THE MECHANOTRANSDUCTION OF HYDROSTATIC PRESSURE BY
MESENCHYMAL STEM CELLS

A Dissertation

Submitted to the Graduate School
of the University of Notre Dame
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

by

Andrew J. Steward

___________________________________

Diane R. Wagner, Director

Graduate Program in Bioengineering
Notre Dame, Indiana
April 2014
THE MECHANOTRANSDUCTION OF HYDROSTATIC PRESSURE BY MESENCHYMAL STEM CELLS

Abstract

by

Andrew J. Steward

Understanding the underlying mechanisms of mesenchymal stem cell (MSC) mechanotransduction has important implications for tissue engineering and regenerative medicine. Hydrostatic pressure (HP) is the dominant load bearing mechanism in joints; however, the mechanotransductive pathways utilized in response to HP are not well understood. Cell-matrix interactions, cytoskeletal organization, and calcium ion (Ca++) signaling have all been proposed to regulate MSC mechanotransduction. This dissertation aims to provide insight into the roles of these pathways in the response of MSCs to HP.

To test whether the response of MSCs to HP depends on cell-matrix interactions, MSCs were seeded into either agarose or fibrin hydrogels and exposed to cyclic HP. Agarose hydrogels were found to support a spherical cellular morphology, while MSCs seeded into fibrin hydrogels attached and spread. While agarose hydrogels better supported chondrogenesis of MSCs, HP only enhanced chondrogenesis in fibrin hydrogels. This study demonstrates that a complex relationship between cell-matrix
interactions and HP mechanotransduction plays a key role in regulating the chondrogenic differentiation of MSCs.

Next, the roles of integrins and the cytoskeleton in the mechanotransduction of HP were examined. Matrix stiffness and/or density modulated the development of the pericellular matrix and consequently integrin binding and cytoskeletal structure. This study suggests that physiological cues such as HP enhance chondrogenesis of MSCs as the pericellular environment matures and the cytoskeleton adapts, and points to a novel role for vimentin in the mechanotransduction of HP.

Intracellular Ca\(^{++}\) concentrations have been found to increase with application of HP, yet the role of Ca\(^{++}\) signaling in the mechanotransduction of HP had yet to be investigated. Ca\(^{++}\) signaling was found to regulate the chondrogenic response of MSCs and modulate the reorganization of vimentin in response to HP. Further, HP was found to initiate Ca\(^{++}\) signaling by activating the purinergic signaling pathway.

These results suggest a complex interplay between cell-matrix interactions and Ca\(^{++}\) signaling may transduce HP via vimentin adaptation to loading, indicating that the mechanotransduction of HP is distinct from other loading modalities. Overall, the understanding gained about the mechanisms regulating the chondrogenic response to HP could have important implications for tissue engineering and regenerative medicine.
To my parents for always being there for me and giving me the tools I’ve needed to succeed, and to Courtney for all of the love and support she has given me.
CONTENTS

Figures................................................................................................................................. vi
Abbreviations....................................................................................................................... xi
Acknowledgments............................................................................................................. xiii

Chapter 1:  Introduction ..................................................................................................... 1
  1.1 Background and significance.................................................................................. 1
  1.2 Cartilage................................................................................................................... 2
  1.3 Mesenchymal stem cells ....................................................................................... 5
  1.4 Biophysical cues ....................................................................................................... 6
    1.4.1 Substrate/Matrix stiffness and cell shape...................................................... 6
    1.4.2 Hydrostatic pressure..................................................................................... 9
  1.5 Mechanotransduction ............................................................................................. 11
    1.5.1 Integrin binding and focal adhesion formation .......................................... 12
    1.5.2 Cytoskeleton ............................................................................................... 14
    1.5.3 Calcium signaling ....................................................................................... 16
    1.5.4 Purinergic signaling .................................................................................... 17

Chapter 2:  Cell-Matrix Interactions Regulate Mesenchymal Stem Cell Response to
Hydrostatic Pressure ........................................................................................................ 19
  2.1 Introduction ............................................................................................................. 19
  2.2 Materials and Methods ......................................................................................... 21
    2.2.1 Materials...................................................................................................... 21
    2.2.2 Cell isolation, expansion, and encapsulation .......................................... 21
    2.2.3 Application of hydrostatic pressure........................................................... 22
    2.2.4 Biochemical analysis ................................................................................... 23
    2.2.5 Confocal microscopy, histology, and immunohistochemistry .................. 24
    2.2.6 Experimental design ................................................................................. 25
    2.2.7 Statistical analysis ...................................................................................... 25
  2.3 Results....................................................................................................................... 25
    2.3.1 Cellular morphology, contraction, and proliferation.................................. 25
    2.3.2 Extracellular matrix synthesis in fibrin and agarose hydrogels.............. 27
    2.3.3 Donor variability in response to HP ............................................................ 29
    2.3.4 Markers of endochondral ossification ....................................................... 30
  2.4 Discussion.................................................................................................................. 33
Chapter 3: The Pericellular Environment Regulates Cytoskeletal Development and the Differentiation of Mesenchymal Stem Cells and Determines their Response to Hydrostatic Pressure

3.1 Introduction

3.2 Materials and Methods

3.2.1 Materials

3.2.2 Cell isolation, expansion, and encapsulation

3.2.3 Application of hydrostatic pressure

3.2.4 Biochemical analysis

3.2.5 Confocal microscopy, histology, and immunohistochemistry

3.2.6 RNA isolation and quantitative real-time polymerase chain reaction

3.2.7 Statistical analysis

3.3 Results

3.3.1 Influence of matrix stiffness on MSC differentiation in a permissive environment

3.3.2 Chondrogenesis of MSCs is regulated by matrix stiffness, integrin binding, and cytoskeletal organization

3.3.3 Influence of hydrostatic pressure on chondrogenesis and the cytoskeletal organization of MSCs

3.3.4 Integrin binding is necessary for mechanotransduction of hydrostatic pressure

3.4 Discussion

Chapter 4: Calcium Signaling Regulates the Chondrogenic Response of Mesenchymal Stem Cells to Hydrostatic Pressure

4.1 Introduction

4.2 Materials and Methods

4.2.1 Materials

4.2.2 Cell isolation, expansion, and encapsulation

4.2.3 Application of hydrostatic pressure

4.2.4 Biochemical analysis

4.2.5 Confocal microscopy and immunohistochemistry

4.2.6 Statistical analysis

4.3 Results

4.3.1 Ca^{2+} mobility is a key regulator of the mechanotransduction of HP

4.3.2 Ca^{2+} mobility is required for changes in vimentin architecture in response to HP

4.3.3 CaM, CaMKII, and Cn are all key regulators of the mechanotransduction of HP

4.3.4 CaM, CaMKII, and Cn are required for changes in vimentin architecture in response to HP

4.4 Discussion
Chapter 5: Purinergic Signaling Regulates the Chondrogenic Response of Mesenchymal Stem Cells to Hydrostatic Pressure

5.1 Introduction ........................................... 79
5.2 Materials and Methods ................................ 82
  5.2.1 Materials ........................................... 82
  5.2.2 Cell isolation, expansion, and encapsulation .......... 82
  5.2.3 Application of hydrostatic pressure ..................... 82
  5.2.4 Biochemical analysis ................................ 82
  5.2.5 ATP release assay .................................. 83
  5.2.6 Statistical analysis .................................. 83
5.3 Results .................................................. 83
  5.3.1 ATP is released by MSCs in response to HP via hemichannels 83
  5.3.2 Purinergic signaling regulates the chondrogenic response of MSCs to HP 84
  5.3.3 Purinergic signaling is required for changes in vimentin architecture in response to HP 85
  5.3.4 Purinergic signaling acts upstream of matrix stiffness in the mechanotransduction of HP 87
5.4 Discussion .............................................. 88

Chapter 6: Conclusions ...................................... 92

Appendix A: Exploring the Roles of Integrin Binding and Cytoskeletal Reorganization during Mesenchymal Stem Cell Mechanotransduction in Soft and Stiff Hydrogels Subjected to Dynamic Compression

A.1 Introduction ........................................... 97
A.2 Materials and Methods ................................. 99
  A.2.1 Materials ........................................... 99
  A.2.2 Cell isolation, expansion, and encapsulation .......... 99
  A.2.3 Application of dynamic compression ................. 100
  A.2.4 Confocal microscopy and histology ................... 100
  A.2.5 RNA isolation and qRT-PCR reaction ................ 100
  A.2.6 Statistical Analysis ................................ 100
A.3 Results .................................................. 101
  A.3.1 MSCs display a temporal response to dynamic compression despite minimal changes to their gross actin, vimentin, and tubulin networks during chondrogenesis in 3D hydrogels 101
  A.3.2 A well developed PCM appears important for dynamic compression to promote chondrogenesis of MSCs 104
  A.3.3 Integrin mediated binding by MSCs to their PCM is involved in mechanotransduction of dynamic compression 105
  A.3.4 Tubulin rapidly reorganizes within the cytoskeleton of MSCs in response to DC 107
A.4 Discussion .............................................. 109

Bibliography ................................................... 113
FIGURES

Figure 1.1: Chondrocyte and collagen fiber organization in articular cartilage.

Figure 1.2: Horizontal view of circumferential collagen organization in the deep layer showing chondrocyte (C), pericellular matrix (*), pericellular capsule (arrowheads), territorial matrix ™ and interterritorial matrix (IM). Figure adapted from Poole et al.

Figure 1.3: MSCs sense and respond to their biochemical and biophysical environment.

Figure 1.4: Soluble factors, cell shape, substrate/matrix stiffness, integrin binding, and cytoskeletal contractility all influence the differentiation of MSCs.

Figure 1.5: Integrins, focal adhesions, the cytoskeleton, and ion channels interact with the PCM to transmit signals ‘outside-in’ and ‘inside-out.’

