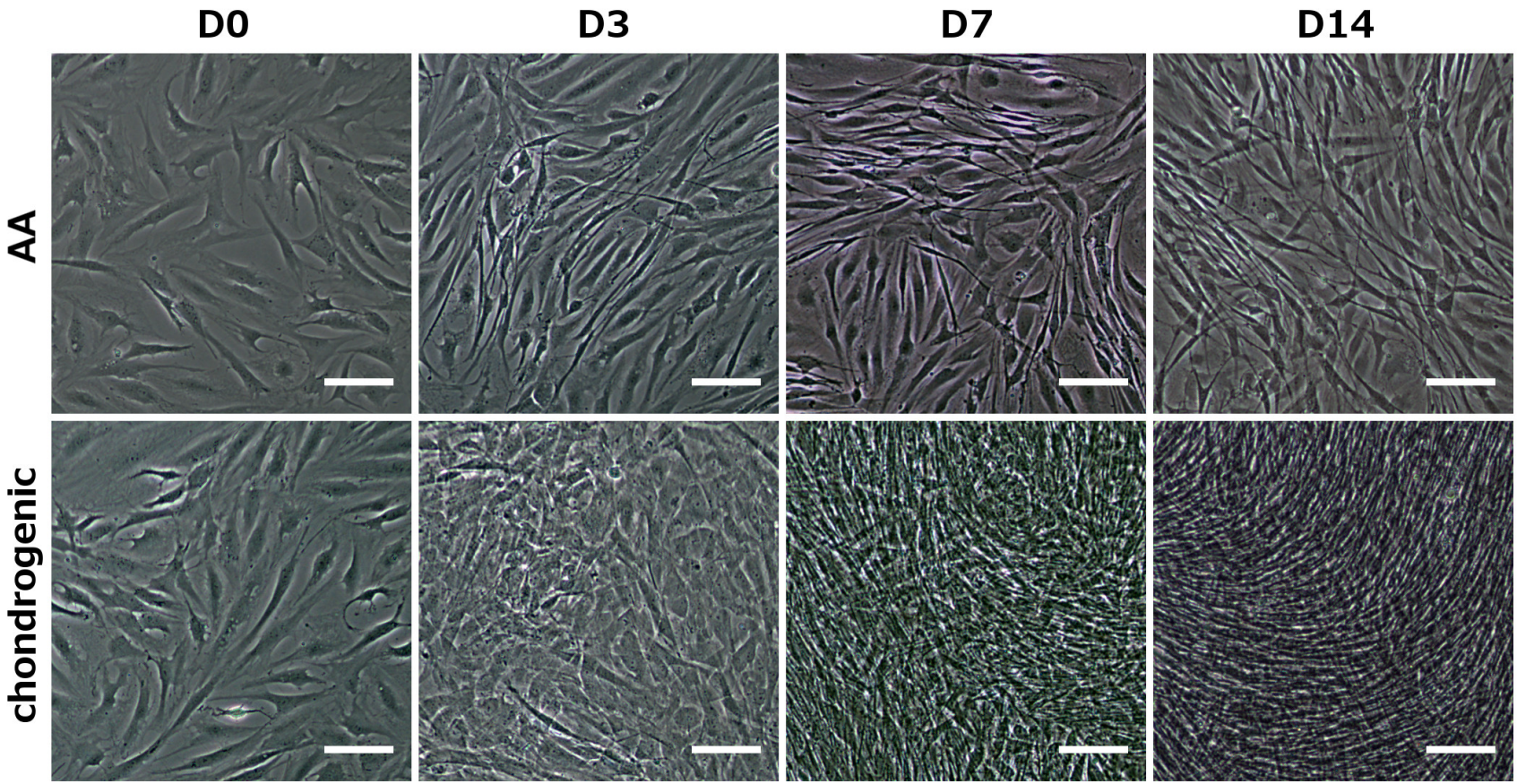


Figure 3.8: Right-limb SF MPCs (Donor 6) undergoing dramatic morphological changes during construct generation from Day 0 to Day 14. Scale bar 100 µm.

### 3.5.2 Time Course of Auto-aggregation

In order to assess the variation in auto-aggregation of the constructs, they were observed daily over the course of construct generation (Fig. 3.9). After approximately ten days in culture the cell monolayers in chondrogenic differentiation media began to exhibit varying levels of contraction and auto-aggregation, evidenced by the edges of the monolayers receding from the walls of the culture dishes. By day 11, all of the constructs from one of the left synovial membrane sets had fully detached and aggregated into the three-dimensional construct form, as well as one construct from one of the other left synovial membrane sets.

By day 16, all of the constructs generated from cells from synovial fluid and membrane of the right knee had detached and aggregated, while the remaining synovial membrane constructs from the left knee detached over days 17 and 18. Overall, the constructs from a single tissue source took between 11 and 18 days to completely detach and aggregate, a variation of more than 60%. It's worth noting that the first and last complete set of synovial membrane constructs to aggregate were both generated from cells obtained from the same limb, which may suggest substantial variation within the tissue.

Notably, the cell monolayers grown from the left-limb synovial fluid cells did not auto-aggregate by day 18 and had to be detached manually. As well, although many of the monolayers treated with ascorbic acid media demonstrated some contraction in their culture wells, none underwent auto-aggregation and all were detached manually on day 18.

### 3.5.3 Construct Aggregation Variation

Before constructs were prepared for mechanical loading, pictures were taken and subsequently analyzed in ImageJ, an open-source image analysis program, in order to determine the relative area of the constructs with respect to the area of their culture wells. This provided an approximate measure of the extent of aggregation of the constructs. While this method of measurement cannot be used to make strong conclusions with respect to the ac-

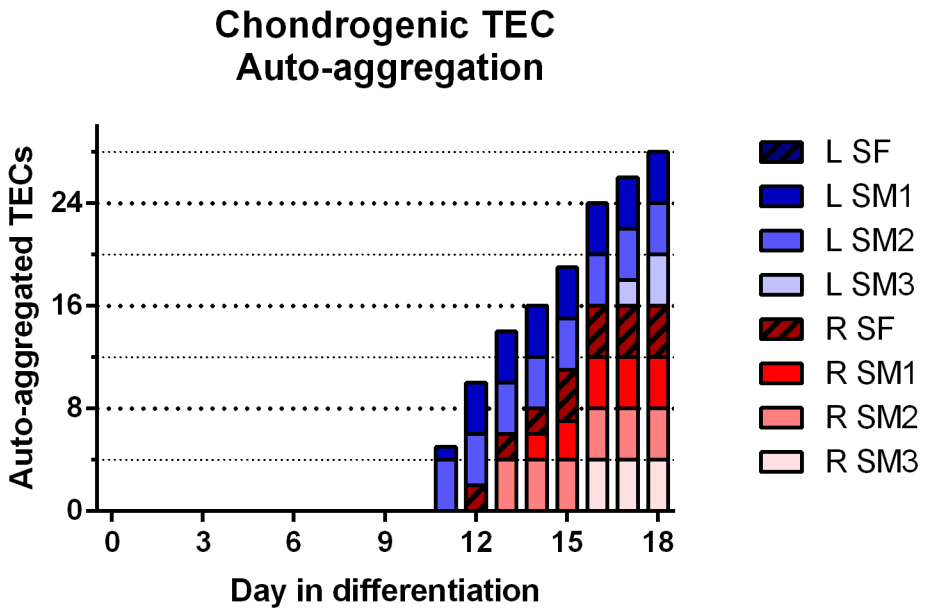


Figure 3.9: Time course of auto-aggregation in Donor 6 chondrogenic constructs.

tual sizes of the constructs generated by the different methods used in these experiments, the AA constructs were observably thinner in general. Attempts to weigh the constructs were unsuccessful as the masses of the constructs appeared to be less than the margin of error in the weigh scales used.

The constructs produced with chondrogenic differentiation media had a mean construct area more than double that of the AA constructs (Fig. 3.10). As, there was a substantially greater variability in the aggregation of the AA constructs. There appeared to be little, if any, relation between the timing of construct aggregation and the extent of aggregation by day 21.

### 3.5.4 Gene Expression

In order to examine the variability of the constructs, their gene expression was analyzed for genes relating to chondrogenic, osteogenic, and adipogenic differentiation, in order to investigate the activity of MPCs at the transcriptional level (Fig. 3.11). As a complement to these data, an overview of detected and non-detected (i.e. amplified and non-amplified)

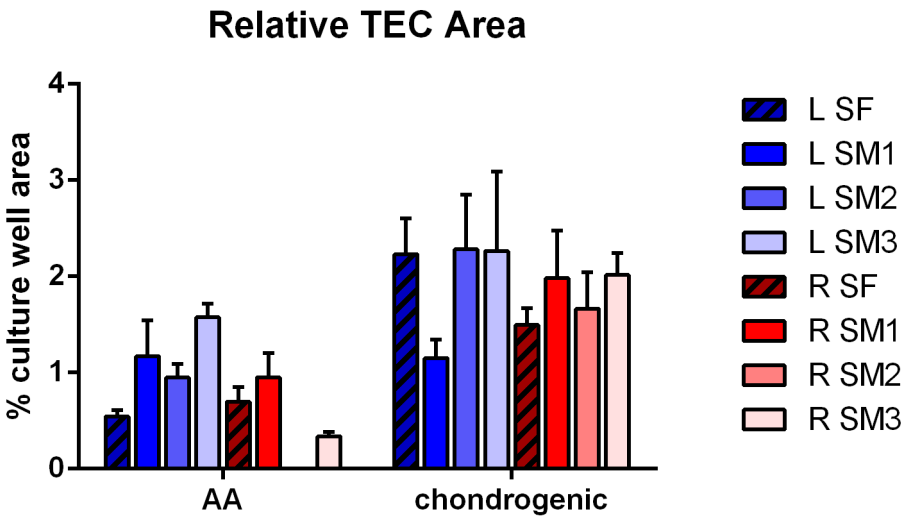


Figure 3.10: Relative aggregation of Donor 6 chondrogenic constructs prior to mechanical loading. One set (R SM2) absent due to technical issues.

genes is provided in Table 3.5.

The gene expression of constructs generated from both SM and SF MPCs was investigated. In general, the observed gene expression was highly variable between samples across almost all genes, although a number of trends were observed.

Type-I collagen expression varied across all treatment groups. There was a statistically significant non-zero median difference between the AA and chondrogenic unloaded constructs. Except for one SM set (R SM2) and one SF set (L SF), the baseline type-I collagen expression in chondrogenic constructs was substantially higher than the expression observed in the AA constructs. However, there was no clear influence of mechanical loading. Although some differences were observed, such as approximately 50-fold to 100-fold decreases in expression in R SM2 AA constructs and L SF chondrogenic constructs, as well as some modest decreases in expression in some synovial membrane chondrogenic constructs, the expression of type-I collagen in loaded constructs was generally similar to that of unloaded constructs of the same generation method.

Type-II collagen was not frequently detected during these experiments. It was not de-

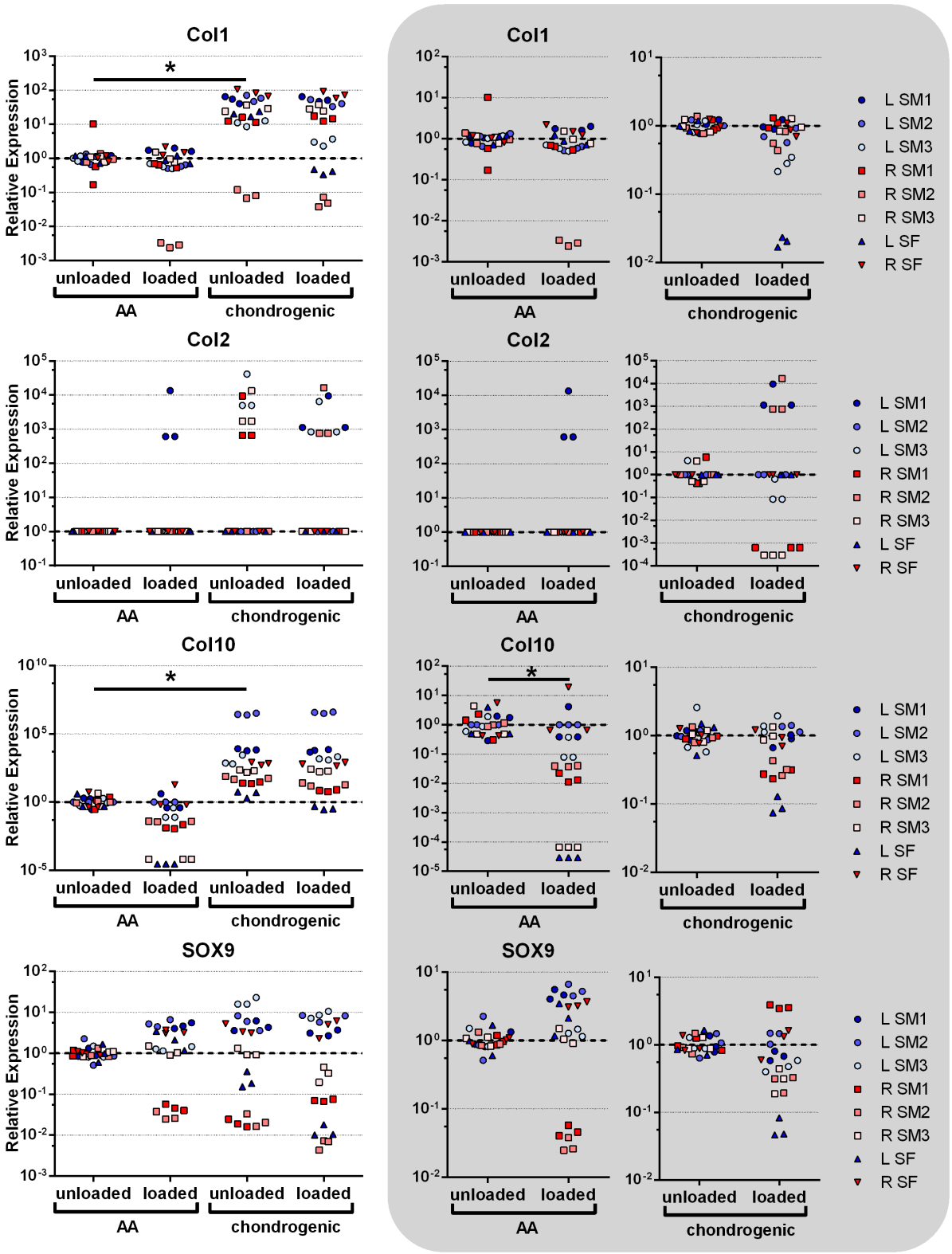


Figure 3.11: Gene expression of SM and SF MPC constructs assessed by kqPCR (Donor 6, n = 8 constructs per treatment group, 1 construct each from 6 SM and 2 SF biopsies). * - significant non-zero median difference at $p$ <0.05.