Figure 1.6: An integrin with α and β subunits attaches to the scaffold, and to the cytoskeleton through linking proteins talin (tal) and vinculin (vin). Next, focal adhesions containing multiple integrins form in response to integrin binding and are a concentrated site of cytoskeletal filaments and proteins such as talin, vinculin, paxillin (pax), focal adhesion kinase (FAK) and Src.

Figure 2.1: Representative confocal microscopy images of MSCs from Donor 1 seeded in either agarose (top) or fibrin (bottom) hydrogels at day 21 for both FS and HP groups.

Figure 2.2: (A) Total sGAG/DNA of FS constructs. (B) Total collagen/DNA of FS constructs. Data is for Donor 1 (n=4). *p<0.05 indicates a significant difference between agarose and fibrin groups.

Figure 2.3: (A) Total sGAG/DNA normalized to FS condition. (B) Total collagen/DNA normalized to FS condition. Data is for Donor 1 (n=4). *p<0.05 indicates a significant difference between FS and HP groups.

Figure 2.4: Representative Alcian blue and picro-sirius red images of MSCs from Donor 1 seeded in either agarase (top) or fibrin (bottom) hydrogels at day 21 for both FS and HP groups.
Figure 2.5: Representative collagen types I and II images of MSCs from Donor 1 seeded in either agarose (top) or fibrin (bottom) hydrogels at day 21 for both FS and HP groups.

Figure 2.6: (A) Diameters, (B) DNA content, (C) total sGAG produced normalized to DNA content, and (D) total collagen produced normalized to DNA content for fibrin hydrogels. Data shown for all three donors and HP groups were normalized to FS groups. *p<0.05 indicates a significant difference between FS and HP groups.

Figure 2.7: (A) Quantity of ALP activity (normalized to DNA) released to the media over the three week culture period for Donors 1 and 3 in both agarose and fibrin hydrogels in 10 ng/ml TGF-β3. (B) Quantity of ALP activity (normalized to DNA) for HP groups normalized to FS groups for two replicates cultured in 1 and 10 ng/ml TGF-β3. For both Donor 1 and 3, the media was analyzed in triplicate for these assays. However, as constructs were pooled during loading, it can be argued that these triplicates are not independent and hence a statistical analysis was not performed. Insufficient media was available from donor 2 to facilitate undertaking this specific assay. (C) Representative sections stained for ALP enzyme activity for MSCs from Donor 3 seeded in either agarose (left) or fibrin (right) hydrogels at day 21 for both FS and HP groups supplemented with 10ng/ml of TGF-β3. No noticeable differences were observed for constructs supplemented with 1 ng/ml of TGF-β3.

Figure 3.1: Representative Alcian blue and picro-sirius red images of MSCs cultured in a 16% FBS permissive media (Scale bar = 30 µm).

Figure 3.2: (A) Total sGAG and (B) total collagen retained in the construct (white) and released to the media (black). (C) Total sGAG and (D) total collagen produced when cultured either with (RGDS+, black) or without (RGDS-, white) RGDS peptide. (E) Representative Alcian blue, picro-sirius red, (F) collagen type I, and collagen type II histological and immunohistochemical images of 1% and 4% scaffolds (Scale bar = 10 µm). a: p<.05.

Figure 3.3: Representative confocal images of vinculin, actin, vimentin, and tubulin in 1%, 4%, and 4% +RGDS constructs (Scale bar = 10 µm/40 µm).

Figure 3.4: (a) Total sGAG and (b) total collagen normalized to the FS condition. (c) Sox9, (d) Agc, and (e) Col2A1 relative gene expression normalized to the FS condition. (f) Representative confocal images of vinculin, actin, vimentin, and tubulin in 4% FS and HP groups (Scale bar = 10 µm/40 µm). a: p<.05.

Figure 3.5: Representative images of vimentin in MSCs encapsulated in fibrin gels in either FS or HP conditions.
Figure 3.6: (a) Total sGAG and (b) total collagen in 4% gels normalized to the FS condition. (c) Sox9, (d) Agc, and (e) Col2A1 relative gene expression in 4% gels normalized to the FS condition. (f) Representative confocal images of vinculin, actin, vimentin, and tubulin in 4% FS and HP groups cultured with RGDS peptide (Scale bar = 10 µm/40 µm). a: p < 0.05.

Figure 4.1: Diagram displaying the calcium signaling pathways examined and the inhibitors utilized in the current study.

Figure 4.2: A custom MATLAB code converts a raw confocal image of vimentin structure to an RGB image and subsequently averages over a 2x2 grid of pixels to smooth the image. Finally, a distribution of intensities within each cell is plotted and the standard deviation is used as a measure of homogeneity within each cell.

Figure 4.3: A) DNA and B) sGAG/DNA in constructs exposed to various calcium signaling inhibitors. *p ≤ 0.05, relative to control.

Figure 4.4: A) Total sGAG/DNA normalized to the FS condition. B) Representative collagen type II immunohistological images. Scale Bars = 50 µm. *p < 0.05, relative to FS condition.

Figure 4.5: Representative confocal images of vinculin, actin, and tubulin staining of constructs in either the HP or FS groups. Scale Bars = 10 µm.

Figure 4.6: A) Representative confocal images of vimentin in constructs in either the HP or FS groups. B) Semi-quantitative analysis using custom MATLAB code to determine differences in vimentin structures in FS constructs normalized to those exposed to HP. Scale Bars = 10 µm. *p ≤ 0.05, relative to HP condition.

Figure 4.7: A) DNA and B) sGAG/DNA in constructs exposed to various inhibitors of downstream calcium targets. *p ≤ 0.05, relative to control.

Figure 4.8: A) Total sGAG/DNA normalized to the FS condition. B) Representative collagen type II immunohistological images. Scale Bars = 50 µm. *p < 0.05, relative to FS condition.

Figure 4.9: Representative confocal images of vinculin, actin, and tubulin staining of constructs in either the HP or FS groups. Scale Bars = 10 µm.

Figure 4.10: A) Representative confocal images of vimentin in constructs in either the HP or FS groups. B) Semi-quantitative analysis using custom MATLAB code to determine differences in vimentin structures in FS constructs normalized to those exposed to HP. Scale Bars = 10 µm. *p ≤ 0.05, relative to HP condition.
Figure 5.1: Diagram displaying the purinergic signaling pathways examined and the inhibitors utilized in the current study.

Figure 5.2: ATP released by MSCs cultured either with or without an inhibitor of hemichannels after 4 hours of exposure to HP on days 7, 14, and 21. *p<0.05, relative to FS condition.

Figure 5.3: A) Total sGAG/DNA normalized to the FS condition. B) Representative collagen type II immunohistological images. Scale Bars = 50 µm. *p<0.05, relative to FS condition.

Figure 5.4: Representative confocal images of vinculin, actin, and tubulin staining of constructs in either the HP or FS groups. Scale Bars = 10 µm.

Figure 5.5: Representative confocal images of vimentin in constructs in either the HP or FS groups. Scale Bars = 10 µm.

Figure 5.6: A) ATP released by MSCs cultured in either soft or stiff hydrogels after 4 hours of exposure to HP on days 7, 14, and 21. B) Total sGAG/DNA of constructs cultured with various pharmacological inhibitors or in control conditions.

Figure 6.1: Diagram displaying the purinergic and calcium signaling pathways proposed to act in the mechanotransduction of HP. 1) HP initiates release of ATP via hemichannels. 2) ATP activates P-receptors to allow Ca^{++} to enter the cell directly or via IP3-mediated release from SERCS. 3) The increase in intracellular Ca^{++} concentration triggers VGCCs which in turn 4) induce calcium-induced calcium release from SERCS. 5) This overall increase in Ca^{++} binds to the downstream targets CaM, CaMKII, and Cn, which are known to trigger a variety of signaling cascades that alter gene expression and differentiation.

Figure 6.2: Diagram displaying the known and proposed interactions between HP induced chondrogenesis, integrin/focal adhesion formation, cytoskeletal organization, Ca^{++} mobility and signaling, and purinergic signaling. Solid arrows indicate relationships that have been investigated, while dashed arrows indicate relationships that require further research to fully understand.

Figure A.1: (A) Sox9, Agc, and Col2A1 gene expression in stiff hydrogels normalized to FS condition. (B) Representative Alcian blue and picro-sirius red images, and (C) representative vinculin, actin, vimentin and tubulin confocal images of stiff hydrogels on days 7, 14, and 21 (Scale bar = 50 µm; 12.5 µm for inset images). *p < 0.05 relative to FS.
Figure A.2: (A) Sox9, Agc, and Col2A1 gene expression in soft and stiff hydrogels normalized to FS condition on day 7. (B) Representative Alcian blue and picro-sirius red images, and (C) representative vinculin, actin, vimentin and tubulin confocal images of soft and stiff hydrogels on days 7 (Scale bar = 50 μm; 12.5 μm for inset images). *p < 0.05 relative to soft hydrogels.

Figure A.3: (A) Sox9, Agc, and Col2A1 gene expression in stiff hydrogels on day 7, with or without the RGDS peptide, normalized to FS condition. (B) Representative Alcian blue and picro-sirius red images, and (C) representative vinculin, actin, vimentin and tubulin confocal images of stiff hydrogels on day 7, with or without the RGDS peptide (Scale bar = 50 μm; 12.5 μm for inset images). *p < 0.05 relative to groups cultured with RGDS peptide.

Figure A.4: Representative tubulin confocal images on days 7, 14, and 21 in either the FS, DC, or DC with RGDS conditions (Scale bar = 50 μm; 12.5 μm for inset images).
ABBREVIATIONS

Agc: Aggrecan
ADP: Adenosine diphosphate
ALP: Alkaline phosphatase
ATP: Adenosine triphosphate
BSA: Bovine serum albumin
Ca\(^{++}\): Calcium ions
CaM: Calmodulin
CaMKII: Calmodulin kinase type II
cDNA: Complimentary deoxyribonucleic acid
Cn: Calcineurin
Col I: Collagen type I
Col II: Collagen type II
Col2A1: Collagen type II alpha 1
DC: Dynamic compression
DMMB: Dimethylmethylene blue
DNA: Deoxyribonucleic acid
ECM: Extracellular matrix
FAK: Focal adhesion kinase
FBS: Fetal bovine serum
FS: Free swelling
GAPDH: Glyceraldehyde-three-phosphate dehydrogenase
hgDMEM: High-glucose Dulbecco’s modified eagle medium
HP: Hydrostatic pressure
MAPK: Mitogen-activated protein kinase
MSC: Mesenchymal stromal (stem) cell
PCM: Pericellular matrix
qRT-PCR: Quantitative real-time polymerase chain reaction
RGD: Arginine-glycine-aspartic acid
RGDS: Arginine-glycine-aspartic acid-serine
RGE: Arginine-glycine-glutamic acid
RhoA: RhoA GTPase
RNA: Ribonucleic acid
ROCK: Rho kinase
RyR: Ryanodine receptor
SACC: Stretch-activated calcium channel
SERCS: Sarcoendoplasmic reticulum stores
sGAG: Sulphated glycosaminoglycan
Sox9: SRY (Sex determining region Y)-box 9 gene
TGF-β3: Transforming growth factor-beta 3
VGCC: Voltage-gated calcium channel
ACKNOWLEDGMENTS

This dissertation would not have been possible without the aid of many mentors, friends, and family members. I would like to thank my advisers, Dr. Diane Wagner and Dr. Daniel Kelly, for the opportunity to pursue my interest in mechanobiology, the advice and guidance I’ve received through the years, and the chance to perform my research in two different countries. The cooperation and patience exhibited by both of you made the entire process much easier. I would also like to thank my committee members, Dr. Glen Niebur and Dr. Phillipe Sucosky, as well as all of the other countless faculty and staff at the University of Notre Dame and Trinity College Dublin who provided irreplaceable assistance with my research.

I also owe much of my success to my friends and colleagues who have helped train me in several lab techniques, as well as be sounding boards for ideas. I’d like to especially thank Thomas A. Metzger for many stimulating coffee break conversations. I want to thank all of my teammates on Brotre Dame for letting “Old Reliable” be a part of many fun times and great memories. I want to thank all of my friends in Ireland for making my transition to another country so smooth and enjoyable. I want to specifically thank Dr. Stephen Thorpe for buying my first pint of Guinness and making me feel welcome from the first day I arrived. I also want to thank Dr. Thomas Nagel for being such a close friend and sharing our common experiences as foreigners. Further I want to
thank Dr. Holly Weiss for all of her guidance in my research and for making my transition back to Notre Dame as easy as possible.

I would also like to thank the following funding sources: University of Notre Dame Stem Cell Initiative, U.S. Army Medical Research and Material Command (W81XWH-07-0662 and W81XWH-09-1-0741), NSF grant 1335007 (BMMB/CMMI), President of Ireland Young Researcher Award from Science Foundation Ireland (08/Y15/B1336), and the European Research Council Starter Grant (No. 258463). Further, I would like to acknowledge the Naughton Graduate Fellowship program, and specifically Mr. Martin Naughton, which provided the means to not only perform research at the University of Notre Dame and Trinity College Dublin, but to grow and have experiences all over the world I never dreamed of having. I would also like to thank Fergal Naughton for all of his help in making my move to a new country a smooth one, and always making me feel welcome in Ireland.

Finally, none of this would be possible if it weren’t for my two amazing parents. You both taught me the value of having a strong work ethic and having the perseverance to keep going in the face of adversity. These have aided me, and will continue to aid me, in everything I do. You have also supported and encouraged me in everything I have done, and I am extremely grateful and blessed to have you as my parents. I also want to thank Courtney; you have always provided an ear to listen to my problems, the encouragement to relieve my stresses, and the patience to wait on me while I went to Ireland. The support of my family has been the most important factor in my successful completion of this project.
CHAPTER 1:
INTRODUCTION

1.1 Background and significance

Cellular response to mechanical signals is thought to play a key role in tissue healing and repair. Understanding how mesenchymal stem cells (MSCs) respond to mechanical signals is a major area of research in the field of mechanobiology and has important implications for tissue engineering and regenerative medicine. While some progress has been made in understanding how mechanical signals are sensed by MSCs, the mechanotransduction of hydrostatic pressure (HP) is not well understood relative to deforming loads such as tension, compression, and fluid flow. Further, the effects of HP on cellular behavior (such as proliferation and differentiation) are not fully understood. Extracellular molecules, membrane-bound proteins (integrins, ion channels, etc.) and intracellular molecules (cytoskeletal components, focal adhesion complexes, etc.) are all believed to play key roles in determining how MSCs sense and transmit mechanical signals, leading to extremely complex mechanotransduction pathways. This dissertation adds to current knowledge about how integrins, the cytoskeleton, calcium signaling, and purinergic signaling cooperate to elicit a chondrogenic response in MSCs in response to HP. This information will provide insight into the mechanoregulation of the chondrogenic differentiation of MSCs, as well as possibly provide mechanisms that could be targeted in the future to improve chondrogenesis in tissue engineering applications.
1.2 Cartilage

Articular cartilage is found on the ends of all diarthrodial joints and is critical for joint motion. The ability to resist compression and distribute loads allows cartilage to decrease the peak stresses on subchondral bone. Cartilage is an aneural, avascular, connective tissue comprised mostly of extracellular matrix (ECM) molecules and water. Approximately 70-80% of cartilage consists of water, which is vital for nutrient transfer and load distribution. Chondrocytes, the cellular component of cartilage, make up approximately 1% by volume of articular cartilage. The main function of chondrocytes is to produce and organize the ECM, which is composed of collagen (60 percent of dry weight), proteoglycans (25-35 percent of dry weight), and non-collagenous proteins (15-20 percent of dry weight). Collagen is a fibrous molecule that increases the tensile strength and organization of the tissue. Proteoglycans are composed of a protein core and numerous sulphated glycosaminoglycans (sGAG) branches. The branches are made up of disaccharide units; the type and number of these units determine the specific properties of the proteoglycan. The major types of proteoglycans are chondroitin sulphate, keratan sulphate, dermatan sulphate, heparan sulphate, and hyaluronan. Hyaluronan is not sulphated or attached to a protein core, but it is the most abundant proteoglycan in cartilage and plays a role in binding other proteoglycans in order to form larger complexes. Proteoglycans possess a negative charge that attracts positive ions, causing an osmotic imbalance. This imbalance leads to absorption of water which helps hydrate the tissue and induces a swelling pressure that increases the tissue’s resistance to compression. Cartilage also has a unique zonal organization (Fig. 1.1). Chondrocytes and collagen fibers at the surface of the cartilage are aligned parallel to the surface to
resist shear stresses in the superficial zone. The chondrocytes and collagen fibers are more randomly aligned in the middle zone in order to distribute the load throughout the tissue. Finally, the cells and fibers are aligned perpendicular to the surface to resist compressive forces in the deep zone. Clearly, although cartilage appears to be a simple aneural, avascular, connective tissue, there are many levels of complexity in the composition and structure.

![Figure 1.1: Chondrocyte and collagen fiber organization in articular cartilage.](image)

While cartilage has vertical organizational variation, differences in matrix composition that radiate from the chondrocytes are also important. Three regions of the ECM are distinguished by different proteins and functions: the interterritorial, territorial, and pericellular matrices (Fig. 1.2). The interterritorial matrix occupies the spaces furthest from the cells and consists of keratin sulphate-rich proteoglycans and thick collagen bundles. The territorial matrix contains chondroitin sulphate-rich proteoglycans and smaller more radially organized collagen bundles. Finally, the pericellular matrix (PCM) is made up of small diameter collagen fibers that form a tightly woven capsule.
immediately adjacent to the cell. Type II collagen is the most abundant collagen in articular cartilage, but it is accompanied by type XI, IX, VI, III, XII, XIV, and X collagen as well. Although type VI collagen is a small percentage of the total collagen content of articular cartilage, it has been shown to be highly concentrated in the PCM. Type VI collagen is believed to provide pericellular architecture and also to improve signaling between the cell and its microenvironment. The PCM also consists of sGAG, and an assortment of glycoproteins, hyaluronan, biglycan, fibronectin, and laminin. In native cartilage tissue, cells interact with the distinct extracellular matrix in the pericellular microenvironment. Providing an environment in which the cells reproduce this surrounding structure may be critical to the success of cartilage tissue engineering.

Figure 1.2: Horizontal view of circumferential collagen organization in the deep layer showing chondrocyte (C), pericellular matrix (*), pericellular capsule (arrowheads), territorial matrix (TM) and interterritorial matrix (IM). Figure adapted from Poole et al.
1.3 Mesenchymal stem cells

Musculoskeletal tissue is constantly remodeling itself in order to grow and adapt to its environment. Musculoskeletal cells such as osteocytes (bone cells), chondrocytes (cartilage cells), adipocytes (fat cells), myocytes (muscle cells), and others arise from a common progenitor known as a mesenchymal stromal (or stem) cell (MSC). MSCs are multipotent progenitor cells found in bone marrow, fat, and synovial fluid among others. MSCs have the capability to sense a variety of stimuli and respond accordingly. MSC differentiation is a complex process that is regulated by the cells biochemical and biophysical environment. Environmental factors such as hypoxia, growth factors, mechanical loading, and matrix stiffness are all integrated by MSCs and determine whether they remain quiescent, proliferate, or differentiate down a particular lineage (Fig. 1.3).
Figure 1.3: MSCs sense and respond to their biochemical and biophysical environment.