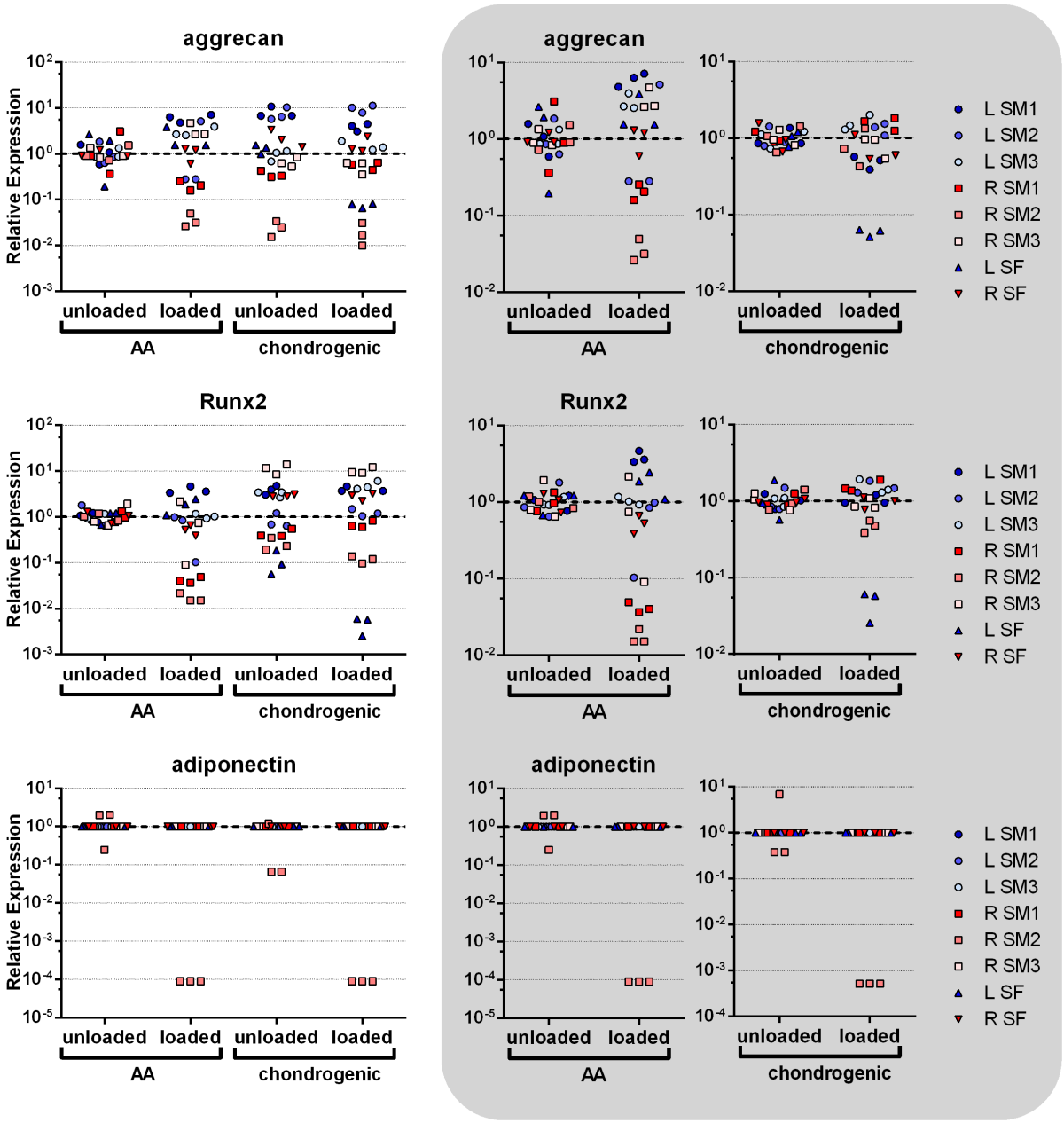


Figure 3.11: Gene expression of Donor 6 SM MPC constructs (cont.)

Table 3.5: Overview of Donor 6 kqPCR data

| **Col1** | | **AA L-** | **AA L+** | **chondrogenic L-** | **chondrogenic L+** |
|---|---|---|---|---|---|
| L SM | 1 | pos | pos | pos | pos |
| | 2 | pos | pos | pos | pos |
| | 3 | pos | pos | pos | pos |
| R SM | 1 | pos | pos | pos | pos |
| | 2 | pos | pos | pos | pos |
| | 3 | pos | pos | pos | pos |
| SF | L | pos | pos | pos | pos |
| | R | pos | pos | pos | pos |

| **Col2** | | **AA L-** | **AA L+** | **chondrogenic L-** | **chondrogenic L+** |
|---|---|---|---|---|---|
| L SM | 1 | neg | mix | neg | mix |
| | 2 | neg | neg | neg | neg |
| | 3 | neg | neg | mix | mix |
| R SM | 1 | neg | neg | mix | neg |
| | 2 | neg | neg | neg | mix |
| | 3 | neg | neg | mix | neg |
| SF | L | neg | neg | neg | neg |
| | R | neg | neg | neg | neg |

| **Col10** | | **AA L-** | **AA L+** | **chondrogenic L-** | **chondrogenic L+** |
|---|---|---|---|---|---|
| L SM | 1 | mix | mix | pos | pos |
| | 2 | neg | neg | pos | pos |
| | 3 | pos | mix | pos | pos |
| R SM | 1 | mix | pos | pos | pos |
| | 2 | pos | pos | pos | pos |
| | 3 | mix | neg | pos | pos |
| SF | L | mix | neg | pos | pos |
| | R | mix | mix | pos | pos |

| **SOX9** | | **AA L-** | **AA L+** | **chondrogenic L-** | **chondrogenic L+** |
|---|---|---|---|---|---|
| L SM | 1 | pos | pos | pos | pos |
| | 2 | pos | pos | pos | pos |
| | 3 | pos | pos | pos | pos |
| R SM | 1 | pos | pos | pos | pos |
| | 2 | pos | pos | pos | pos |
| | 3 | pos | pos | pos | pos |
| SF | L | pos | pos | pos | pos |
| | R | pos | pos | pos | pos |

Overview of Donor 6 kqPCR data, indicating whether genes were positively (pos), partially (mix), or not detected (neg) in a particular sample. Partially detected genes were amplified in some but not all technical replicates.

Table 3.5: Overview of Donor 6 kqPCR data (cont.)

| **Aggrecan** | | **AA L-** | **AA L+** | **chondrogenic L-** | **chondrogenic L+** |
|---|---|---|---|---|---|
| L SM | 1 | pos | pos | pos | pos |
| | 2 | pos | mix | pos | pos |
| | 3 | pos | pos | pos | pos |
| R SM | 1 | pos | pos | pos | pos |
| | 2 | pos | pos | pos | pos |
| | 3 | pos | pos | pos | pos |
| SF | L | mix | pos | pos | pos |
| | R | pos | pos | pos | pos |

| **Runx2** | | **AA L-** | **AA L+** | **chondrogenic L-** | **chondrogenic L+** |
|---|---|---|---|---|---|
| L SM | 1 | pos | pos | pos | pos |
| | 2 | pos | mix | pos | pos |
| | 3 | pos | pos | pos | pos |
| R SM | 1 | pos | pos | pos | pos |
| | 2 | pos | pos | pos | pos |
| | 3 | pos | mix | pos | pos |
| SF | L | pos | pos | pos | pos |
| | R | pos | pos | pos | pos |

| **Adiponectin** | | **AA L-** | **AA L+** | **chondrogenic L-** | **chondrogenic L+** |
|---|---|---|---|---|---|
| L SM | 1 | neg | neg | neg | neg |
| | 2 | neg | neg | neg | neg |
| | 3 | neg | neg | neg | neg |
| R SM | 1 | neg | neg | neg | neg |
| | 2 | mix | neg | mix | neg |
| | 3 | neg | neg | neg | neg |
| SF | L | neg | neg | neg | neg |
| | R | neg | neg | neg | neg |

tected at all in any of the synovial fluid constructs, and in only a few of the membrane constructs. This pattern of expression appears as dramatic differences in expression, on the order of 1000-fold, in the cases where it was detected. Given such large swings in relative expression, it is not surprising that no statistically significant differences were observed. Interestingly, only one set of constructs (L SM2) showed consistent upregulation of type-II collagen in chondrogenic constructs compared to AA constructs, regardless of the application of mechanical loading. With one exception (L SM1), none of the AA constructs exhibited type-II collagen expression, where it was upregulated in the loaded constructs. Among the chondrogenic constructs there was substantial variability, with differences between loaded and unloaded constructs ranging from 1000-fold increased expression, to a 1000-fold decrease, to no observable effect at all.

The expression of type-X collagen in constructs was much more significantly affected by the method of generation than whether mechanical loading was applied. The synovial membrane chondrogenic constructs exhibited increased expression ranging from $10^2$-fold to over $10^5$-fold that of AA constructs. In contrast, only one set of synovial fluid constructs exhibited the same behaviour, while the expression of the other set was varied. Overall, the were was a statistically significant non-zero median difference in type-X collagen expression between the AA and chondrogenic unloaded constructs. The application of mechanical loading in the AA constructs also resulted in statistically significant findings. It appeared to lead to a downregulation of type-X collagen expression in both AA and chondrogenic constructs, but was of a larger magnitude in the AA constructs. However, detection of type-X collagen was not consistent amongst replicates in the AA treatment groups. While this may affect the validity of the statistical findings, it also serves to emphasize the substantial difference in expression of the gene between the AA and chondrogenic groups.

No statistically significant differences in SOX9 expression were found in either the synovial membrane or fluid samples. Interestingly, among the synovial membrane samples the

method of generation appeared to have an effect on expression, but varied according to the limb from which the constructs were derived. In general, the chondrogenic constructs showed upregulation of SOX9 if they originated in the left limb, but with one exception were downregulated in those from the right limb. This sort of divergence was also somewhat shown in the loaded constructs in the AA set. With the exception of one construct from each limb, the loaded left limb constructs exhibited upregulated SOX9 expression, while it was the opposite case in the right limb constructs. This trend was not seen in the chondrogenic constructs. In general, the synovial fluid constructs exhibited a similar trend of divergence.

The expression of aggrecan was highly variable amongst the constructs and therefore no statistically significant differences were observed. No trend could be observed relating to the method of generation. With respect to the effect of loading, amongst the AA constructs the response appeared polarized between upregulation and downregulation, however there was no observable pattern in how the data clustered. No such trends were evident amongst the loaded chondrogenic constructs, which appeared to have a much more moderate response to loading.

The expression of Runx2 did not follow any particularly evident trends and showed no statistically significant behaviour in either the synovial membrane nor the synovial fluid constructs. As with other genes, the method of generation in some cases caused an upregulation of expression, while in other constructs it led to downregulation. This also occurred following mechanical loading in the AA synovial membrane constructs, while the chondrogenic membrane constructs exhibited only a minor response to mechanical loading. The synovial fluid constructs were similarly variable.

Expression of adiponectin was non-existent in almost all samples. With the exception of one set of constructs it was not detected at all. In the one set of constructs that did exhibit adiponectin expression (R SM2), it was very weakly detected and was close to exceeding the threshold value.

### 3.5.5 Histological Analysis

Histological sections from these experiments were stained using Alcian blue, Alizarin red, and Oil Red O, as indicators of possible chondrogenesis, osteogenesis, and adipogenesis, respectively.

All constructs from this set, from both generation methods and regardless of mechanical loading, did not stain positively for Alizarin red or Oil Red O, indicating an absence of calcium deposits or lipids that might otherwise imply osteogenic or adipogenic differentiation (Fig. 3.12). Therefore, only H&E and Alcian blue staining will be presented here.

Constructs generated from synovial membrane and synovial fluid MPCs exhibited very similar patterns of staining in terms of morphology, intensity, and overall construct structure (Fig. 3.13, 3.14).

There are a number of visible features that appear to differentiate the ascorbic acid media constructs from the chondrogenic constructs. In general, the AA constructs exhibited uniformly sparse staining, with voids spread evenly throughout the construct sections (Fig. 3.15), giving them a porous appearance. In contrast, the chondrogenic constructs appeared more monolithic, although feature large gaps in the sections that likely coincide with the construct configuration.

As well, the relative spatial density of cell nuclei appeared to be higher in the AA constructs based on visual observation. In many regions in the chondrogenic constructs, the nuclei appear to be more spread out, which may be attributable to a smaller ratio of cell nuclei to matrix. As well, cell nuclei appear to be uniformly distributed throughout the AA constructs, whereas in the chondrogenic constructs the cell nuclei appear to be distributed unevenly. Some regions in the chondrogenic constructs appear to be sparsely populated by cells, with nearby regions apparently densely packed with cell nuclei (Fig. 3.16).