1.4 Biophysical cues

1.4.1 Substrate/Matrix stiffness and cell shape

Adult stem cells are potentially exposed to a diverse range of mechanical environments \textit{in vivo} ranging from soft brain tissue to stiff cortical bone. \textit{In vitro}, matrix or substrate stiffness has been shown to play a role in regulating the differentiation of MSCs towards specific lineages.\textsuperscript{18,21} When cultured on 2D substrates that mimicked the stiffness of physiologic neurogenic, myogenic, and osteogenic environments, MSCs adopted a phenotype corresponding to the tissue stiffness, as demonstrated by cellular morphology, transcript markers and protein production.\textsuperscript{18} In a similar experiment, MSCs
seeded onto soft substrates were shown to have a greater adipogenic and chondrogenic potential, while those on stiffer substrates had a stronger myogenic potential.\textsuperscript{21} In such 2D culture systems, substrate stiffness is generally found to affect cellular morphology, while in 3D hydrogel culture MSCs can retain a spherical morphology irrespective of the hydrogel stiffness.\textsuperscript{22,23} In spite of this, the fate of encapsulated MSCs is still generally dependent on the stiffness of the hydrogel, with stiffer gels supporting osteogenesis and softer gels supporting adipogenesis.\textsuperscript{22} Integrin binding has been shown to be necessary in order for osteogenic differentiation to occur on stiff substrates, while an absence of integrin binding has little to no effect on MSC differentiation down adipogenic or neurogenic lineages when cultured on soft substrates.\textsuperscript{22–24} The number of integrin bonds formed was dependent upon the stiffness of the matrix, and the degree of osteogenic differentiation correlated with the number of integrin bonds formed. Correspondingly, inhibition of integrin binding decreased the osteogenic potential of the cells, suggesting that matrix stiffness-mediated integrin binding has a direct effect on MSC lineage commitment.\textsuperscript{22}

As already described, matrix stiffness appears to be a key determinant of cell shape, particularly in 2D systems, due perhaps to substrate stiffness-mediated changes in integrin binding, adhesion strength and cellular stiffness/contractility\textsuperscript{21,25–27}. Cell shape is determined by both the internal configuration of the cytoskeleton and external interactions with the extracellular matrix (ECM) and adjacent cells. Cell shape has been shown to be a potent stimulator of differentiation.\textsuperscript{19} It has been possible to directly determine the role of cell shape on MSC differentiation by seeding cells on micropatterned fibronectin-coated islands of differing size and then stimulating the cells
with a mixed media that potentially permits differentiation along multiple lineages. On small islands where MSCs adopted a rounded morphology, adipogenesis was predominant, while on larger islands where MSCs adopted a spread morphology, osteogenesis was favored. This study also demonstrated that cell shape regulates RhoA GTPase (RhoA) and Rho kinase (ROCK) activity. ROCK is a Rho effector involved in myosin contraction, and RhoA is a key regulator of contractility. Inhibition of ROCK switched lineage commitment of cells from an osteogenic to an adipogenic phenotype, while activation of RhoA in cells exposed to adipogenic media promoted an osteogenic phenotype, indicating that cellular contractility controls MSC lineage commitment down either an osteogenic or adipogenic lineage. Further, this study suggests that RhoA can fully replace the signals mediated by the soluble differentiation factors. In a similar study, MSCs stimulated with the growth factor TGF-β3 were either allowed to flatten and spread, or to maintain a rounded cell morphology. MSCs allowed to spread proceeded down a myogenic lineage, while those kept rounded committed to a chondrogenic lineage. RhoA was not upregulated; however Rac1 (a member of the Rho GTPase family) was upregulated in the spread cells, and was sufficient to induce myogenesis and inhibit chondrogenesis.

Together these studies suggest the following hypothesis: integrin binding allows MSCs to probe the stiffness of its surrounding matrix and then cytoskeletal tension adapts to its surrounding substrate. These changes in cytoskeletal tension lead to changes in a myriad of cell signaling cascades that control cell behavior. Decoupling the effects of substrate stiffness, cell shape, integrin binding, stress fiber formation/tension, and the
effects of growth factors is experimentally difficult, and more research in this area is needed in order to better understand the complex nature of these interactions (Fig. 1.4).

1.4.2 Hydrostatic pressure

MSCs respond not only to the biological and mechanical properties of the surrounding matrix, but also to external mechanical signals such as fluid flow, hydrostatic pressure, compression, and tension. The type, frequency, magnitude, and duration of such cues have all been shown to affect MSC differentiation. This dissertation will focus on the response of MSCs to hydrostatic pressure.

Hydrostatic pressure (HP) has been shown to increase both chondrogenic gene expression (e.g., Sox9, aggrecan, collagen type II) and matrix production (proteoglycan, collagen) in MSCs maintained in pellets, collagen I sponges, and synthetic scaffolds.
Furthermore, HP has been shown to play a role in the maintenance of the chondrogenic phenotype for chondrocytes and joint tissue-derived stem cells by suppressing the expression of hypertrophic markers.\textsuperscript{36–38} In contrast, other studies have demonstrated that HP has little to no effect on chondrogenic gene expression and matrix accumulation.\textsuperscript{39,40} Therefore, uncertainty exists in the literature as to the role HP plays in regulating chondrogenic differentiation of MSCs. Cyclic HP was found to increase chondrogenesis in chondrocyte pellets, but HP had no effect in chondrocytes embedded in alginate hydrogels.\textsuperscript{41} Consequently, differences in cell-matrix interactions between the various studies may explain the differential response to HP.

Hydrostatic pressure has also been shown to affect components of the cytoskeleton.\textsuperscript{42–45} High magnitude static HP was observed to inhibit microtubule and actin fiber formation in epithelial cells, leading to cell rounding.\textsuperscript{42} High magnitude static HP was correlated with a decrease in matrix synthesis in chondrocytes regardless of the presence of functional microtubules; however, intact, dynamic microtubules were needed for mechanotransduction of cyclic HP by chondrocytes.\textsuperscript{43} Pharmacological inhibition of actin and microtubule polymerization has separately been shown not to abrogate the pressure-stimulated increases in chondrogenic gene expression.\textsuperscript{45} Due to these contradictions further research is needed to determine the role of cytoskeletal remodeling in the chondrogenic response of MSCs to HP.

Hydrostatic pressure generates little to no deformation, which makes putative mechanotransduction pathways less obvious than other loading regimes. Similar to oscillatory fluid flow, intracellular ion concentrations have been suggested as a possible mechanotransductive cue in chondrocytes.\textsuperscript{46–50} Static HP inhibits the Na/K and Na/K/2Cl
pump, but enhances Na/H exchange. Also, 30 seconds of static HP has been shown to triple intracellular calcium concentration, mainly by promoting release from intracellular calcium stores. Addition of gadolinium was also found to inhibit the increase in intracellular calcium, indicating that stretch-activated ion channels as a pathway for calcium influx and subsequent calcium induced calcium release. However, to date, no study has investigated whether calcium signaling is a key component in the mechanoresponse of MSCs to HP.

1.5 Mechanotransduction

There is strong evidence that both intrinsic and extrinsic mechanical signals are key regulators of MSC differentiation. Understanding how MSCs sense and respond to these signals is currently a highly researched area. MSCs have the ability to take such “outside-in” signals, transmit the signal to the nucleus and alter gene expression. The response may alter the cells’ surrounding matrix, therefore causing “inside-out” signals. Integrin binding, focal adhesion formation, cytoskeletal organization, calcium signaling, and purinergic signaling (among others) play key roles in this “outside-inside-out” signaling and will be reviewed in more detail in the following sections (Fig. 1.5).
Figure 1.5: Integrins, focal adhesions, the cytoskeleton, ion channels, and P-receptors interact with the PCM to transmit signals ‘outside-in’ and ‘inside-out.’

1.5.1 Integrin binding and focal adhesion formation

The primary linkage between the extracellular environment and the interior of the cell occurs through integrin molecules in the plasma membrane. Integrins are made up of α and β subunits, with the specific combination of the various available α and β subunits determining the specific ligand the integrin binds to. A single integrin can bind to a variety of extracellular matrix components, and a single type of matrix component can be bound by a variety of integrins. Integrins themselves may have little direct control of cellular behavior, rather when mechanical signals are transmitted to integrins from the extracellular matrix, large protein complexes (known as focal adhesions) form which trigger signaling cascades within the cell.

Focal adhesion proteins are involved in numerous signaling pathways and are also an anchorage site for the actin cytoskeleton. Focal adhesions are composed of many
structural proteins including, but not limited to, β-subunits of integrins, vinculin, talin, and the actin cytoskeleton. The assembly of focal adhesions helps to stabilize integrin binding, which (as previously discussed) is a key regulator of MSC differentiation.\textsuperscript{19} Focal adhesion assemblies can also provide a platform for numerous other proteins involved in signaling cascades to bind and transmit signals to the nucleus.\textsuperscript{58} Tyrosine kinases such as FAK and paxillin,\textsuperscript{59} serine-threonine kinases such as MAPK,\textsuperscript{60} GTPases such as Rho,\textsuperscript{61} and intracellular calcium concentration\textsuperscript{62–64} are all activated by the formation of focal adhesions, and they, along with their downstream signals, have all been implicated in MSC differentiation (Fig. 1.6).\textsuperscript{19,65–68}

![Figure 1.6: An integrin with α and β subunits attaches to the scaffold, and to the cytoskeleton through linking proteins talin (tal) and vinculin (vin). Next, focal adhesions containing multiple integrins form in response to integrin binding and are a concentrated site of cytoskeletal filaments and proteins such as talin, vinculin, paxillin (pax), focal adhesion kinase (FAK) and Src.\textsuperscript{69}](image-url)
Integrin binding and focal adhesion formation also help transduce extrinsic mechanical signals (Appendix A).\textsuperscript{70–74} Tensile loading of chondrocytes decreased chondrogenesis and increased osteogenesis, integrin expression and FAK; inhibition of integrin binding completely rescued chondrogenesis.\textsuperscript{73} Therefore, although integrin binding suppressed chondrogenesis, integrin binding was necessary for the mechanotransduction of tensile loading. Integrin binding has also been found to mediate the osteogenic response of various cell lines to fluid shear stress.\textsuperscript{71,72} Further, inhibition of integrin binding decreased the chondrogenic response of MSCs to dynamic compression (Appendix A).\textsuperscript{70} Clearly, formation of focal adhesions, or lack thereof, plays a critical role in the mechanotransduction and regulation of MSC differentiation.

1.5.2 Cytoskeleton

The cytoskeleton is comprised of microfilaments, microtubules, and intermediate filaments that provide structure and support to the cell. The cytoskeleton actively generate isometric tension within the cell by an actomyosin filament sliding mechanism similar to muscle.\textsuperscript{75,76} Therefore cells are pre-stressed, and since the cytoskeleton anchors at integrin/focal adhesion sites, mechanical loads can be transferred through the cell.\textsuperscript{77} The tension generated by the cytoskeleton depends on matrix stiffness, ligand type and density, and intracellular signals.\textsuperscript{78} This cytoskeletal tension can also determine cell morphology and affect the activity of focal adhesions and cell-cell junctions;\textsuperscript{79,80} therefore, the mechanical state of the cytoskeleton plays a prominent role in MSC differentiation.\textsuperscript{81} RhoA and ROCK have been implicated frequently as key signals during mechanotransduction and differentiation. RhoA’s main function is to regulate focal
adhesions and microfilament stress fibers through downstream phosphorylation cascades which effect myosin contractility. Although the fact that RhoA affects focal adhesions and stress fibers is well established, the downstream effects of these on MSC differentiation are less clear. For instance, RhoA/ROCK signaling was found to both enhance and suppress chondrogenesis by regulating Sox9 expression depending upon the cell culture model used.

Intermediate filaments, such as vimentin, are also known to regulate chondrogenesis of MSCs, with siRNA-mediated knockdown of vimentin inhibiting cartilage-specific ECM production. These intermediate filaments also contribute to the stiffness of chondrocytes. Previous research has shown that vimentin can directly interact with actin microfilaments, integrins αvβ3 and α2β1, and their associated focal adhesions. Intermediate filaments also regulate focal contact size and help to stabilize cell-matrix interactions. Vimentin has also been found to mechanically couple integrins to the nucleus, allowing extrinsic forces to be propagated through the cell and alter cellular behavior. Together, this suggests that intermediate filaments may play a role in the transduction of mechanical cues.

Inhibition of microtubules has also been found to decrease proteoglycan synthesis. Microtubules have previously been demonstrated to play a role in resisting cell deformation. The role of microtubules in mechanotransduction differs depending on cell type, culture conditions, and load applied. Microtubules were found to reorganize in MSCs exposed to DC, demonstrating that the microtubule network is mechanosensitive; however, this network did not appear to be involved in the chondrogenic mechanotransduction of DC that is mediated by integrin binding as
microtubule reorganization in response to DC was not inhibited by abrogating integrin binding (Appendix A).  

1.5.3 Calcium signaling

Ionic concentrations within a cell control many cellular functions; ions act as second messengers in many signaling pathways, regulate osmosis and therefore cell volume and a variety of other homeostatic functions. The cellular membrane is impermeable to most ions, and therefore calcium ion (Ca\(^{++}\)) signaling is triggered by Ca\(^{++}\) entering the cell through a variety of ion channels, such as stretch-activated calcium channels (SACCs) and voltage-gated calcium channels (VGCCs), or release of Ca\(^{++}\) from sarcoendoplasmic reticulum calcium stores (SERCS). Ca\(^{++}\) signaling plays a key role in differentiation down multiple lineages.\(^{98-102}\) Some ion channels have been found to either activate or deactivate in response to mechanical cues,\(^{103}\) and Ca\(^{++}\) signaling plays a key role in the response of MSCs to a variety of loading types.\(^{62,104-108}\) Cyclic tensile loading of MSCs has been shown to increase proteoglycan production, but this response was inhibited when stretch-activated ion channel activity was blocked, indicating stretch-activated ion channels are important elements of the MSC mechanotransductive pathway.\(^{109}\) Other studies have also demonstrated that fluid flow stimulation increases the expression of osteogenic markers in adipose derived MSCs\(^{110}\) and bone marrow derived MSCs.\(^{62,111}\) Oscillatory fluid flow has been shown to increase intracellular Ca\(^{++}\) and activate MAP kinases (specifically ERK 1/2),\(^{62,111}\) which have been shown to influence MSC differentiation.\(^{112,113}\) Further, both osmotic pressure and dynamic compression have
been found to increase intracellular Ca\(^{++}\) concentrations via SACCs,\(^{104,114,115}\) and influence chondrogenesis.\(^{17,46,70,116–118}\)

1.5.4 Purinergic signaling

While the involvement of ion channel activity has been proposed to be a key mechanism of MSC mechanotransduction, there are other mechanisms that regulate calcium signaling as well. In the purinergic signaling pathway, ATP is released by the cells via hemichannels and then activates P-receptors; P-receptors then increase intracellular Ca\(^{++}\) via either direct or indirect methods.\(^{119,120}\) This increased Ca\(^{++}\) concentration potentially activates VGCCs and the subsequent pathways described above.\(^{120,121}\) For example, previous studies demonstrated that intracellular Ca\(^{++}\) concentrations increased in chondrocytes in response to loading. However, the increased Ca\(^{++}\) concentration persisted after loading finished, and Ca\(^{++}\) waves propagated between cells that had no physical connection.\(^{122–124}\) Together, this suggested that an autocrine/paracrine factor was being released to sustain and propagate the response to loading. Inhibition of purinergic signaling was found to suppress both the Ca\(^{++}\) signal persistence and propagation, indicating that release of ATP and subsequent activation of P-receptors may play a key role in mechanotransduction.\(^{115,120–125}\) Further, addition of exogenous ATP was found to initiate Ca\(^{++}\) signaling in chondrocytes in both monolayer and agarose scaffolds.\(^{125,126}\) Purinergic signaling has also been implicated in MSC differentiation, as differential regulation of P-receptors was found to regulate the lineage commitment of MSCs.\(^{127,128}\) Additionally, supplementation with exogenous ATP increased the mechanical properties of MSC-agarose constructs.\(^{129}\) Although ATP release
has been found to be upregulated in response to fluid flow in bone and vascular cells, there is little research in the literature correlating purinergic signaling and MSC differentiation in response to loading.\textsuperscript{121,130–132}
CHAPTER 2:

CELL-MATRIX INTERACTIONS REGULATE MESENCHYMAL STEM CELL 
RESPONSE TO HYDROSTATIC PRESSURE

2.1 Introduction

As described in Chapter 1, environmental cues, both biochemical and biophysical, regulate chondrogenesis of mesenchymal stem cells (MSCs). Various forms of mechanical loading, such as dynamic compression, tension, hydrostatic pressure and fluid flow have been shown to play a key role in determining the differentiation pathway of MSCs.\textsuperscript{14} This has led to increased interest in the use of hydrostatic pressure (HP) in the field of cartilage tissue engineering.\textsuperscript{133} Cyclic hydrostatic pressure has been shown to increase chondrogenic gene expression (Sox9, aggrecan, collagen type II) and proteoglycan and collagen synthesis in MSCs.\textsuperscript{15,16,30–35,37,134–136} In contrast, other studies have demonstrated that HP has no significant effect on chondrogenesis of MSCs.\textsuperscript{39,40} Therefore, uncertainty exists in the literature as to the role HP plays in regulating chondrogenic differentiation of MSCs.

Cell-matrix interactions also play a key role in the chondrogenic differentiation of MSCs.\textsuperscript{69} Arginine-glycine-aspartic acid (RGD) is an amino acid sequence that integrins are known to bind to, and it is commonly utilized to allow cells to adhere to scaffolds that do not have any binding sites. Previous studies have demonstrated that when MSCs are seeded in RGD-modified alginate hydrogels chondrogenic gene expression and matrix
accumulation is inhibited relative to arginine-glycine-glutamic acid (RGE)-modified controls to which MSCs cannot adhere.\textsuperscript{137} The inhibitory effect of RGD can be blocked with the addition of soluble RGD or cytochalasin D (an actin cytoskeleton inhibitor), demonstrating a role for cell attachment and actin cytoskeleton formation in suppressing chondrogenic differentiation.\textsuperscript{138} While the interplay between MSCs and the extracellular environment may inhibit chondrogenesis, such interactions and the development of a functional actin cytoskeleton may also be necessary for mechanotransduction to occur.\textsuperscript{53,89,139} For example, an intact, dynamic actin cytoskeleton under tension has been shown to be necessary for fluid flow-induced changes in Sox-9 gene expression in MSCs.\textsuperscript{140} Furthermore, chondrocytes in pellet culture respond more favorably to HP relative to cells embedded in alginate hydrogels,\textsuperscript{41} suggesting that cellular interactions with the local environment also regulate stem cell response to HP. These studies suggest that the response of MSCs to HP may depend on cell-matrix interactions unique to the scaffold or hydrogel within which they are embedded.

The objective of this study was to examine the interplay of cell-matrix interactions and hydrostatic pressure on chondrogenesis of MSCs. We specifically sought to compare the response of MSCs to HP following encapsulation in fibrin, where cells are known to directly adhere and spread within the hydrogel,\textsuperscript{141,142} to that in agarose, where cells do not adhere and hence maintain a spherical morphology when encapsulated within the hydrogel.\textsuperscript{116,143,144} Our hypothesis was that cells seeded in fibrin hydrogels would be more mechanosensitive and hence demonstrate a more robust response to HP, while cells seeded in agarose hydrogels would show little to no response to HP.
2.2 Materials and Methods

2.2.1 Materials

Porcine femurs were obtained from the Trinity College Dublin Bioresources Unit. Dulbecco’s modified Eagle’s Medium (hgDMEM GlutaMAX), fetal bovine serum, and penicillin-streptomycin were all purchased from GIBCO, Biosciences. Agarose, fibrinogen, the secondary antibody for immunohistochemistry, all media supplements, and all other chemicals (unless indicated otherwise) were purchased from Sigma-Aldrich, Ireland. Aprotinin was purchased from Nordic Pharma, UK. Transforming growth factor-β3 was purchased from ProSpec-Tany TechnoGene Ltd, Israel. The hydraulic cylinder was purchased from PHD, Inc., USA. The DMMB assay kit was purchased from Blyscan, Biocolor Ltd., Northern Ireland. The pNPP ALP colorimetric assay was purchased from Cambridge Bioscience, Ltd., United Kingdom. Rhodamine 110-phalloidin was purchased from VWR International, Ireland. Primary antibodies were purchased from Abcam, while the ABC staining and DAB peroxidase kits were purchased from Vector Labs.