Visible differences could be observed in the borders of the constructs. The AA constructs generally lacked a distinct border, instead having a more ragged and uneven appearance,

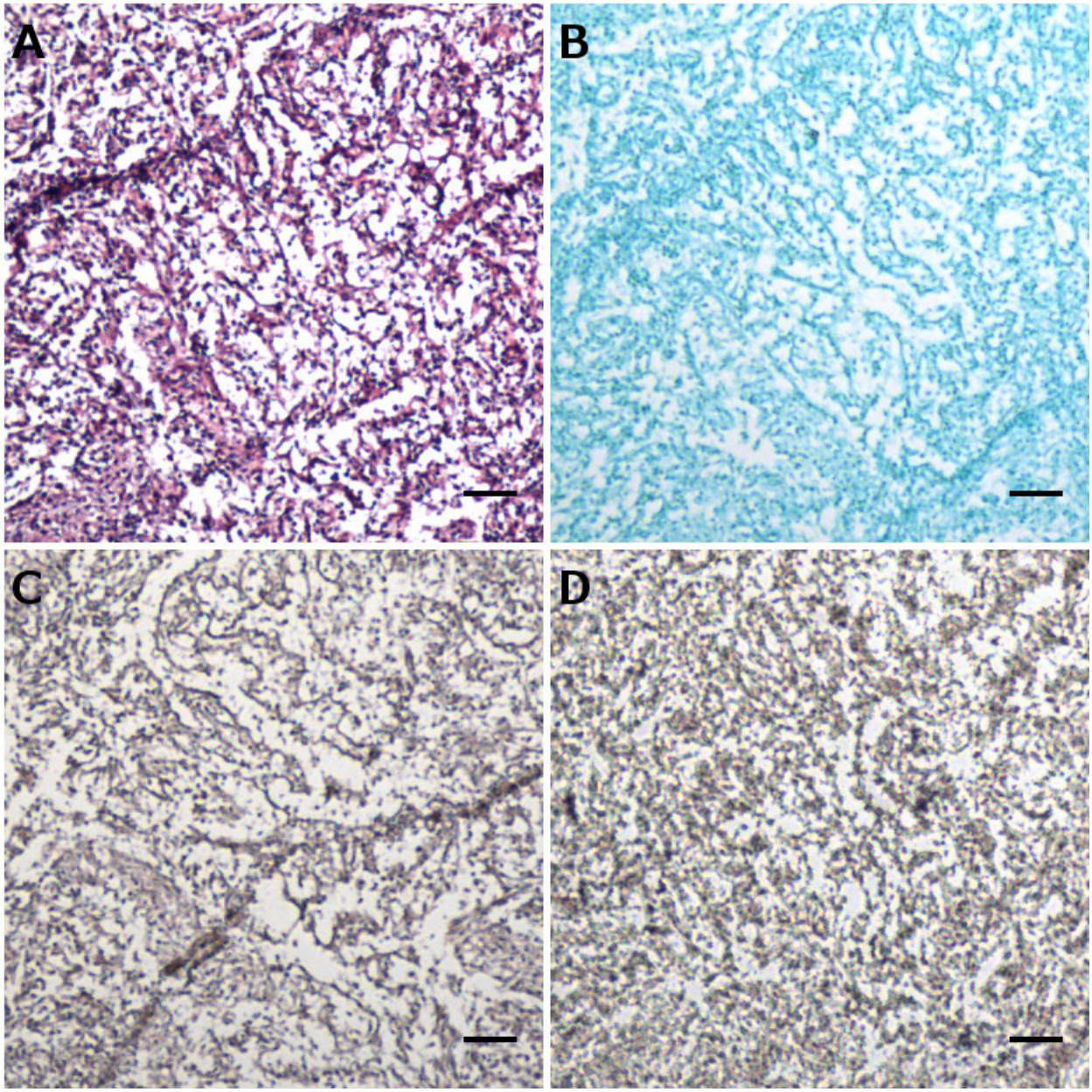


Figure 3.12: Representative histological sections of Donor 6 constructs stained with H&E (A), Alcian blue (B), Alizarin red (C), and Oil Red O (D). Scale bars 100 µm.

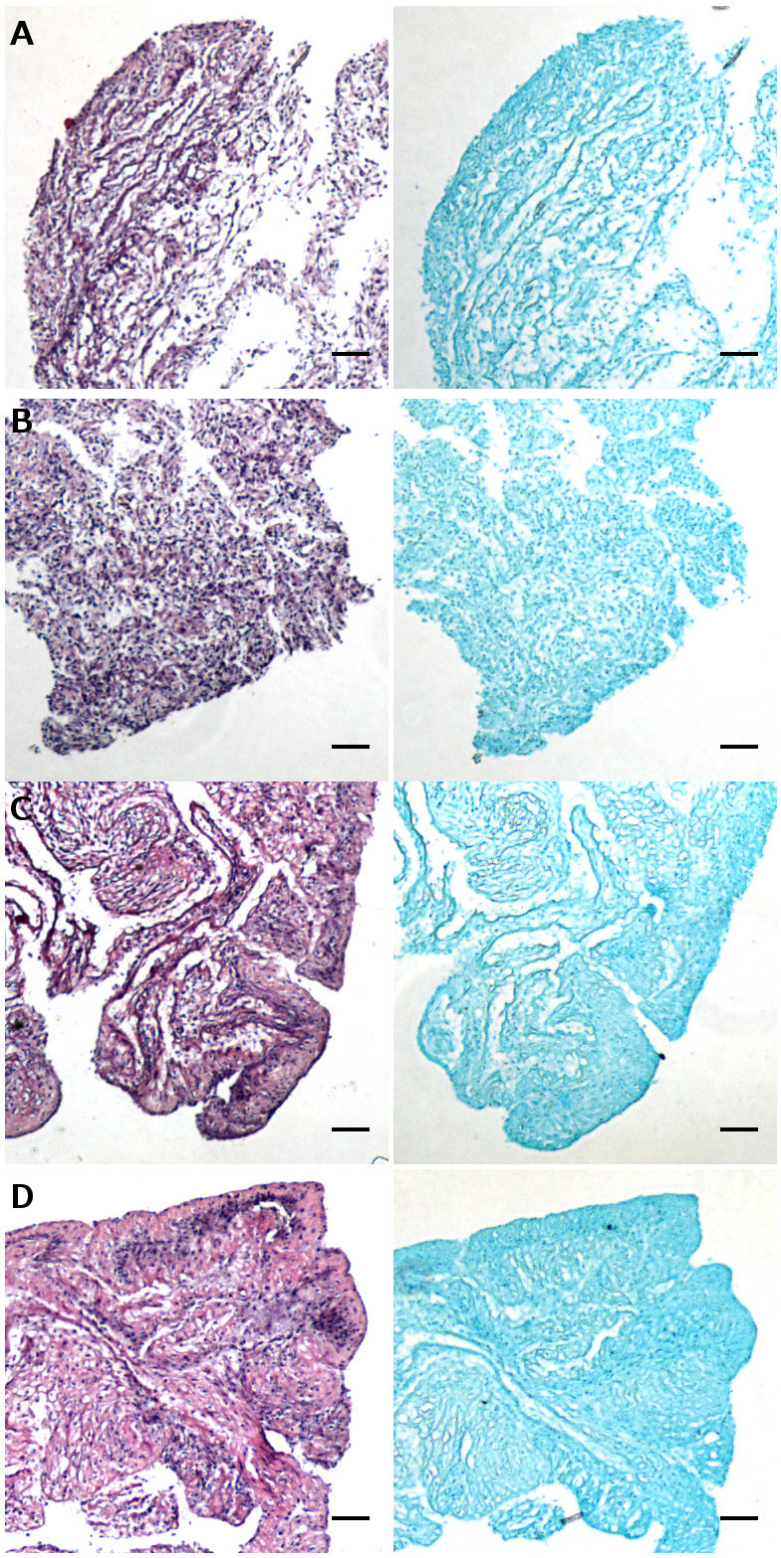


Figure 3.13: Donor 6 SM MPC constructs stained with H&E (left) and Alcian blue (right). AA constructs, unloaded (A) and loaded (B), and chondrogenic constructs, unloaded (C) and loaded (D). Scale bars 100 µm.

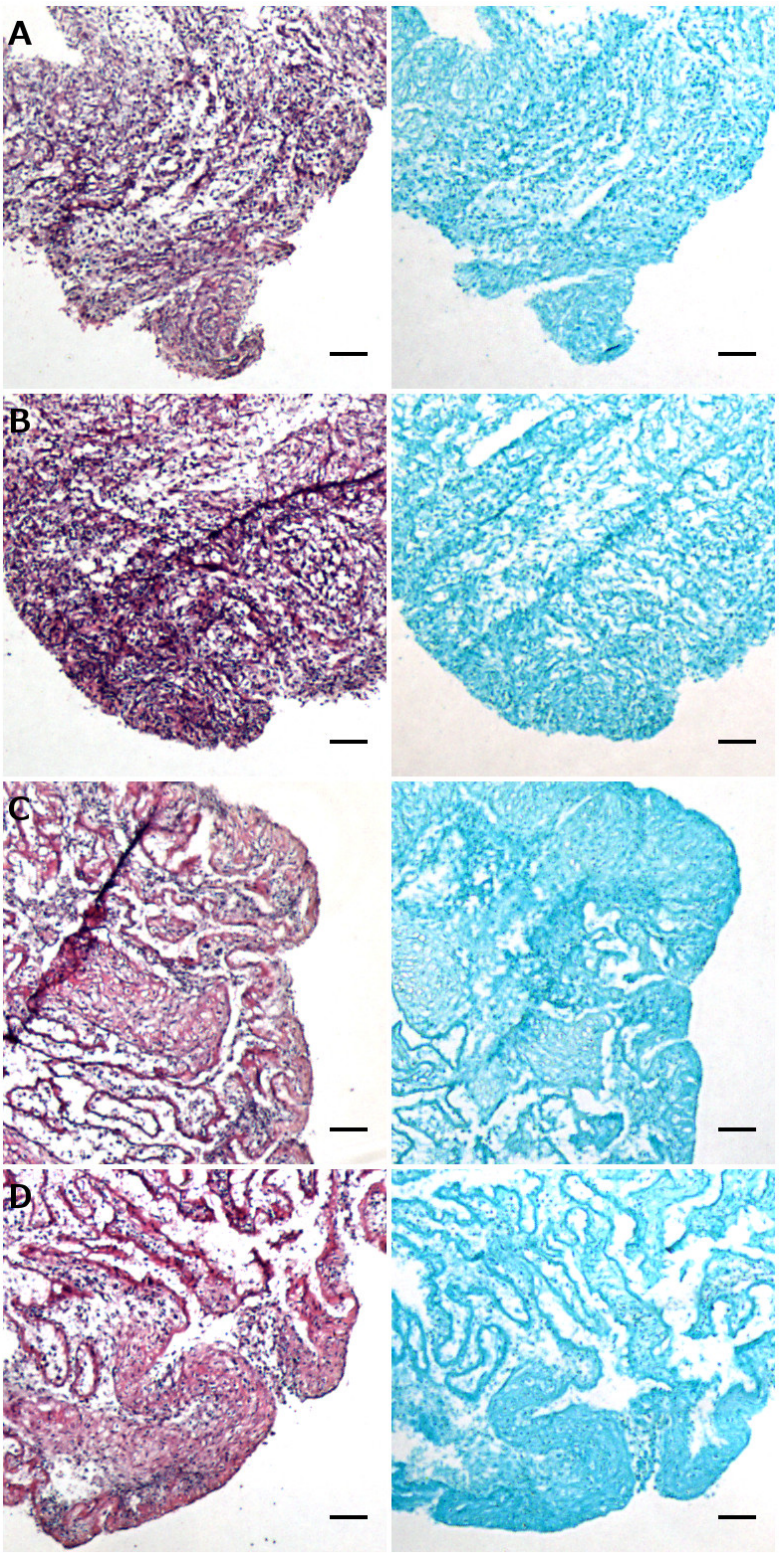


Figure 3.14: Donor 6 SF MPC constructs stained with H&E (left) and Alcian blue (right). AA constructs, unloaded (A) and loaded (B), and chondrogenic constructs, unloaded (C) and loaded (D). Scale bars 100 µm.

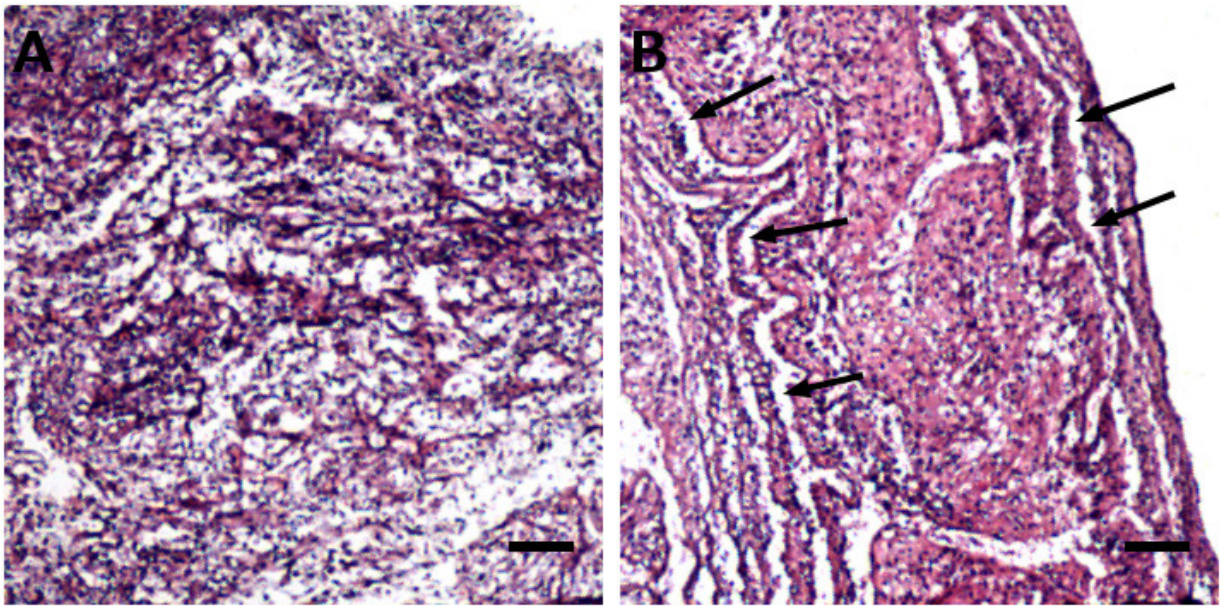


Figure 3.15: Differences in bulk configuration between AA (A) and chondrogenic (B) Donor 6 constructs. Arrows show continuous voids that may be indicative of construct configuration following aggregation. Scale bar 100 µm

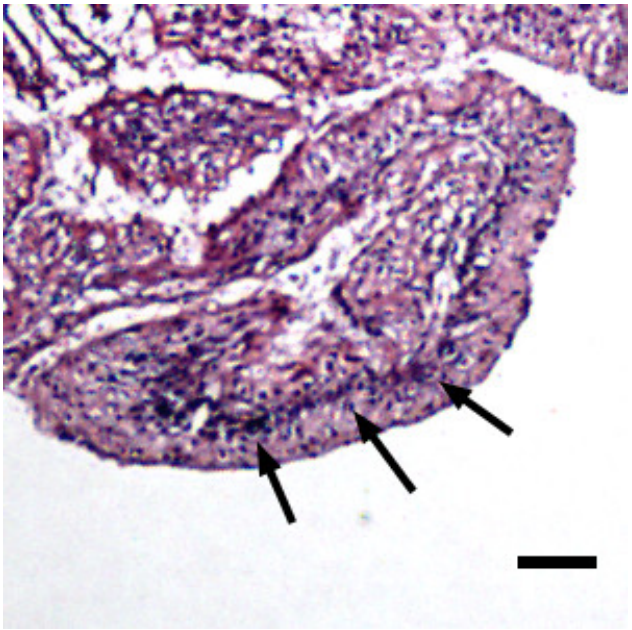

Figure 3.16: Clustering of cell nuclei (arrows) in non-surface regions of a Donor 6 chondrogenic construct. Scale bar 100 µm.

and in many cases are relatively homogeneous from the surface to the near-surface regions (Fig. 3.17A). Conversely, the chondrogenic constructs generally possessed a clear and distinct border that was rounded and smooth. In some cases, the chondrogenic constructs appeared to possess a distinct border layer characterized by intense Alcian blue staining, which looked like a darkened band under H&E staining (Fig. 3.17B). Cell nuclei could also be found within this border layer, although they appeared compressed and elongated parallel to the construct border.

Differences were also observed in how individual cells appeared to be situated in the chondrogenic constructs. In many areas the cells appeared to reside within distinct, rounded voids (Fig. 3.18). This was not observed in the AA constructs.

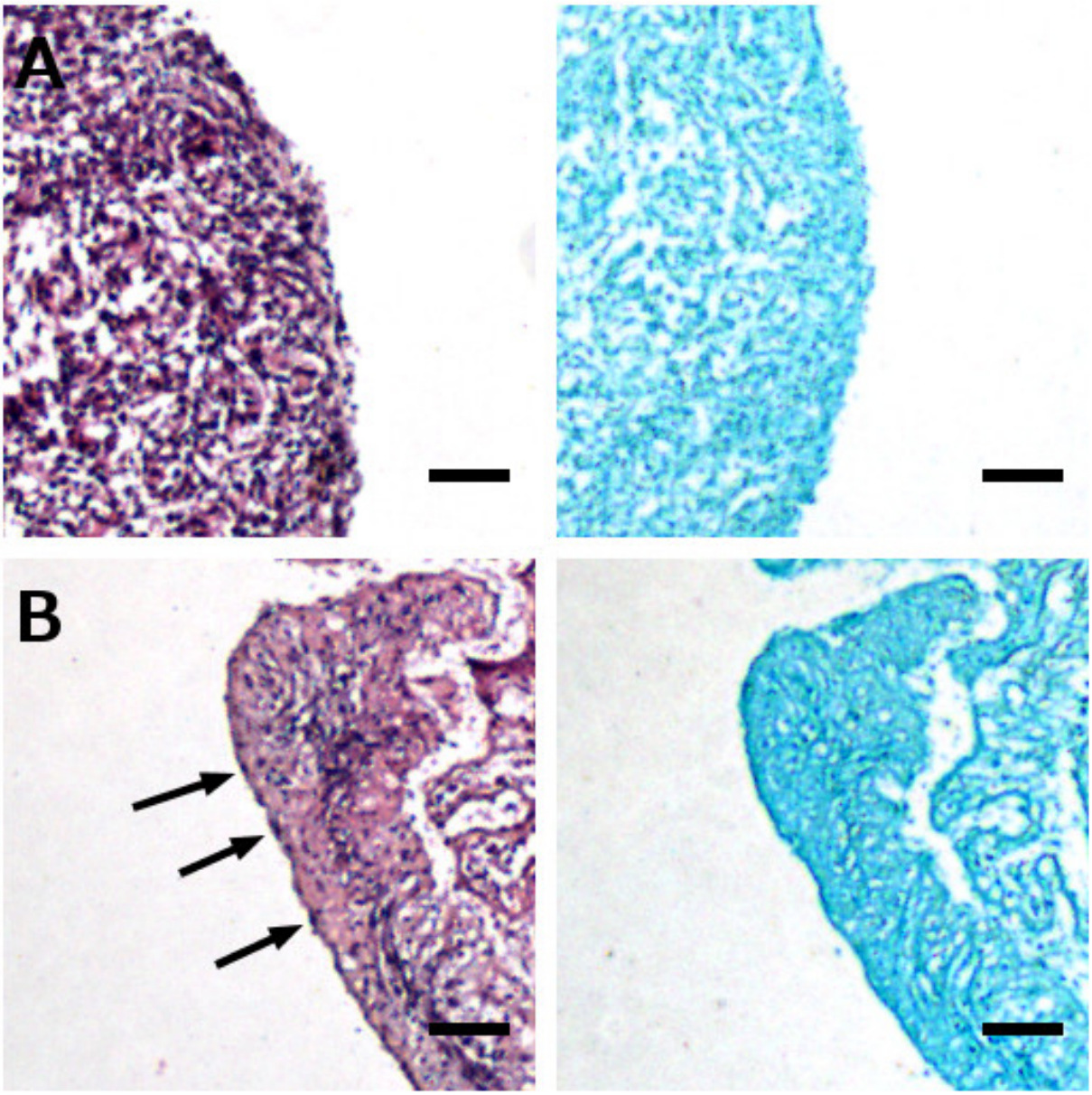


Figure 3.17: AA (A) and chondrogenic (B) Donor 6 constructs exhibit differences in their borders under H&E (left) and Alcian blue (right) staining. Arrows indicate elongated cell nuclei parallel to construct border. Scale bar 100 µm.

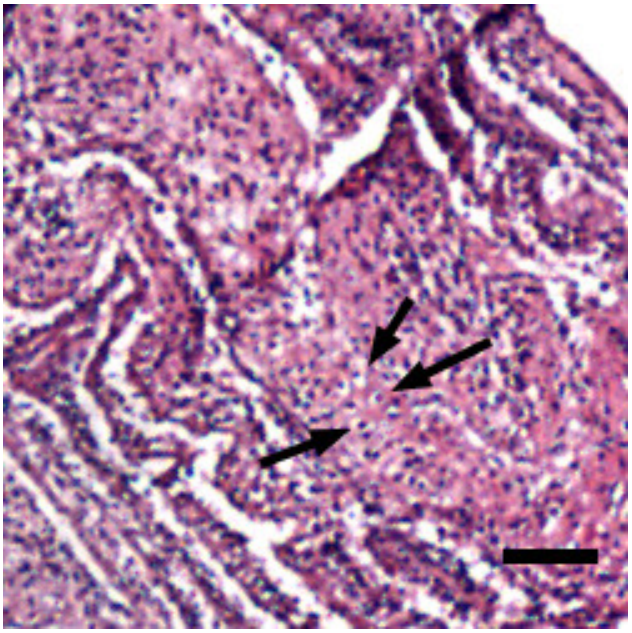

Figure 3.18: Section of Donor 6 chondrogenic construct stained with H&E apparently showing lacunae (arrows). Scale bar 100 µm.

# Chapter 4

# Discussion

## 4.1 MPC Variability and Heterogeneity

Variability was frequently encountered throughout this study in a number of forms, including variability in gene expression, cell morphology during the generation of constructs, and in histological findings. Coupled with the relatively low number of biological replicates used in experiments, it is difficult to make firm conclusions with respect to the role of mechanical loading in modulating the behaviour of the constructs investigated here.

There are a number of potential sources of variability that may have played a role in this study, as well as a number of ways that they could be addressed in future studies, including donor variability, tissue heterogeneity, and variability in mechanical loading.

### 4.1.1 Donor Variability

The variation that exists from person to person is an important issue that becomes especially relevant when translating a new technology to a clinical application. Tissue from several donors of different age and sex were utilized in this study, making donor-to-donor variability an important consideration.

One study utilizing human bone marrow MPCs derived from two different donors found that clonal populations could possess dramatically different combinations of differentiation potency (e.g. tripotent, bipotent, etc.) in a hierarchical organization that was not consistent between donors (Russell *et al.*, 2010). Other studies investigating bone marrow MPCs from a variety of donors have found substantial variability in their capacity for different types of differentiation (DiGirolamo *et al.*, 1999) and growth kinetics (Phinney *et al.*, 1999).

Another study examined human bone marrow MPCs from over 50 donors of both gen-

ders across a broad age range (Siegel *et al.*, 2013), and subsequently analyzed them for gender and age differences with respect to phenotype, growth kinetics, colony formation, and differentiation. The investigators found several statistical differences in MPC growth and proliferation based on age and gender, but no consistent differences in differentiation capacity. The gene expression of several differentiation markers as presented in the study exhibits such substantial variation that little can be concluded in terms of the effects of age and gender on MPC differentiation potential, other than that they may exist.

Donor-dependent variability in the response of MPCs to mechanical loading is another important factor that has been recently investigated. A recent study using MPC-laden constructs subjected to hydrostatic pressure reported substantially different collagen and GAG deposition between MPCs derived from different donors (Meyer *et al.*, 2011).

The findings of these studies highlight the possible variability that can be introduced into a study by utilizing tissue from multiple donors. MPC function can vary unpredictably from donor to donor, and it is therefore difficult to make broad conclusions given the small number of donors used in the present study. However, the findings of the present study do serve to emphasize the fact that it may be necessary to approach this problem on a patient-to-patient basis and the potential importance of personalized medicine.

### 4.1.2 Cell Heterogeneity

Even within a single donor, it is known that there is heterogeneity amongst MPCs, in terms of their morphology, differentiation potential, and cell surface markers. One of the significant obstacles in stem cell research is the absence of a clear definition or a means of consistently isolating a homogeneous population of cells. The most frequently cited criteria for MSCs were formulated by the International Society for Cellular Therapy (Dominici *et al.*, 2006) and defined MSCs as plastic-adherent cells that express CD105, CD73, and CD90, while not expressing CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface markers. They also need to be capable of osteogenic, chondrogenic, and adipogenic differentiation.

Consequently, MSCs are often functionally isolated based on the expansion of plastic-adherent cells derived from stem cell-containing tissues such as bone marrow, adipose tissue, SM, etc. (Sivasubramaniyan *et al.*, 2012). Indeed, the SM and SF cells used in this study generally fit the above criteria. They readily adhered to plastic culture flasks and both SM and SF MPCs have a demonstrated capacity for trilineage differentiation (De Bari *et al.*, 2001; Jones *et al.*, 2004). As well, the cells were depleted for several of the indicated antigens and subsequently enriched for $CD90^+$ cells, and therefore it's likely that the cells used in these experiments comprise a sizeable progenitor population. However, even ignoring the non-MPC cell types potentially present, there is still a substantial capacity for heterogeneity.

The criteria specified above are useful in the isolation of MPCs, yet they are not entirely specific to a homogeneous population of MPCs (Pevsner-Fischer *et al.*, 2011) but rather likely describe a heterogeneous cell population that one might find within a native tissue. A growing area of research is focused on the investigation of stem cell subpopulations. Several studies have investigated the possibility of MPC subpopulations that may possess unique properties or be particularly suited for certain applications, such as enhanced capacities for certain types of differentiation. It is well-known that MPCs exhibit functional differences based on the tissue of origin, such as the increased tendency toward chondrogenic differentiation in SM MPCs (Sakaguchi *et al.*, 2005) or differences in immunomodulatory effects (Melief *et al.*, 2013).

There is growing interest in the investigation of MPC subpopulations within individual tissues. Identifying these subpopulations is typically performed through the use of cell surface markers. One such study found that $CD73^+$, $CD106^+$, and $CD271^+$ cells isolated from synovial membrane MPCs exhibited differing potentials for differentiation, and concluded that the CD271- and CD73-enriched population may be especially suited for cartilage tissue-engineering applications (Arufe *et al.*, 2010). Another study found functional differences in bone marrow MPCs sub-grouped based on proliferation characteristics that were reflected

in gene expression profiles (Tormin *et al.*, 2009).

It has been demonstrated that clonal populations of cells in MSC-containing tissues, such as bone marrow, can differ substantially in their ability to undergo trilineage differentiation (Pittenger, 1999). Moreover, changes in differentiation potential have been observed following extended time in culture (Muraglia *et al.*, 2000). This has also been demonstrated in SM MPCs, where a recent study found an increased chondro-osteogenic differentiation ability in $CD73^+/CD39^+$ culture-expanded synovial membrane MPCs (Gullo and De Bari, 2013). However, the same enhanced differentiation capacity was not observed in the same subpopulation isolated from fresh SM tissue. While this particular finding may be potentially advantageous in the clinical application of culture-expanded MPCs, such as in regenerative medicine, it likely also represents another dimension of variability that could hamper subsequent studies and thus requires further investigation.

There is also evidence to suggest that there may be a certain amount of heterogeneity inherent in MPCs. Colonies of single cell-derived bone marrow MPCs have been shown to yield cells with different differentiation capacities (DiGirolamo *et al.*, 1999).

In the context of the present study, there is strong evidence of MPC subpopulations within synovial tissue (Karystinou *et al.*, 2009) that may partially explain the variability in gene expression observed. Although similar heterogeneity has not been demonstrated in SF MPCs, they are theorized to originate in the synovial tissue (Jones *et al.*, 2008) and would likely exhibit the same heterogeneity seen in SM MPCs.

### 4.1.3 Other Factors

Besides the heterogeneity that exists between donors and the possible intrinsic heterogeneity found within an individual donor, there are a number of other factors that could conceivably have contributed to the variability observed in the present study. Most significantly, these would likely be related to cell culture and cell derivation methods.