2.2.2 Cell isolation, expansion, and encapsulation

Bone marrow was harvested from the femoral diaphysis of three 4-month-old pigs (~50 kg) under sterile conditions. MSCs were isolated and expanded according to a modified method developed for human MSCs. Cultures were expanded in hgDMEM GlutaMAX supplemented with 10% fetal bovine serum (FBS), and penicillin (100 U/ml)-streptomycin (100 µg/ml). After expansion (third passage) MSCs were encapsulated in
either agarose (Type VII) or fibrin hydrogels at a density of $15 \times 10^6$ cells/ml. MSCs were mixed with agarose at ~40°C to yield a final gel concentration of 2%. The agarose-cell suspension was cast in a stainless steel mold, and cored using a biopsy punch to produce cylindrical scaffolds ($\phi$ 5 x 3 mm thickness). Fibrinogen was dissolved in aprotinin solution. Cells were mixed with this solution and thrombin was added to crosslink the gel. The solution was immediately injected into an agarose mold to yield fibrin cylindrical hydrogels (50 mg/ml fibrinogen, 5000 KIU/ml aprotinin, and 2.5 U/ml thrombin final concentrations) with the same dimensions as the agarose hydrogels. Constructs were maintained in 2.5 ml/construct of a chemically defined chondrogenic media consisting of hgDMEM GlutaMAX supplemented with penicillin (100 U/ml)-streptomycin (100 µg/ml), 100 µg/ml sodium pyruvate, 40 µg/ml L-proline, 50 µg/ml L-ascorbic acid-2-phosphate, 1.5 mg/ml BSA, 1 x insulin-transferrin-selenium, 100 nM dexamethasone and either 1 or 10 ng/ml recombinant human transforming growth factor-β3 (TGF-β3). Constructs were allowed to equilibrate overnight before the initiation of hydrostatic pressure.

2.2.3 Application of hydrostatic pressure

Constructs were sealed into sterile bags with 2 mL of medium per construct during the daily loading period. After loading constructs were removed from bags and returned to culture dishes containing 2.5 mL of medium per construct to allow gases to equilibrate overnight. Cyclic HP was applied in a custom bioreactor filled with water within a 37 °C incubator as described previously. The sealed bags exposed to HP were placed into the pressure vessel while the free swelling (FS) controls were placed into an
open water bath next to the pressure vessel. The pressure vessel was connected to a hydraulic cylinder that was loaded using a computer controlled Instron 8874 materials testing machine. The pressure inside the vessel was measured using a pressure gauge. The load applied to the hydraulic cylinder by the Instron was set such that the HP inside the vessel reached an amplitude of 10 MPa at a frequency of 1 Hz, 4 h/d, 5 d/week for 3 weeks. Half-medium exchanges were performed biweekly and media samples were collected for biochemical analysis.

2.2.4 Biochemical analysis

Constructs’ (n=4) wet weight and diameters were measured, and digested with papain (125 µg/ml) in 0.1 M sodium acetate, 5 mM L-cysteine-HCl, and 0.05 M EDTA (pH 6.0) at 60°C under constant rotation for 18 hours. DNA content was quantified using the Hoechst Bisbenzimide 33258 dye assay as described previously, with a calf thymus DNA standard. Sulphated glycosaminoglycan (sGAG) content was quantified using the dimethylmethylene blue dye-binding assay (DMMB) with a chondroitin sulphate standard. Collagen content was determined by measuring the hydroxyproline content. Briefly, samples were hydrolyzed at 110°C for 18 hours in 38% HCl and assayed using a chloromine-T assay with a hydroxyproline:collagen ratio of 1:7.69. Media samples were also analyzed using the DMMB and hydroxyproline assays, and subsequently added to that accumulated within constructs to yield the total sGAG and collagen produced. Total sGAG and collagen values were normalized to DNA values; subsequently the HP groups were normalized to the FS groups. Media samples were analyzed for alkaline phosphatase (ALP) activity using a pNPP ALP colorimetric assay.
2.2.5 Confocal microscopy, histology, and immunohistochemistry

At day 21, constructs (n=2) were cut in half and fixed in 4% paraformaldehyde overnight at 4°C and rinsed with PBS. In order to examine cellular morphology and the actin cytoskeleton, samples were permeabilized in a 0.5% Triton-X solution, washed in PBS, incubated in a solution containing 1.5% BSA and 5 U/ml rhodamine 110-phalloidin for 1 hour, and then imaged using a Zeiss 510 Meta confocal microscope at 20x magnification.

The remaining halves were dehydrated and embedded in paraffin wax. Constructs were sectioned perpendicular to the disc face yielding 5 µm thick sections. Sections were stained with either 1% Alcian blue 8GX in 0.1 M HCl for sGAG, picro-sirius red for collagen, or Fast Red TR Salt 1,5-naphthalenedisulfonate for ALP enzyme activity. Collagen types I, II, and X were further identified through immunohistochemistry. Briefly, sections were treated with peroxidase, followed by chondroitinase ABC in a humidified environment at 37°C for one hour to permeabilize the extracellular matrix. Samples were then blocked with goat serum, and afterwards the primary antibodies for collagen types I, II, and X (mouse monoclonal) were applied for one hour. Next, the secondary antibody (Anti-Mouse IgG biotin conjugate) was added for one hour followed by incubation with ABC reagent (Vectastain PK-4000) for 45 minutes. Finally the slides were developed with DAB peroxidase for 4 minutes. Samples were washed with PBS between each step, and negative and positive controls of porcine ligament (positive for type I collagen, negative for type II collagen) and cartilage (positive for type II collagen, negative for type I collagen) were also assessed.
2.2.6 Experimental design

Porcine bone marrow from one donor was cultured, exposed to HP, and analyzed. In order to examine the effects of possible donor to donor variability, the experiment was independently repeated two more times with bone marrow derived MSCs from two additional donors. Data presented in figures 1-5 is from the first donor. Figure 6 is a compilation comparing key data for all three donors. Figure 7 compares ALP activity in the media from donors 1 and 3 (insufficient media prevented such an analysis for donor 2).

2.2.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 5.00, GraphPad Software) with 3-4 samples analyzed for each experimental group for every donor. Biochemical results, both numerical and graphical, are expressed in the form of mean ± standard deviation. Differences between HP and FS samples or between agarose and fibrin were determined using a Student’s t-test. A level of $p < 0.05$ was considered significant.

2.3 Results

2.3.1 Cellular morphology, contraction, and proliferation

HP had no effect on cellular morphology, proliferation, construct contraction, sGAG or collagen production when MSCs were cultured in 10 ng/ml TGF-β3, and therefore unless otherwise stated the data shown is for MSCs cultured in the presence of
1 ng/ml TGF-β3. MSCs encapsulated in agarose hydrogels retained a spherical morphology, while MSCs in fibrin hydrogels attached and spread (Fig. 2.1). Actin stress fiber formation was clearly evident within MSCs embedded in fibrin hydrogels. While a few attachments did form in the agarose by day 21, this is due to the MSCs interacting with their own pericellular matrix (PCM) rather than any direct attachment between the MSCs and agarose, as these attachments were not observed if TGF-β3 was absent from the culture media (data not shown).

Figure 2.1: Representative confocal microscopy images of MSCs from Donor 1 seeded in either agarose (top) or fibrin (bottom) hydrogels at day 21 for both FS and HP groups.
There was no contraction observed in any of the agarose hydrogels. HP was observed to suppress contraction of fibrin constructs (FS: 4.67±0.06 mm, HP: 4.83±0.06 mm). HP did not influence the DNA content in either agarose or fibrin hydrogels after 21 days in culture.

2.3.2 Extracellular matrix synthesis in fibrin and agarose hydrogels

The sum of the extracellular matrix retained within the scaffold and released into the media was used to determine the total amount of sGAG and collagen synthesized. MSCs encapsulated in agarose synthesized significantly higher amounts of sGAG (normalized to DNA) than those seeded in fibrin (fibrin: 5.23±1.03; agarose: 23.20±3.00); however, MSCs in fibrin synthesized significantly more collagen (normalized to DNA) (fibrin: 109.02±7.03; agarose: 81.55±11.92) (Fig. 2.2).

![Figure 2.2: (A) Total sGAG/DNA of FS constructs. (B) Total collagen/DNA of FS constructs. Data is for Donor 1 (n=4). *p<0.05 indicates a significant difference between agarose and fibrin groups.](image)

The application of HP significantly increased sGAG synthesis in fibrin hydrogels (1.4 fold increase; p<0.05), however it had no effect in agarose constructs (Fig. 2.3). HP did not significantly influence collagen synthesis in either fibrin or agarose (Fig. 2.3).
Alcian blue and picro-sirius red were used to qualitatively assess sGAG and collagen production and distribution within the hydrogels (Fig. 2.4). MSC seeded agarose constructs stained more intensely for Alcian Blue than MSC seeded fibrin constructs, particularly in the pericellular region. MSC seeded fibrin hydrogels stained more intensely and diffusively for picro-sirius red, with staining in the agarose hydrogels again more intense in the pericellular region.