It is commonly believed that, in general, MPCs exhibit declining differentiation potential

with extended time in culture. This has been demonstrated in human bone marrow MPCs in a number of investigations (DiGirolamo *et al.*, 1999; Conget and Minguell, 1999; Bonab *et al.*, 2006). However, some studies have suggested that this may not occur in all cells equally (Muraglia *et al.*, 2000) and may in some cases lead to enhanced differentiation potential in certain MPC subpopulations, as has been shown in synovial tissue MPCs (Gullo and De Bari, 2013).

Heterogeneity could also be introduced as a result of the methods used in cell derivation. The methodology used to obtain MPCs has previously been suggested to be a source of variation in the resulting cells (Muschler *et al.*, 1997). There is more than one method used to derive cells from synovial tissue, including enzymatic digestion (Harvanová *et al.*, 2011) and the direct culture of SM explants (Harris *et al.*, 2013). Even SF MPC isolation can be performed in more than one way, including cell pellet resuspension following centrifugation (Harvanová *et al.*, 2011) and direct plating of synovial fluid samples (Krawetz *et al.*, 2012).

The effect of cell culture and cell isolation methods may play a major role in modifying the behaviour of the resulting cells and result in the inadvertent selection of a particular MPC subpopulation that may vary from donor to donor. Further studies may need to investigate this potential confounding factor in order to improve synovial MPC outcomes.

## 4.2 Construct Characterization

The main objective of the present study was to investigate the effects of mechanical loading on exogenous scaffold-free tissue-engineered constructs generated from human synovial MPC monolayers. Constructs were generated through one of two methods of generation: AA-supplemented media, or chondrogenic differentiation media. Constructs were successfully generated by both methods, from both synovial membrane (SM) and synovial fluid (SF) MPCs derived from several different donors. The constructs were subjected to confined

compressive loading and subsequently analysed. For the purposes of this discussion, the main focus will be on the data obtained from the final, largest set of experiments (§3.5).

In order to characterize the constructs and the constituent MPCs two main types of analysis were performed. First, the gene expression of the constructs was analyzed in order to ascertain the presence of various differentiation markers, including those for chondrogenesis (SOX9, aggrecan, type-II collagen), osteogenesis (Runx2, type-I and type-X collagen) and adipogenesis (adiponectin). Secondly, histological staining was used to investigate the structure of the constructs as well as possible differentiation. Attempts were made to characterize the mechanical properties of the constructs through mechanical testing. These tests were largely unsuccessful and are described in greater detail in the appendix (§A.1)

### 4.2.1 Macroscopic Appearance

On a macroscopic level, all of the constructs shared a number of similarities, including a gel-like appearance and roughly spherical shape. The AA constructs were noticeably thinner and more delicate than the chondrogenic constructs, and also did not exhibit the auto-aggregation behaviour seen in many of the chondrogenic constructs. The AA constructs generally did not exceed 1-2 mm in diameter, whereas the chondrogenic constructs ranged from 3-5 mm in diameter. Given that the construct scaffold consisted entirely of endogenous matrix proteins, this likely indicates that there was significantly more ECM protein deposition in the chondrogenic constructs. This could be confirmed by weighing the constructs directly, which was not done in the present study due to the risk of contamination and due to technical issues. AA is known to play a major role in collagen protein production (Geesin *et al.*, 1991), and therefore is commonly used as part of most chondrogenic and osteogenic differentiation media compositions (Na *et al.*, 2007; Choi *et al.*, 2008; Fernandes *et al.*, 2010). However, other factors used in most chondrogenic differentiation media are known to induce comparatively more matrix protein deposition. In the present study, these included TGF-β3, BMP-2, and dexamethasone. The difference in size can therefore reasonably be attributed

to the additional matrix protein expression induced by these extra factors. In particular, TGF-β3 is likely a main contributor (Yoo *et al.*, 1998). This is somewhat reflected in the gene expression data (Fig. 3.11) which show substantial upregulation in some ECM protein mRNA expression levels in some of the chondrogenic constructs. However, the snapshot of gene expression showed by those figures may not be representative of the patterns of expression that may have existed during the 21 days of construct generation.

### 4.2.2 MPC Differentiation

The differentiation of the MPCs in the constructs was investigated through gene expression of various markers and histological staining. Several early- and later-stage markers were investigated.

In contrast to some earlier results (i.e. Donor 5) the expression of adiponectin mRNA was largely non-existent in the Donor 6 constructs. Taken together with the absence of positive staining with Oil Red O staining or observed lipid globules during monolayer culture, it is unlikely that adipogenic differentiation took place in these constructs. The findings from the Donor 5 constructs remain largely unexplained and may be an outlier that can be attributed to donor variability.

The MPCs in the constructs most likely differentiated along chondrogenic or osteogenic lineages. Transcription factors commonly used as markers of these types of differentiation (SOX9 and Runx2, respectively) were detected to varying degrees throughout most of the constructs. Differences were present both between the AA and chondrogenic constructs, as well as between the loaded and unloaded constructs. There were no consistent differences in either case, but the differences often exceeded 10-fold.

Similarly, there were inconsistent differences in mRNA expression for various ECM proteins often used as markers of chondrogenic and osteogenic differentiation. Type-II collagen is one of the main markers of chondrogenesis. It was detected intermittently in the SM constructs, but was absent in the SF constructs. It is not exactly clear why type-II col-

lagen was generally weakly expressed because, with the possible exception of the loading, the conditions present in the experiments likely were amenable to chondrogenesis. While it has been shown that chondrogenesis is supported by three-dimensional culture of MPCs (Caterson *et al.*, 2001), it has also recently been shown that $CD90^+$ SF MPCs are capable of chondrogenesis while remaining in a two-dimensional monolayer (Krawetz *et al.*, 2012). This would likely have permitted MPC chondrogenesis *in vivo*. In future studies, the presence of type-II collagen could be more definitively determined via immunostaining.

Aggrecan is another ECM protein used as a marker of chondrogenesis. Its gene expression was detected much more strongly than type-II collagen, although to similarly varying degrees. However, its value as a marker was arguably diminished by the absence of significant type-II collagen expression. As described earlier, the function of articular cartilage depends on the combined activity of aggrecan and type-II collagen. The presence of GAGs based on positive Alcian blue staining in both AA and chondrogenic constructs regardless of loading support the presence of aggrecan throughout the constructs.

Type-I and Type-X collagen can both be used as markers of osteogenesis. There was found to be a statistically significant non-zero median difference in gene expression of both of these proteins between AA and chondrogenic constructs. Type-I collagen forms the basis of many tissues throughout the body, and together with type-X collagen may indicate osteogenesis via endochondral ossification. It may suggest that the MPCs are likely to undergo hypertrophy and eventually drive ECM mineralization. However, matrix mineralization can be a lengthy process, and although none was observed under Alizarin staining, it is unclear if mineral deposits may appear after an extended period of time. This does raise the possibility of the applicability of these constructs in bone tissue engineering and may be worth further study.

Interestingly, the constructs did not generally show either chondrogenic markers or osteogenic markers exclusively. Since kqPCR analysis makes use of total RNA and thus mRNA from all of the MPCs within the constructs, it's possible that both modes of differentiation

were taking place in a given construct. While this may be useful evidence in the potency of the MPCs in the constructs, future investigations may find it desirable to look at methods of better controlling the differentiation of the construct MPCs, such through the use of clonal cell cultures.

### 4.2.3 Construct Structure

A number of noticeable features were identified in the histological sections of the constructs generated during the course of these experiments. They were generally observed in both the loaded and unloaded constructs, suggesting that their appearance is not related to the application of mechanical loading.

In general, the constructs exhibited varying degrees of porosity. This may be attributable to the manner of ECM deposition in monolayer culture, as well as a consequence of the monolayer folding and configurational changes that took place during construct aggregation. The resulting three-dimensional constructs had large voids where there were visible gaps between different parts of the original monolayer. This is a potential limitation of the aggregated monolayer approach, as it does not currently offer the same control or homogeneity that exogenous three-dimensional scaffolds do. In the present study, it was not possible to control how the MPC monolayers aggregated, and thus dictate the final three-dimensional configuration of the constructs. However, these issues may be mitigated over longer periods of culture and mechanical loading post-aggregation. There is evidence that cartilage undergoes remodelling in response to mechanical loading *in vivo* (Grodzinsky *et al.*, 2000), and that MPCs can modify the mechanical properties of a construct scaffold (Awad *et al.*, 2004). It is possible that similar remodelling could be replicated in the AA and chondrogenic constructs investigated here.

The constructs also exhibited substantial structural heterogeneity, based on variations in staining intensity, the non-uniform distribution of cell nuclei, and the appearance of lacunae-like structures in various regions of the chondrogenic constructs. Some chondrogenic con-

structs appeared to have an approximately 10-20 µm thick border containing elongated cell nuclei in some outward-facing regions. This phenomenon was not observed in all chondrogenic constructs and was notably absent in the AA constructs. It is unlikely that this is a technical artifact occurring as a result of histological analysis. It is well-known that native articular cartilage has a heterogeneous and anisotropic structure that is intimately related to the complex mechanical environment developed during physiological loading. The distinct superficial region of cartilage is also known to play an important role in joint function. Many different tissue engineering approaches attempt to create spatially-varying mechanical properties in constructs through the use of different scaffold materials and manufacturing techniques (Woodfield *et al.*, 2004; Moroni *et al.*, 2007; Nguyen *et al.*, 2011). However, the role of the border region observed in these constructs is unclear. Future studies that investigate the collagen structure within the constructs, such as through the use of polarized light microscopy (Rieppo *et al.*, 2009), may indicate if there is a systematic variation in collagen structure and orientation. As well, immunostaining for proteins known to be expressed in articular cartilage surface layers, such as lubricin (Swann *et al.*, 1981; Schumacher *et al.*, 1994), may give an indication of the unique activity, if any, of the observed boundary region.

## 4.3 Mechanical Loading

### 4.3.1 The Effect of Mechanical Loading

One of the major focuses of the present study was to investigate the potential role of mechanical loading in modulating the behaviour of MPC-laden exogenous scaffold-free tissue-engineered constructs. The data gathered during these experiments suggest that the application of mechanical loading in the form of confined compression did indeed elicit observable differences in the behaviour of the MPCs in the constructs. However, the differences generally did not follow clear trends. As well, the difference in gene expression between loaded and unloaded constructs tended to orders of magnitude smaller in comparison to the difference

between AA and chondrogenic constructs, regardless of loading (Fig. 3.11). Some stand-out examples include type-I and type-X collagen expression in SM constructs.

One of the most likely explanations for this apparently weak influence of mechanical loading has to do with the loading protocol utilized in these experiments. Due to a number of constraints and obstacles, including limited cell numbers, cost, the limited number of constructs that could be loaded concurrently, and the difficulties associated with extended loading protocols, a very limited amount of time was spent optimizing the loading protocol for the experiments used in this study.

It is unlikely that the type of mechanical loading used in these experiments was a major contributor to the lack of clear response. Dynamic compression is one of the most commonly used types of mechanical loading in the context of cartilage tissue engineering, having been used previously to great effect in articular chondrocyte-based tissue-engineering efforts (Kisiday *et al.*, 2004; Waldman and Spiteri, 2004). Many studies have investigated the potential for compressive loading to enhance the chondrogenic differentiation of MPCs (Campbell *et al.*, 2006; Angele *et al.*, 2004). However, it has also been shown in porcine MPC-seeded agarose hydrogels that intermittent dynamic compression can lead to a suppression of chondrogenesis (Thorpe *et al.*, 2008) based on Alcian blue staining and immunostaining for type-II collagen. These related but diverse findings highlight the sensitivity of MPCs to their mechanical environment, and the value of the optimization of mechanical loading protocols.

As well, investigators unfortunately do not always explicitly or clearly describe the method by which mechanical compression was applied (Pelaez *et al.*, 2009). This can in turn obfuscate details that may give insight to other aspects of the mechanical environment, such as fluid movement or potential hydrostatic forces, that develop during loading. This is significant because it has been suggested that fluid shear developed during dynamic compressive loading may play a dominant role in some circumstances (Tanaka *et al.*, 2005).

Therefore, understanding the nature of the loading developed during an experiment is a critical component in making any conclusions with respect to the effect of the loading applied.

Confined compression is a commonly used mode of loading for promoting chondrogenic differentiation in MPCs, however the compressive strain utilized in the present study was approximately 5%, whereas many studies utilize a 10% strain. Chondrocytes are known to respond to compression over a range of strains, including strains on the order of 5% (Guilak *et al.*, 1994; Buschmann *et al.*, 1995). The applied strain may not have been optimal for promoting chondrogenesis, but it is unlikely that the loading parameters used for experiments failed to meet a minimum threshold in order to elicit a response. Therefore, the findings still adequately demonstrate that loading can induce differences compared to unloaded controls. With the exception of the strain magnitude, the loading protocol used in this study is in line with a number of studies. A 1 Hz frequency is by far the most commonly used in compressive loading for chondrogenesis in MPCs, and is rooted in a variety of studies that emphasize its physiological relevance (Lee and Bader, 1997; Hung *et al.*, 2004).

It could be argued that hydrostatic pressure would be a more appropriate form of mechanical loading, given that articular cartilage is primarily subjected to cyclic hydrostatic pressure *in vivo* during normal joint activity. Several studies have examined the effect of cyclic hydrostatic pressure on MPCs. Angele *et al.* (2003) found that cyclic hydrostatic pressure led to increases in proteoglycan and collagen in human bone marrow MPC aggregates that had been cultured in chondrogenic media. Another study found that hydrostatic pressure could lead to increases in chondrogenic gene expression markers in MPC aggregates, but was not as effective a stimulus as TGF-β3 supplementation (Miyanishi *et al.*, 2006). However, contrary results have also been reported in other studies. Zeiter *et al.* (2009) investigated possible synergistic interactions between hydrostatic pressure, BMP-2, and TGF-βin bovine bone marrow MPCs and found that only TGF-βled to increases in gene expression of chondrogenic markers and GAG levels. Therefore, it is unclear whether

hydrostatic pressure would be a more efficacious method of mechanical loading. It could potentially be explored within the current platform with relative ease. By substituting the porous plug with a solid plug or other impermeable component it may be possible to create a hydrostatic environment in the gel. This would likely necessitate additional development and characterization of the system, however.

Another possible factor in the limited response observed in these experiments is the timing of mechanical loading. These experiments utilized a single, 4-hour bout of loading and a 24-hour rest period. Conversely, many studies utilize longer time frames and repeated loading cycles ranging from 7 days (Angele *et al.*, 2004) to 6 weeks (Thorpe *et al.*, 2010). These longer-term loading protocols may have two benefits: the relevant signal pathways have a longer time to convey the necessary signals and elicit change in cell behaviour, and there's an extended period of time over which the resulting changes in gene and protein expression can be observed. In other words, it may simply be that the experiments in this study concluded before significant effects of mechanical loading could be observed. Alternatively, it may be that there is a specific window of time during which the MPCs are most receptive to loading. The importance of the timing of loading has been demonstrated using human bone marrow MPCs subjected to hydrostatic pressure (Hess *et al.*, 2009).

### 4.3.2 Sources of Variability in Mechanical Loading

It has been reported in several studies that the specific parameters of a loading protocol can have a profound effect on MPC behaviour. Other types of loading, such as uniaxial or biaxial tension, and fluid shear are also known to be potent modifiers of MPC behaviour. Tensile loading and fluid shear have both been used to stimulate osteogenic differentiation in MPCs in a number of different studies (Jagodzinski *et al.*, 2004; Sumanasinghe *et al.*, 2006; Yourek *et al.*, 2010).

If the constructs generated in the present study and their constituent MPCs did not experience uniform mechanical loading it may not be surprising if they exhibit a variable

and heterogeneous response. Significant structural and configurational differences could be observed amongst constructs, and the chondrogenic constructs in particular exhibited substantial variation as a result of the way the constructs transitioned from cell monolayers to three-dimensional constructs during aggregation. The cells exhibited directionality that varied regionally, which could result in a difference in the strains they would have been subjected to during loading. Moreover, since construct aggregation may indicate a certain amount of internal tensile loading developed within the constructs, they may be subjected to heterogeneous mechanical loading even before the external compression is applied. However, the effect of any possible internal loading is unclear and may warrant further study.

It could be clearly seen amongst the constructs that they originated as aggregated monolayers, as opposed to a monolithic three-dimensional construct. Relatively large voids were formed in some of the constructs that were retained even when they were cast into agarose gels for loading. These pockets of fluid could conceivably have generated substantial shear during compressive loading in a manner that would have been non-uniform throughout a single construct and likely not consistent with other constructs within the same experiment.

Moreover, due to the complex and unique geometry of the constructs there is likely significant variation in the internal strains developed within the constructs. Since different areas in the constructs were likely free to deform in a variety of ways, including uniaxial or biaxial tension and compression, bending, torsion, etc. the local mechanical environment developed within the constructs may be significantly more complex than the externally-applied global strain would suggest. Therefore, knowing that different types of loading can have dramatically different effects on MPCs, it is not necessarily surprising if the cells within the constructs react inconsistently and in an exceedingly variable way.

## 4.4 Limitations

The main limitation of the study has to do with the limited number of replicates that were utilized during experiments. Due to a variety of factors (e.g. cost, technical limitations, etc.) a limited number of constructs could be generated and subjected to mechanical loading in a single experiment. Consequently, cells from various donors were utilized which introduced the issue of donor-to-donor variability. However, even mitigating this factor by using cells from only one donor didn't eliminate the problem, likely due to the heterogeneity inherent within tissues and cells. These factors significantly affected the statistical analysis, such that very few statistically significant relationships within the data were found. As well, the potential confounding effect of the varied age and sex of the tissue donors in the present study was not assessed.

As well, the experiments in this study took place over a 21-day span, while many studies investigating MPC chondrogenesis take place over twice as long a period of time. The constructs were also only subjected to one bout of loading, while other studies make use of repeated intermittent loading. The relatively short time frame combined with relatively short loading may not have been optimal conditions for studying the effect of mechanical loading on these constructs.

## 4.5 Future Directions

The present study could serve as a useful platform for future studies with a variety of directions.

As described earlier, one of the major obstacles in the present study was the variability in data observed. This may have come from a number of sources. Reducing this variability and examining methods to mitigate the effect of heterogeneity encountered may be useful in future endeavours. One of the main obstacles encountered in this study was the limited number of cells that could be obtained in a reasonable amount of passaging, which is part

of why MPCs derived from different donors were used. As well, many tissue engineering strategies require huge populations of cells. Therefore, methods of enhancing cell expansion without significant losses in MPC function could be of great benefit. Many recent advances in bioreactors and microcarrier technology have made it possible to efficiently culture various types of cells, including MPCs (Malda and Frondoza, 2006). Utilizing these developments for future experiments could eliminate or minimize some of the heterogeneity associated with MPCs from different sources if it would enable generating more cells from a single source. This would permit the generation of not only more constructs, but also larger constructs. This in particular may be useful for more in-depth histological analysis.

Scaling up the generation of constructs presents another major obstacle in the cost of chondrogenic factors. Constructs are generated in a two-dimensional monolayer, and combined with regular media changes this would necessitate the use of large amounts of chondrogenic factors in order to generate chondrogenic constructs. Consequently, it would likely be worthwhile to investigate alternative methods of enhancing chondrogenic activity in the constructs. For example, the use of low oxygen tension may help to improve outcomes. Native articular cartilage is normally relatively hypoxic due to the lack of vasculature, and lower levels of oxygen have been suggested to improve chondrogenic differentiation in MPCs (Buckley *et al.*, 2010; Sheehy *et al.*, 2012). There is even data to indicate that oxygen tension has a more potent effect than compressive loading in inducing chondrogenic differentiation in MPCs (Meyer *et al.*, 2010).

As well, future experiments may be conducted over a longer time frame and use repeated, intermittent loading cycles. Mechanical loading-induced changes in gene expression, matrix deposition, and construct structure may become more pronounced over a longer duration. The long-term changes that may occur in the constructs in the three-dimensional configuration following aggregation were not thoroughly investigated in this study and it may be worthwhile to investigate whether the constituent MPCs remodel the construct, either in

response to aggregation or as a result of repeated cycles of mechanical loading. It may also give a better indication of the heterogeneity of the constructs. More conclusive evidence of differentiation in the constructs may only become apparent in a longer time frame. However, given the potential issues with sterility mentioned previously, this may require modification or additional development of the loading system in order to ensure the long-term sterility that would be required for repeated loading cycles.

Another future direction could pursue more in-depth construct characterization. For instance, immunostaining for a variety of matrix proteins may give more insight into the components of the constructs. This may also provide more information about certain phenomena observed in the constructs, such as the non-uniform distribution of cells and construct borders observed in some of the chondrogenic constructs. Finally, it may be worthwhile investigating the mechanism underlying the response to mechanical loading in the constructs. Compromising integration of the cells with the ECM by inhibiting integrin binding or blocking the TGF-β signalling pathway may give insight into how signals are transformed in an exogenous scaffold-free construct.

# Chapter 5

# Conclusions

In this study, exogenous scaffold-free tissue-engineered constructs were generated from human MPC monolayers by treating them with either AA-supplemented culture media or chondrogenic differentiation media. Constructs were successfully generated from both SM and SF MPCs from different donors by both generation methods. The constructs were then subjected to confined compressive loading using a modified Flexcell system. To the extent of our knowledge, the investigation of scaffold-free constructs under the influence of mechanical loading has not been studied previously, and therefore the present study represents a novel effort to that end.

The generated constructs shared many similarities regardless of cell source. AA constructs were noticeably thinner and more delicate, whereas the chondrogenic constructs were thicker, more robust, and demonstrated a capacity for auto-aggregation into the three-dimensional construct form. The AA constructs did not exhibit auto-aggregation.

In subsequent analyses the constructs exhibited substantial variability and heterogeneity. Constructs derived from different tissue biopsies from a single donor did not exhibit consistent trends in mRNA expression. Notably, commonly used gene expression markers of chondrogenic differentiation were variably expressed both depending on the method used to generate constructs (AA versus chondrogenic) and in response to mechanical loading. Type-II collagen mRNA was not strongly detected throughout this study, likely indicating the absence of a mature articular chondrogenic phenotype. However, this likely also indicates that the constructs retained their chondrogenic potential throughout the duration of the experiments. Also, although the differences in gene expression between loaded and unloaded constructs was smaller than the different between AA and chondrogenic constructs,

the presence of differences due to loading suggest that the method of mechanical loading utilized in this study could conceivably be used to enhance chondrogenic differentiation in these constructs.

Under histological analysis, it could be seen that the constructs contained a complex matrix that, in the case of the chondrogenic constructs, was heterogeneous. The cells appeared to be intimately integrated into the construct matrices, although cell morphology and surrounding matrix structure varied significantly in the chondrogenic constructs.

The heterogeneity observed in this study likely arose from a number of sources. These include the inherent variability present between donors, as well as variability within the synovial tissue of a single donor that may exist due to the presence of cell subpopulations. Although donor-dependent variability in the response to mechanical loading has been shown previously, the findings of this study may also suggest variability in response between MPCs derived from different tissue biopsies from a single donor. The response to mechanical loading may have varied due to the heterogeneity seen in construct configuration following aggregation. Due to the complex geometry developed within the constructs, local stresses and strains throughout may have been substantially different from the global forces exerted by the loading system.

It was hypothesized that the MPCs in the constructs would be responsive to mechanical loading via connections to the ECM deposited during construct generation and subsequently enhance chondrogenic differentiation. Despite the observed differences in chondrogenic markers following mechanical loading, the role of the ECM was not clearly determined, nor was a clear enhancement of chondrogenic differentiation apparent. Loaded articular chondrocytes appeared to show a difference in chondrogenic gene expression following loading despite likely not having sufficient time to establish ECM, possibly suggesting mechanotransduction by a mechanism unrelated to the ECM. Therefore, the findings of the present study neither confirm nor strictly refute the hypothesis. However, this study does provide a platform from

which future studies could continue to investigate the potential role of mechanical loading on the generation of clinically relevant tissue-engineered constructs for cartilage repair.

# Chapter 6

# Bibliography

Akizuki, S, Mow, VC, Muller, F, Pita, JC, and Howell, DS (1987). Tensile properties of human knee joint cartilage. II. Correlations between weight bearing and tissue pathology and the kinetics of swelling. *Journal of Orthopaedic Research*, 5(2):173–86.

Almarza, AJ and Athanasiou, Ka (2004). Design characteristics for the tissue engineering of cartilaginous tissues. *Annals of Biomedical Engineering*, 32(1):2–17.

Ando, W, Tateishi, K, Hart, DA, Katakai, D, Tanaka, Y, Nakata, K, Hashimoto, J, Fujie, H, Shino, K, Yoshikawa, H, and Nakamura, N (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, Tateishi, K, Katakai, D, Hart, DA, Higuchi, C, Nakata, K, Hashimoto, J, Fujie, H, Shino, K, Yoshikawa, H, and Nakamura, N (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 Engineering Part A*, 14(12):2041–9.

Angele, P, Schumann, D, Angele, M, Kinner, B, Englert, C, Hente, R, Füchtmeier, B, Nerlich, M, Neumann, C, and Kujat, R (2004). Cyclic, mechanical compression enhances chondrogenesis of mesenchymal progenitor cells in tissue engineering scaffolds. *Biorheology*, 41(3-4):335–46.

Angele, P, Yoo, J, Smith, C, Mansour, J, Jepsen, K, Nerlich, M, and Johnstone, B (2003). Cyclic hydrostatic pressure enhances the chondrogenic phenotype of human mesenchymal progenitor cells differentiated in vitro. *Journal of Orthopaedic Research*, 21(3):451–7.

Arufe, MC, De La Fuente, A, Fuentes, I, de Toro, FJ, and Blanco, FJ (2010). Chondrogenic potential of subpopulations of cells expressing mesenchymal stem cell markers derived from human synovial membranes. *Journal of Cellular Biochemistry*, 111(4):834–45.

Awad, HA, Wickham, MQ, Leddy, HA, Gimble, JM, and Guilak, F (2004). Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. *Biomaterials*, 25(16):3211–22.

Bian, L, Zhai, DY, Zhang, EC, Mauck, RL, and Burdick, JA (2012). Dynamic Compressive Loading Enhances Cartilage Matrix Synthesis and Distribution and Suppresses Hypertrophy in hMSC-Laden Hyaluronic Acid Hydrogels. *Tissue Engineering Part A*, 18:715–724.

Bombardier, C, Hawker, G, and Mosher, D (2011). The Impact of Arthritis in Canada: Today and Over the Next 30 Years. Technical report, Arthritis Alliance of Canada.

Bonab, MM, Alimoghaddam, K, Talebian, F, Ghaffari, SH, Ghavamzadeh, A, and Nikbin, B (2006). Aging of mesenchymal stem cell in vitro. *BMC Cell Biology*, 7:14.

Brown, TD (2000). Techniques for mechanical stimulation of cells in vitro: a review. *Journal of Biomechanics*, 33(1):3–14.

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

Burns, M and Valdivia, H (2007). Modelling the limit of detection in real-time quantitative PCR. *European Food Research and Technology*, 226(6):1513–1524.

Buschmann, MD, Gluzband, Y, Grodzinsky, A, and Hunziker, EB (1995). Mechanical compression modulates matrix biosynthesis in chondrocyte/agarose culture. *Journal of Cell Science*, 108 ( Pt 4:1497–508.

Campbell, JJ, Lee, DA, and Bader, DL (2006). Dynamic compressive strain influences chondrogenic gene expression in human mesenchymal stem cells. *Biorheology*, 43(3-4):455–70.

Caterson, EJ, Nesti, LJ, Li, WJ, Danielson, KG, Albert, TJ, Vaccaro, aR, and Tuan, RS (2001). Three-dimensional cartilage formation by bone marrow-derived cells seeded in polylactide/alginate amalgam. *Journal of Biomedical Materials Research*, 57(3):394–403.

Choi, KM, Seo, YK, Yoon, HH, Song, KY, Kwon, SY, Lee, HS, and Park, JK (2008). Effect of ascorbic acid on bone marrow-derived mesenchymal stem cell proliferation and differentiation. *Journal of Bioscience and Bioengineering*, 105(6):586–94.

Conget, P and Minguell, J (1999). Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. *Journal of Cellular Physiology*, 73(January):67–73.

Darling, EM and Athanasiou, KA (2005). Rapid phenotypic changes in passaged articular chondrocyte subpopulations. *Journal of Orthopaedic Research*, 23(2):425–32.

De Bari, C, Dell'Accio, F, Tylzanowski, P, and Luyten, F (2001). Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis and Rheumatism*, 44(8):1928–1942.

Delaine-Smith, RM and Reilly, GC (2011). The effects of mechanical loading on mesenchymal stem cell differentiation and matrix production. *Vitamins and Hormones*, 87:417–80.

DiGirolamo, C, Stokes, D, Colter, D, Phinney, D, Class, R, and Prockop, D (1999). Propagation and senescence of human marrow stromal cells in culture: a simple colonyforming assay identifies samples with the greatest potential to. *British Journal of Haematology*, 107:275–281.

Dominici, M, Le Blanc, K, Mueller, I, Slaper-Cortenbach, I, Marini, F, Krause, D, Deans, R, Keating, A, Prockop, D, and Horwitz, E (2006). Minimal criteria for defining multipo-

tent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*, 8(4):315–7.

Felson, DT, Anderson, JJ, Naimark, A, Walker, AM, and Meenan, RF (1988). Obesity and knee osteoarthritis. The Framingham Study. *Annals of Internal Medicine*, 109(1):18–24.

Fernandes, H, Mentink, A, Bank, R, Stoop, R, van Blitterswijk, C, and de Boer, J (2010). Endogenous collagen influences differentiation of human multipotent mesenchymal stromal cells. *Tissue Engineering Part A*, 16(5).

Geesin, JC, Hendricks, LJ, Gordon, JS, and Berg, RA (1991). Modulation of collagen synthesis by growth factors: the role of ascorbate-stimulated lipid peroxidation. *Archives of Biochemistry and Biophysics*, 289(1):6–11.

Grodzinsky, A, Levenston, M, Jin, M, and Frank, E (2000). Cartilage tissue remodeling in response to mechanical forces. *Annual Review of Biomedical Engineering*, 2:691–713.

Guilak, F, Meyer, B, Ratcliffe, a, and Mow, V (1994). The effects of matrix compression on proteoglycan metabolism in articular cartilage explants. *Osteoarthritis and Cartilage*, 2(2):91–101.

Gullo, F and De Bari, C (2013). Prospective purification of a subpopulation of human synovial mesenchymal stem cells with enhanced chondro-osteogenic potency. *Rheumatology*, pages 1–11.

Harris, Q, Seto, J, O'Brien, K, Lee, PS, Kondo, C, Heard, BJ, Hart, DA, and Krawetz, RJ (2013). Monocyte Chemotactic Protein-1 Inhibits Chondrogenesis of Synovial Mesenchymal Progenitor Cells: an In vitro Study. *Stem Cells*, (403).

Harvanová, D, Tóthová, T, and Sarišský, M (2011). Isolation and characterization of synovial mesenchymal stem cells. *Folia Biologica (Praha)*, 57:119–124.

Haugh, MG, Meyer, EG, Thorpe, SD, Vinardell, T, Duffy, GP, and Kelly, DJ (2011). Temporal and spatial changes in cartilage-matrix-specific gene expression in mesenchymal stem cells in response to dynamic compression. *Tissue Engineering Part A*, 17(23-24):3085–93.

Hazenbiller, O (2012). *Microenvironmental factors directing differentiation of murine embryonic stem cells down osteogenic and chondrogenic lineages*. Masters thesis, University of Calgary.

Hess, R, Douglas, T, Myers, KA, Rentsch, B, Rentsch, C, Worch, H, Shrive, NG, Scharnweber, D, and Hart, DA (2009). Hydrostatic Pressure Stimulation of Human Mesenchymal Stem Cells Seeded on Collagen-Based Artificial Extracellular Matrices. *Journal of Biomechanical Engineering*, 132(2):21001.

Huang, A, Farrell, M, Kim, M, and Mauck, R (2010). Long-term dynamic loading improves the mechanical properties of chondrogenic mesenchymal stem cell-laden hydrogels. *European Cells and Materials*, 19(215):72–85.

Hung, CT, Mauck, RL, Wang, CCB, Lima, EG, and Ateshian, GA (2004). A paradigm for functional tissue engineering of articular cartilage via applied physiologic deformational loading. *Annals of Biomedical Engineering*, 32(1):35–49.

Jagodzinski, M, Drescher, M, Zeichen, J, Hankemeier, S, Krettek, C, Bosch, U, and van Griensven, M (2004). Effects of cyclic longitudinal mechanical strain and dexamethasone on osteogenic differentiation of human bone marrow stromal cells. *European Cells and Materials*, 7:35–41; discussion 41.

Jones, BA and Pei, M (2012). Synovium-Derived Stem Cells : A Tissue-Specific Stem Cell for Cartilage Engineering and Regeneration. *Tissue Engineering Part B*, 18(4):301–311.

Jones, EA, Crawford, A, English, A, Henshaw, K, Mundy, J, Corscadden, D, Chapman, T, Emery, P, Hatton, P, and McGonagle, D (2008). Synovial fluid mesenchymal stem cells in

health and early osteoarthritis: detection and functional evaluation at the single-cell level. *Arthritis and Rheumatism*, 58(6):1731–40.

Jones, EA, English, A, Henshaw, K, Kinsey, SE, Markham, AF, Emery, P, and McGonagle, D (2004). Enumeration and phenotypic characterization of synovial fluid multipotential mesenchymal progenitor cells in inflammatory and degenerative arthritis. *Arthritis and Rheumatism*, 50(3):817–27.

Karystinou, A, Dell'Accio, F, Kurth, TBA, Wackerhage, H, Khan, IM, Archer, CW, Jones, EA, Mitsiadis, TA, and De Bari, C (2009). Distinct mesenchymal progenitor cell subsets in the adult human synovium. *Rheumatology*, 48(9):1057–64.

Kisiday, JD, Frisbie, DD, McIlwraith, CW, and Grodzinsky, AJ (2009). Dynamic compression stimulates proteoglycan synthesis by mesenchymal stem cells in the absence of chondrogenic cytokines. *Tissue Engineering Part A*, 15(10):2817–24.

Kisiday, JD, Jin, M, DiMicco, MA, Kurz, B, and Grodzinsky, AJ (2004). Effects of dynamic compressive loading on chondrocyte biosynthesis in self-assembling peptide scaffolds. *Journal of Biomechanics*, 37(5):595–604.

Krawetz, RJ, Wu, YE, Martin, L, Rattner, JB, Matyas, JR, and Hart, DA (2012). Synovial fluid progenitors expressing CD90+ from normal but not osteoarthritic joints undergo chondrogenic differentiation without micro-mass culture. *PLoS One*, 7(8):e43616.

Lee, DA and Bader, DL (1997). Compressive strains at physiological frequencies influence the metabolism of chondrocytes seeded in agarose. *Journal of Orthopaedic Research*, 15(2):181–8.

Li, Z, Kupcsik, L, Yao, SJ, Alini, M, and Stoddart, MJ (2010a). Mechanical load modulates chondrogenesis of human mesenchymal stem cells through the TGF-beta pathway. *Journal of Cellular and Molecular medicine*, 14(6A):1338–46.

Li, Z, Yao, SJ, Alini, M, and Stoddart, MJ (2010b). 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 Engineering Part A*, 16(2):575–84.

Little, C, Bawolin, N, and Chen, X (2011). Mechanical properties of natural cartilage and tissue-engineered constructs. *Tissue Engineering Part B*, 17(4).

Ma, PX, Schloo, B, Mooney, D, and Langer, R (1995). Development of biomechanical properties and morphogenesis of in vitro tissue engineered cartilage. *Journal of Biomedical Materials Research*, 29(12):1587–95.

Malda, J and Frondoza, CG (2006). Microcarriers in the engineering of cartilage and bone. *Trends in Biotechnology*, 24(7):299–304.

Melief, SM, Zwaginga, JJ, Fibbe, WE, and Roelofs, H (2013). Adipose Tissue-Derived Multipotent Stromal Cells Have a Higher Immunomodulatory Capacity Than Their Bone Marrow-Derived Counterparts. *Stem Cells Translational Medicine*, 2(6):455–463.

Meyer, EG, Buckley, CT, Steward, AJ, and Kelly, DJ (2011). The effect of cyclic hydrostatic pressure on the functional development of cartilaginous tissues engineered using bone marrow derived mesenchymal stem cells. *Journal of the Mechanical Behavior of Biomedical Materials*, 4(7):1257–65.

Meyer, EG, Buckley, CT, Thorpe, SD, and Kelly, DJ (2010). Low oxygen tension is a more potent promoter of chondrogenic differentiation than dynamic compression. *Journal of Biomechanics*, 43(13):2516–23.

Miyanishi, K, Trindade, MCD, Lindsey, DP, Beaupré, GS, Carter, DR, Goodman, SB, Schurman, DJ, and Lane-Smith, R (2006). Effects of Hydrostatic Pressure and Transforming

Growth Factor-Beta3 on Adult Human Mesenchymal Stem Cell Chondrogenesis In Vivo. *Tissue Engineering*, 12(6):1–10.

Miyazono, K (2000). Positive and negative regulation of TGF-beta signaling. *Journal of Cell Science*, 113 ( Pt 7:1101–9.

Morito, T, Muneta, T, Hara, K, Ju, YJ, Mochizuki, T, Makino, H, Umezawa, a, and Sekiya, I (2008). Synovial fluid-derived mesenchymal stem cells increase after intra-articular ligament injury in humans. *Rheumatology*, 47(8):1137–43.

Moroni, L, Hendriks, JAA, Schotel, R, de Wijn, JR, and van Blitterswijk, CA (2007). Design of biphasic polymeric 3-dimensional fiber deposited scaffolds for cartilage tissue engineering applications. *Tissue Engineering*, 13(2):361–71.

Mouw, JK, Connelly, JT, Wilson, CG, Michael, KE, and Levenston, ME (2007). Dynamic compression regulates the expression and synthesis of chondrocyte-specific matrix molecules in bone marrow stromal cells. *Stem Cells*, 25(3):655–63.

Muraglia, A, Cancedda, R, and Quarto, R (2000). Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. *Journal of Cell Science*, 113 ( Pt 7:1161–6.

Muschler, GF, Boehm, C, and Easley, K (1997). Aspiration to obtain osteoblast progenitor cells from human bone marrow: the influence of aspiration volume. *The Journal of Bone and Joint Surgery*, 79(11):1699–709.

Na, K, Choi, SJ, Kim, S, Sun, BK, Woo, DG, Chung, HM, and Park, KH (2007). Enhancement of cell proliferation and differentiation by combination of ascorbate and dexamethasone in thermo-reversible hydrogel constructs embedded with rabbit chondrocytes. *Biotechnology Letters*, 29(10):1453–7.

Natsu-Ume, T, Majima, T, Reno, C, Shrive, NG, Frank, CB, and Hart, DA (2005). Menisci of the rabbit knee require mechanical loading to maintain homeostasis: cyclic hydrostatic compression in vitro prevents derepression of catabolic genes. *Journal of Orthopaedic Science*, 10(4):396–405.

Nguyen, LH, Kudva, AK, Saxena, NS, and Roy, K (2011). Engineering articular cartilage with spatially-varying matrix composition and mechanical properties from a single stem cell population using a multi-layered hydrogel. *Biomaterials*, 32(29):6946–52.

O'Donnell, S, Lagace, C, McRae, L, Badley, E, MacKay, C, Johnson, S, Koehn, C, Legare, J, Moore, L, and Webster, G (2010). Life with arthritis in Canada: a personal and public health challenge. Technical report, Public Health Agency of Canada.

Oldershaw, Ra (2012). Cell sources for the regeneration of articular cartilage: the past, the horizon and the future. *International Journal of Experimental Pathology*, 93(6):389–400.

Pelaez, D, Huang, CYC, and Cheung, HS (2009). Cyclic compression maintains viability and induces chondrogenesis of human mesenchymal stem cells in fibrin gel scaffolds. *Stem Cells and Development*, 18(1):93–102.

Pevsner-Fischer, M, Levin, S, and Zipori, D (2011). The origins of mesenchymal stromal cell heterogeneity. *Stem Cell Reviews*, 7(3):560–8.

Phinney, DG, Kopen, G, Righter, W, Webster, S, Tremain, N, and Prockop, DJ (1999). Donor variation in the growth properties and osteogenic potential of human marrow stromal cells. *Journal of Cellular Biochemistry*, 75(3):424–36.

Pittenger, MF (1999). Multilineage Potential of Adult Human Mesenchymal Stem Cells. *Science*, 284(5411):143–147.

Quinn, T, Maung, A, Grodzinsky, A, Hunziker, E, and Sandy, J (1999). Physical and Bio-

logical Regulation of Proteoglycan Turnover around Chondrocytes in Cartilage Explants. *Annals of the New York Academy of Sciences*, 878:420–441.

Rieppo, J, Hyttinen, MM, Halmesmaki, E, Ruotsalainen, H, Vasara, a, Kiviranta, I, Jurvelin, JS, and Helminen, HJ (2009). Changes in spatial collagen content and collagen network architecture in porcine articular cartilage during growth and maturation. *Osteoarthritis and Cartilage*, 17(4):448–55.

Russell, KC, Phinney, DG, Lacey, MR, Barrilleaux, BL, Meyertholen, KE, and O'Connor, KC (2010). In vitro high-capacity assay to quantify the clonal heterogeneity in trilineage potential of mesenchymal stem cells reveals a complex hierarchy of lineage commitment. *Stem Cells*, 28(4):788–98.

Sakaguchi, Y, Sekiya, I, Yagishita, K, and Muneta, T (2005). Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. *Arthritis and Rheumatism*, 52(8):2521–9.

Schätti, O, Grad, S, Goldhahn, J, Salzmann, G, Li, Z, Alini, M, and Stoddart, MJ (2011). A combination of shear and dynamic compression leads to mechanically induced chondrogenesis of human mesenchymal stem cells. *European Cells and Materials*, 22:214–225.

Schumacher, B, Block, J, Schmid, T, Aydelotte, M, and Kuettner, K (1994). A novel proteoglycan synthesized and secreted by chondrocytes of the superficial zone of articular cartilage. *Archives of Biochemistry and Biophysics*, 311(1):144–152.

Sekiya, I, Larson, BL, Vuoristo, JT, Reger, RL, and Prockop, DJ (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 and Tissue Research*, 320(2):269–76.

Sekiya, I, Ojima, M, Suzuki, S, Yamaga, M, Horie, M, Koga, H, Tsuji, K, Miyaguchi, K, Ogishima, S, Tanaka, H, and Muneta, T (2012). Human mesenchymal stem cells in

synovial fluid increase in the knee with degenerated cartilage and osteoarthritis. *Journal of Orthopaedic Research*, 30(6):943–9.

Setton, La, Mow, VC, Müller, FJ, Pita, JC, and Howell, DS (1994). Mechanical properties of canine articular cartilage are significantly altered following transection of the anterior cruciate ligament. *Journal of Orthopaedic Research*, 12(4):451–63.

Sheehy, EJ, Buckley, CT, and Kelly, DJ (2012). Oxygen tension regulates the osteogenic, chondrogenic and endochondral phenotype of bone marrow derived mesenchymal stem cells. *Biochemical and Biophysical Research Communications*, 417(1):305–10.

Siegel, G, Kluba, T, Hermanutz-Klein, U, Bieback, K, Northoff, H, and Schäfer, R (2013). Phenotype, donor age and gender affect function of human bone marrow-derived mesenchymal stromal cells. *BMC Medicine*, 11(1):146.

Sivasubramaniyan, K, Lehnen, D, Ghazanfari, R, Sobiesiak, M, Harichandan, A, Mortha, E, Petkova, N, Grimm, S, Cerabona, F, de Zwart, P, Abele, H, Aicher, WK, Faul, C, Kanz, L, and Bühring, HJ (2012). Phenotypic and functional heterogeneity of human bone marrow- and amnion-derived MSC subsets. *Annals of the New York Academy of Sciences*, 1266:94–106.

Soltz, M and Ateshian, G (2000). Interstitial fluid pressurization during confined compression cyclical loading of articular cartilage. *Annals of Biomedical Engineering*, 28(2):150–9.

Sumanasinghe, R, Bernacki, S, and Loboa, E (2006). differentiation of human mesenchymal stem cells in collagen matrices: effect of uniaxial cyclic tensile strain on bone morphogenetic protein (BMP-2) mRNA expression. *Tissue Engineering*, 12(12).

Swann, DA, Slayter, HS, and Silver, FH (1981). The molecular structure of lubricating glycoprotein-I, the boundary lubricant for articular cartilage. *The Journal of Biological Chemistry*, 256(11):5921–5.

Tanaka, SM, Sun, HB, Roeder, RK, Burr, DB, Turner, CH, and Yokota, H (2005). Osteoblast responses one hour after load-induced fluid flow in a three-dimensional porous matrix. *Calcified Tissue International*, 76(4):261–71.

Thorpe, SD, Buckley, CT, Vinardell, T, O'Brien, FJ, Campbell, Va, and Kelly, DJ (2008). Dynamic compression can inhibit chondrogenesis of mesenchymal stem cells. *Biochemical and Biophysical Research Communications*, 377(2):458–62.

Thorpe, SD, Buckley, CT, Vinardell, T, O'Brien, FJ, Campbell, VA, and Kelly, DJ (2010). The response of bone marrow-derived mesenchymal stem cells to dynamic compression following TGF-beta3 induced chondrogenic differentiation. *Annals of Biomedical Engineering*, 38(9):2896–909.

Tormin, A, Brune, JC, Olsson, E, Valcich, J, Neuman, U, Olofsson, T, Jacobsen, SE, and Scheding, S (2009). Characterization of bone marrow-derived mesenchymal stromal cells (MSC) based on gene expression profiling of functionally defined MSC subsets. *Cytotherapy*, 11(2):114–28.

Vater, C, Kasten, P, and Stiehler, M (2011). Culture media for the differentiation of mesenchymal stromal cells. *Acta Biomaterialia*, 7(2):463–77.

Vico, L, Lafage-Proust, MH, and Alexandre, C (1998). Effects of gravitational changes on the bone system in vitro and in vivo. *Bone*, 22(5 Suppl):95S–100S.

Waldman, S and Spiteri, C (2004). Long-term intermittent compressive stimulation improves the composition and mechanical properties of tissue-engineered cartilage. *Tissue Engineering*, 10(9).

Woodfield, TBF, Malda, J, de Wijn, J, Péters, F, Riesle, J, and van Blitterswijk, CA (2004). Design of porous scaffolds for cartilage tissue engineering using a three-dimensional fiber-deposition technique. *Biomaterials*, 25(18):4149–61.

Yoo, JU, Barthel, TS, Nishimura, K, Solchaga, L, Caplan, AI, Goldberg, VM, and Johnstone, B (1998). The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. *The Journal of Bone and Joint Surgery*, 80(12):1745–57.

Yourek, G, McCormick, SM, Mao, JJ, and Reilly, GC (2010). Shear stress induces osteogenic differentiation of human mesenchymal stem cells. *Regenerative Medicine*, 5(5):713–24.

Yuan, JS, Reed, A, Chen, F, and Stewart, CN (2006). Statistical analysis of real-time PCR data. *BMC Bioinformatics*, 7:85.

Zeiter, S, Lezuo, P, and Ito, K (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.

# Appendix A

# Additional Considerations

## A.1 Mechanical Testing

As part of construct characterization, in early experiments some constructs were set aside for mechanical testing. Due to technical limitations only one construct of each treatment (i.e. AA and chondrogenic) could be tested. However, this was not considered a major limitation, as it was not likely that substantial differences in construct mechanical properties would appear over such a short time frame following mechanical loading.

Mechanical testing was performed using a MicroSquisher (CellScale). The MicroSquisher system uses a cantilever combined with a displacement sensor and optical tracking in order to obtain force and displacement data in real-time. As part of the optical tracking component, the system has the added advantage of collecting real-time imagery data that can be useful in combination with the collected force and displacement data. During operation, the system is actuated by a number of piezoelectric motors.

A number of issues prevented the collection of useful and reliable mechanical testing data. These are discussed below.

### A.1.1 Specimen Suitability

The earliest tests involved directly compressing the constructs in the isolated system (Fig. A.1). This led to the same issue addressed by the use of secondary agarose scaffolds when loading the constructs in the Flexcell system. Specifically, since the constructs generally do not have a consistent or uniform shape, and visibly retained a complex configuration indicative of their origins in a two-dimensional monolayer, loading constructs directly yielded inconsistent results of questionable validity. The constructs could be seen to deform, shift,

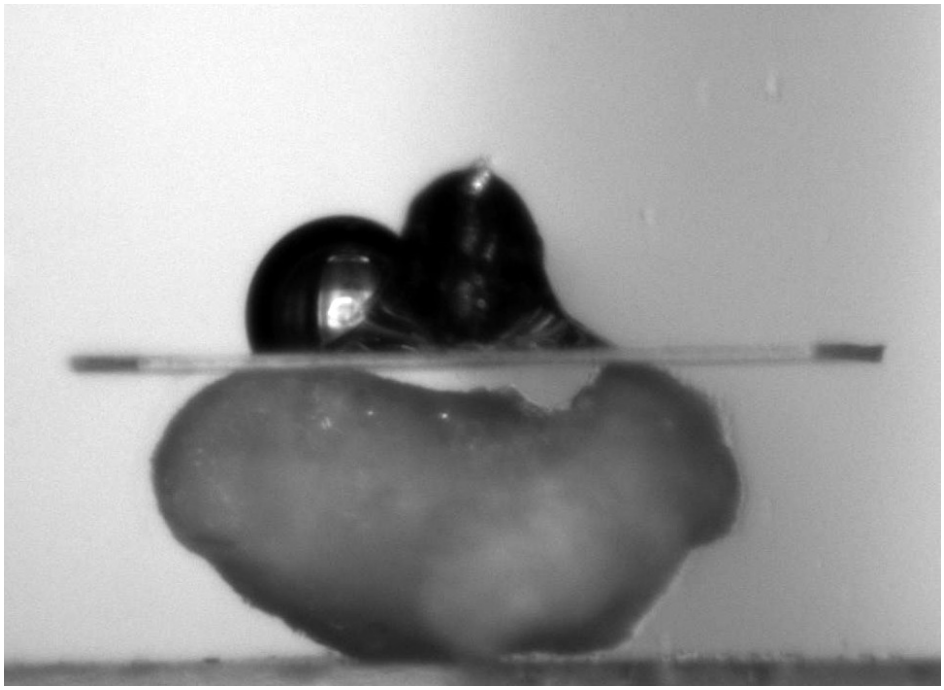

Figure A.1: Initial tests using the MicroSquisher system used the constructs directly.

and distort in a variety of ways during mechanical testing, thus making any collected data suspect.

Consequently, subsequent mechanical tests utilized the same secondary agarose scaffold used previously (Fig. A.2). This permitted the constructs to be positioned in a relatively cylindrical configuration that would be maintained by the agarose, which would in theory by amenable to later calculation of a compression modulus of the construct. However, there was a great deal of difficulty associated with the preparation of the specimens. The constructs were prepared similar to how they would have been for loading in the Flexcell system, except in a culture dish rather than in the modified platen. Plugs of agarose with the embedded constructs were extracted using biopsy punches and then transferred to the MicroSquisher. However, in some cases, most likely through user error, small defects would be created in the agarose gels. These had a tendency to lead to partial or complete specimen failure during testing and rendering the test invalid. Data from those tests could not be obtained as the tests were cancelled before completion.

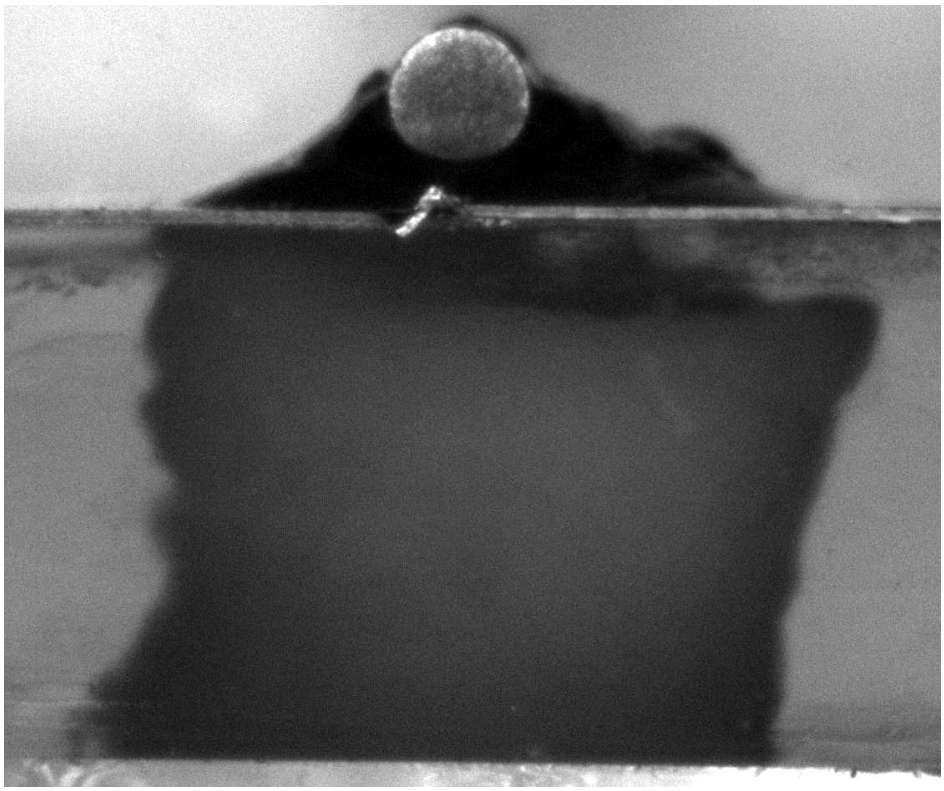

Figure A.2: Implementing the secondary agarose scaffold used for loading in the Flexcell system helped to improve the quality of tests using the MicroSquisher system.

Lastly, preparing adequate specimens for these tests can be exceptionally difficult. Due to the nature and sensitivity of the system, small defects in the specimen can lead to drastically altered data. For example, if the top of the gel isn't very smooth, for example as a result of poor specimen preparation or due to a burr from the biopsy punch, it can be difficult to properly initialize the test in terms of specimen height. More dramatic imperfections can lead to the specimen shifting during the test, thus affecting the validity of the data.

### A.1.2 Testing Protocol

For each test cycle the samples were compressed to 70% of their original height (i.e. a 30% compressive strain) followed by a 10 second hold phase, an unloading phase, and a 30 second rest phase. The loading and unloading phases utilized a ramping strain with a 1% strain/second strain rate was used for both loading and unloading. The test was then repeated for several cycles. One issue that arose was that for larger samples, such as some of the chondrogenic constructs, the specimen was too stiff for the MicroSquisher system. This would generally result in either the system being incapable of achieving the target strain due to the piezoelectric motors reaching their limits, or failing to maintaining the specified strain rate again due to reaching the limits of the motors.

One related issue that occasionally developed during testing was likely due to unequal rates of recovery in the construct and the gel. For unknown reasons, in some of the tests the construct appeared to recover more quickly than the gel, and would come loose or protrude from the gel. This understandably compromised subsequent test cycles.

### A.1.3 Future Considerations

While it may be possible to address and solve many of the issues described here, it may also be argued that there is little value in determining the mechanical properties of the constructs at this stage. The constructs in these experiments are developed over a relatively small amount of time and, as discussed earlier, it is possible they may undergo additional

remodelling following aggregation of the constructs. In that case, it may not make sense to be overly concerned with the mechanical properties at this intermediate stage.

Alternatively, different methods of mechanical testing may be considered. For example, atomic force microscopy could potentially be used to study the mechanical properties of the constructs without needing to address the issue of the construct structure.

# Appendix B

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I hereby give Geoff Buckley-Herd permission to reproduce Figure 3.5 on page 45 from my thesis entitled "Micro-environmental factors directing differentiation of murine embryonic stem cells down osteogenic and chondrogenic lineages" for use in his thesis, entitled "The response of human synovial progenitor cells in a tissue-engineered construct to mechanical loading".

[redacted]

Olesja Hazenbiller

16 September 2013