Figure 2.4: Representative Alcian blue and picro-sirius red images of MSCs from Donor 1 seeded in either agarose (top) or fibrin (bottom) hydrogels at day 21 for both FS and HP groups.
In order to determine the specific types of collagen being produced, immunohistochemistry was performed for collagen types I and II (Fig. 2.5). There was strong pericellular staining for both collagen types in the agarose constructs. Interestingly, HP appeared to decrease collagen type I production by MSCs seeded in fibrin hydrogels, while maintaining at least comparable levels of collagen II production. HP had little to no effect on the staining intensity for either type I or II collagen within the agarose hydrogels.

![Figure 2.5: Representative collagen types I and II images of MSCs from Donor 1 seeded in either agarose (top) or fibrin (bottom) hydrogels at day 21 for both FS and HP groups.](image)

2.3.3 Donor variability in response to HP

In order to further examine the effects of HP on MSCs, this experiment was repeated using MSCs isolated from two additional donors. No significant response to loading was observed for one of the three donors (Fig. 2.6). Significantly less fibrin contraction was observed for donors 1 and 2 when exposed to HP, but donor 3 showed no difference due to loading. HP also significantly inhibited proliferation in donor 2. The two donors (1 and 2) in which HP inhibited fibrin contraction also demonstrated
significant increases in sGAG synthesis in response to loading. Only donor 2 demonstrated a significant increase in collagen production due to HP.

Figure 2.6: (A) Diameters, (B) DNA content, (C) total sGAG produced normalized to DNA content, and (D) total collagen produced normalized to DNA content for fibrin hydrogels. Data shown for all three donors and HP groups were normalized to FS groups. *p<0.05 indicates a significant difference between FS and HP groups.

2.3.4 Markers of endochondral ossification

In order to assess if HP suppressed markers of hypertrophy, ALP activity in the media was first analyzed. MSCs seeded in agarose demonstrated higher levels of ALP activity than in fibrin for constructs maintained at either 1 or 10 ng/ml TGF-β3 (data for 10ng/ml provided in Fig. 2.7A where activity is higher). ALP activity increased with time in agarose constructs, peaking towards the end of the 21 day culture period, while ALP activity peaked at earlier time points in the fibrin hydrogels. Also, when maintained in 10 ng/ml TGF-β3, HP acted to suppress ALP activity in both fibrin and agarose hydrogels.
(Fig. 2.7B). Evidence for reduced ALP enzyme activity was also observed in histological sections stained for naphthol phosphate with fast red (Fig. 2.7C). In spite of this, there was no noticeable difference in collagen type X staining with the application of HP in either hydrogel (data not shown).
Figure 2.7: (A) Quantity of ALP activity (normalized to DNA) released to the media over the three week culture period for Donors 1 and 3 in both agarose and fibrin hydrogels in 10 ng/ml TGF-β3. B) Quantity of ALP activity (normalized to DNA) for HP groups normalized to FS groups for two replicates cultured in 1 and 10 ng/ml TGF-β3. For both Donor 1 and 3, the media was analyzed in triplicate for these assays. However, as constructs were pooled during loading, it can be argued that these triplicates are not independent and hence a statistical analysis was not performed. Insufficient media was available from donor 2 to facilitate undertaking this specific assay. (C) Representative sections stained for ALP enzyme activity for MSCs from Donor 3 seeded in either agarose (left) or fibrin (right) hydrogels at day 21 for both FS and HP groups supplemented with 10ng/ml of TGF-β3. No noticeable differences were observed for constructs supplemented with 1 ng/ml of TGF-β3.
2.4 Discussion

Hydrostatic pressure is a key regulator of stem cell fate and has important applications in the field in cartilage tissue engineering. The hypothesis under investigation in this study was that the response of MSCs to HP would depend on the hydrogel into which they were embedded. To test this hypothesis, MSCs were encapsulated into hydrogels that either prevent (agarose) or promote (fibrin) cell attachment and spreading within the construct. Since it is heavily involved with blood clots, MSC seeded fibrin hydrogels subjected to physiological levels of loading can be considered as an *in vitro* model of the spontaneous healing that occurs within osteochondral defects. Confocal microscopy revealed that MSCs embedded in fibrin hydrogels attained a spread morphology with distinct stress fiber formation, while MSCs in agarose hydrogels retained a spherical morphology. When subjected to TGF-β3 stimulation, bone marrow derived MSCs embedded in agarose hydrogels underwent more robust chondrogenesis than those in fibrin hydrogels. In spite of this, it was observed that HP only enhanced chondrogenesis of MSCs embedded in fibrin hydrogels, as evident by decreased staining for type I collagen, increased sGAG synthesis and a decrease in ALP activity. Together these results suggest that the interaction of MSCs with their local substrate regulates their response to HP.

In the current study, HP increased sGAG synthesis when MSCs were cultured in the presence of 1 ng/ml TGF-β3 but not 10 ng/ml TGF-β3. It has previously been demonstrated that MSCs exhibit a stronger chondrogenic response to mechanical cues at lower concentrations of TGF-β. It has been proposed that mechanical loading and TGF-β act via similar pathways, such that high concentrations of TGF-β mask the effects
of mechanical loading. Further work is required to help elucidate the molecular mechanisms by which HP enhances chondrogenesis of MSCs.

Cell-matrix interactions alone are known to play a key role in determining stem cell fate. Fibrin better supported MSC proliferation and collagen production relative to those in agarose; however MSCs in agarose produced significantly more sGAG. The spherical cell morphology supported by agarose may help to explain this result. Also, unlike agarose, fibrin facilitates integrin mediated cellular attachment through two pairs of RGD sites. In a study comparing bone marrow MSCs seeded in hydrogels conjugated with RGE (no cell attachment) or RGD (cell attachment) peptides, chondrogenic gene expression and protein synthesis were significantly decreased in the RGD modified gel. In addition, there was a significant increase in collagen I in the presence of serum. This agrees with the finding of this study where cell attachment in fibrin, and subsequent cell spreading, correlates with an increase in collagen type I synthesis and a decrease in the chondrogenic phenotype of bone marrow-derived MSCs.

While integrin mediated cell adhesion influences chondrogenesis of MSCs, it also plays a key role in mechanotransduction. As previously discussed, fibrin facilitates integrin-mediated cellular attachment through two pairs of RGD sites. Integrins bind both with the extracellular matrix as well as the cytoskeleton, and they help transmit mechanical signals “outside-in” and “inside-out.” Therefore integrin mediated cell attachment is thought to be a prerequisite in order for a cell to respond to mechanical forces. Previous studies where HP has been shown to enhance chondrogenesis of bone marrow derived MSCs have generally used a culture system in which either cell-cell or cell-matrix attachments are facilitated, which may also explain the pro-
chondrogenic response to HP observed in fibrin hydrogels in this study. Conversely, HP had little to no effect on cartilage specific matrix synthesis for MSCs seeded in agarose, which agrees with the findings of a previous study.\textsuperscript{40} This supports our contention that formation of integrin-mediated adhesions and subsequent focal adhesion assembly is necessary for HP to be sensed by MSCs. Interestingly, previous studies in our lab have shown that long term application of HP (6 weeks) can enhance chondrogenesis of MSCs embedded in agarose hydrogels,\textsuperscript{135} which is potentially explained by cells generating and attaching to their own PCM, which in turn facilitates integrin-mediated mechanotransduction of HP. An alternative (although related) explanation for the substrate dependent response to mechanical loading is that HP is interfering with integrin mediated attachment of MSCs to the local extracellular matrix, which, as has already been discussed, can inhibit chondrogenesis of MSCs.\textsuperscript{44} This hypothesis is supported by the finding that HP also suppressed MSC mediated contraction of fibrin hydrogels, which presumably is at least partially driven by integrin-mediated attachment of MSCs to their local extracellular environment.

Some donor variability in the response of MSCs to HP was observed. HP-mediated suppression of fibrin contraction was greatest for donor 2, and this donor also demonstrated the largest differences in DNA, sGAG synthesis, and total collagen synthesis due to the application of HP. HP also decreased contraction for donor 1 and led to an increase in sGAG synthesis. Conversely, HP had no effect on any of the properties investigated for donor 3. This variable response could be due to batch to batch variability in fibrin properties, as well as differences between each donor. Previous studies have also shown a donor dependent response to HP when seeded in agarose hydrogels, providing
evidence that there is inherent differences in the response of MSCs from various donors to loading.\textsuperscript{135} Further experimentation is needed to better elucidate the extent of the donor dependent response to HP.

Hypertrophy and mineralization of cartilaginous grafts engineered using bone marrow-derived MSCs has been observed in an ectopic environment,\textsuperscript{9} which lacks the mechanical signals present within synovial joints. Hydrostatic pressure, a key component of the \textit{in vivo} joint environment, has previously been shown to suppress markers of hypertrophy and to help maintain the chondrogenic phenotype of synovial membrane derived MSCs.\textsuperscript{37} While HP did not influence the initiation of chondrogenesis in agarose, as evident by the finding that the mechanical stimulus did not influence ECM accumulation, it did appear to suppress ALP activity, a marker of endochondral ossification. Alkaline phosphatase expression by bone marrow-derived MSCs \textit{in vitro} has been shown to correlate with endochondral ossification \textit{in vivo}.\textsuperscript{151,152} With increasing time in culture, MSCs embedded in agarose begin to produce their own PCM (Fig. 2.1, Fig. 2.4), which may be the mechanism by which this stimulus is transduced. Therefore, the decrease in ALP activity when exposed to HP provides further support for the hypothesis that this stimulus may play a role in inhibiting hypertrophy and terminal differentiation of chondrogenically primed MSCs. Understanding how joint specific factors, such as mechanical cues and the local oxygen tension, regulate the endochondral phenotype is a key challenge for the successful translation of stem cell based therapies for articular cartilage repair.\textsuperscript{153}

In conclusion, we have demonstrated that hydrostatic pressure can enhance chondrogenesis of bone marrow-derived MSCs in a biomaterial substrate dependant
manner. Furthermore, not only does HP effect chondrogenic induction of MSCs, it also appears to play a key role in the maintenance of a chondrogenic phenotype. Further understanding of the interplay between cell-matrix interactions and the extrinsic mechanical environment regulating chondrogenesis of MSCs is needed in order to improve stem cell based cartilage regeneration therapies.
CHAPTER 3:

THE PERICELLULAR ENVIRONMENT REGULATES CYTOSKELETAL DEVELOPMENT AND THE DIFFERENTIATION OF MESENCHYMAL STEM CELLS AND DETERMINES THEIR RESPONSE TO HYDROSTATIC PRESSURE

3.1 Introduction

Multiple soluble and insoluble cues are known to regulate the differentiation of mesenchymal stem cells (MSCs), although we are only beginning to understand how these factors interact to regulate cell fate.\textsuperscript{14,17–19,69,154} The matrix or substrate stiffness has been shown to play a role in regulating the differentiation of MSCs down specific lineages in both 2D\textsuperscript{18,21} and 3D environments.\textsuperscript{22–24} Softer substrates tend to guide MSCs down neurogenic, adipogenic and chondrogenic pathways, while stiffer substrates have been shown to support myogenesis and osteogenesis depending on the specific composition of the culture media,\textsuperscript{18,21,22,155} although the underlying mechanisms by which stem cells sense and respond to substrate stiffness are not fully understood. Integrins form the linkage between a cell and its extracellular matrix (ECM) and have long been associated with mechanotransduction,\textsuperscript{156} with matrix stiffness known to regulate integrin binding as well as the organization of adhesion ligands.\textsuperscript{22} Inhibition of integrin binding disrupts modulus driven differentiation in both 2D and 3D culture systems,\textsuperscript{21–23} confirming the role of integrin bonds in determining stem cell fate. It has also been shown that cytoskeletal-integrin linkage strength becomes stronger with increasing
matrix elasticity, implicating the cytoskeleton as a possible downstream target of matrix stiffness mechanotransduction. For example, myosin and actin generated cytoskeletal tension are integral to mechanotransduction of substrate stiffness in 2D, although the effects of actomyosin generated cytoskeletal tension are less clear in 3D.

In addition to cues generated in response to alterations in the stiffness and composition of the pericellular environment in vivo, stem cells are also exposed to extrinsic mechanical loading which is known to influence their ultimate fate. The type (i.e. compression, fluid flow, tension, hydrostatic pressure), frequency, magnitude, and duration of loading all affect MSC lineage commitment. As described in Chapters 1 and 2, hydrostatic pressure (HP) specifically is a key regulator of chondrogenesis. HP has been shown to increase chondrogenic gene expression and matrix production in MSCs, HP also plays a role in maintaining the chondrogenic phenotype by suppressing the expression of type I collagen, alkaline phosphatase (ALP), matrix metalloproteinase 13 (MMP-13), type X collagen, and Indian hedgehog (Ihh). HP has been shown to disrupt actin stress fiber assembly in chondrocytes, and inhibition of microtubules has been shown to suppress the beneficial effect of HP on chondrocyte matrix production in 2D culture. The exact mechanism through which HP is transduced is unknown, although it has been proposed that HP leads to an increase in entropy (under constant temperature and volume) by depolymerizing cytoskeletal polymers into free monomers, therefore disrupting stress fiber assembly. Cell-matrix interactions have been shown to influence MSC response to HP during chondrogenesis in 3D culture, with a more robust response to loading observed in hydrogels that promoted stronger actin stress fiber formation. Similar to
studies exploring stem cell response to matrix stiffness, these studies point to a role for integrins and cytoskeletal adaption in the mechanotransduction of HP.

The objective of this study was to examine the interplay between matrix stiffness and HP in regulating chondrogenesis of MSCs. Bone marrow derived MSCs were encapsulated in hydrogels of differing stiffness and subjected to intermittent HP. Integrin binding was inhibited in some hydrogels in order to determine the role of integrin binding in the mechanotransduction of both matrix stiffness and HP. Actin microfilaments, microtubules, and intermediate filaments were also examined to determine their prospective roles in mechanotransduction. Our hypothesis was that softer hydrogels would support a more chondrogenic phenotype, but that changes in cell-matrix interactions and cytoskeletal development in the stiffer hydrogels would result in a more robust response to the application of HP.

3.2 Materials and Methods

3.2.1 Materials

All materials were acquired from the same companies as in Section 2.2.1 unless otherwise noted. RGDS was purchased from Tocris Bioscience. Monoclonal anti-vinculin FITC conjugate and chloroform were purchased from Sigma-Aldrich, Ireland, while 1 µg/mL anti-vimentin FITC and 1 µg/mL anti-alpha tubulin eFluor® 615 was purchased from eBioscience, Inc. The Ultra-turrax IKA T10 basic homogenizer was purchased from Fisher Scientific, Ireland. The TRIZOL reagent and PureLink™ RNA Mini kit were purchased from Invitrogen, Ireland. The ND 1000 NanoDrop Spectrophotometer was
purchased from LabTech International. All other qRT-PCR supplies were purchased from Applied Biosystems, Ireland.

3.2.2 Cell isolation, expansion, and encapsulation

MSCs were isolated and expanded as described in section 2.2.2. After expansion (third passage) MSCs were encapsulated in agarose (Type VII) at a density of 15 x 10^6 cells/mL. Briefly, MSCs were mixed with 5% agarose at ~40°C to yield final gel concentrations of 1%, 2% or 4% (with equilibrium moduli of 0.5, 10, and 25 MPa, respectively). The agarose-cell suspensions were cast in a stainless steel molds, and cored using biopsy punches to produce cylindrical scaffolds (Ø 5 x 3 mm thickness). Constructs were maintained in 2.5 mL/construct of a chemically defined media (CDM) consisting of hgDMEM GlutaMAX supplemented with penicillin (100 U/ml)-streptomycin (100 µg/ml), 100 µg/ml sodium pyruvate, 40 µg/ml L-proline, 50 µg/ml L-ascorbic acid-2-phosphate, 1.5 mg/ml BSA, 1 x insulin-transferrin-selenium, 100 nM dexamethasone and either 16% FBS (permissive) or 10 ng/ml recombinant human transforming growth factor-β3 (chondrogenic, TGF-β3). Some groups were also cultured with the addition of a 167 µM RGDS peptide (RGDS+) in order to inhibit integrin binding. Cells that were to be cultured with the RGDS peptide were equilibrated in the chondrogenic media supplemented with RGDS for 2 hours prior to encapsulation in agarose, and RGDS was added to the media during each further media change. Constructs were allowed to equilibrate overnight before the initiation of hydrostatic pressure.
3.2.3 Application of hydrostatic pressure

Hydrostatic pressure was applied as described in Section 2.2.3.

3.2.4 Biochemical analysis

DNA, sGAG, and collagen content was analyzed as described in Section 2.2.4.

3.2.5 Confocal microscopy, histology, and immunohistochemistry

Samples were fixed and prepared for confocal as described in Section 2.2.5. In addition to being incubated in a 1.5 % BSA solution containing 5 U/mL rhodamine phalloidin, additional samples were also incubated with either 60 µg/mL monoclonal anti-vinculin FITC conjugate, 1 µg/mL anti-vimentin FITC, or 1 µg/mL anti-alpha tubulin eFluor® 615 for 1.5 hours, and then imaged using a Zeiss 510 Meta confocal microscope at 40x magnification. In order to determine if cytoskeletal reorganization in response to HP was dependent on cell shape and/or scaffold material, fibrin gels were prepared as described in Section 2.2.2, but incubated with 1 µg/mL anti-vimentin FITC and imaged using a Nikon A1R confocal microscope at 40x magnification.

The remaining halves were either histologically analyzed for sGAG and collagen content, or immunohistochemically analyzed for collagen types I and II content as described in Section 2.2.5.

3.2.6 RNA isolation and quantitative real-time polymerase chain reaction

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was used to determine relative gene expression changes in chondrogenic specific genes
with respect to both application of loading and supplementation with RGDS peptide. Total RNA was extracted from agarose constructs (n=3) directly after loading on day 14 of culture. Total RNA was extracted from each construct by homogenizing each construct with an Ultra-turrax IKA T10 basic homogenizer in 1 mL of TRIZOL reagent, followed by a chloroform extraction. The extracted solution was incubated with an equal volume of isopropanol and 5 µL glycogen in a -20°C freezer overnight. The solution was then centrifuged and the precipitate was washed once with 70% ethanol. The precipitate was resuspended in 200 µL of 35% ethanol and the RNA was then extracted with a PureLink™ RNA Mini kit as per manufacturer’s instructions. Total RNA yield and purity were analyzed using an ND 1000 NanoDrop Spectrophotometer and adjusted to a standard concentration prior to cDNA synthesis. To quantify mRNA expression, 50 ng of total RNA was reverse transcribed into cDNA using a high capacity reverse transcription cDNA kit as per manufacturer’s instructions. TaqMan® gene expression assays which contain forward and reverse primers, and a FAM-labeled TaqMan probe for porcine Sox9 (Ss03392406_m1), aggrecan (Agc, Ss03374822_m1), collagen type II alpha 1 (Col2A1, Ss03373344_g1), and Glyceraldehyde-three-phosphate dehydrogenase (GAPDH, Ss03373286) were used in this study. qRT-PCR was performed using an ABI 7500 Sequence Detection system. 5 µL of cDNA preparation (diluted 1:5 with RNase free water), 1 µL of gene assay, 10 µL TaqMan Universal PCR Master mix and 4 µL RNase free water (20 µL total volume) were added to each well. Samples were assayed in triplicate in one run (40 cycles). qRT-PCR data were analyzed using the ΔΔCₜ method as described previously¹⁶¹ with GAPDH as the endogenous control. Relative quantification values are presented as fold changes in gene expression relative to the control group.
3.2.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 5.00, GraphPad Software). Biochemical results, both numerical and graphical, are expressed in the form of mean ± standard deviation. Differences between HP and FS samples or between samples cultured with or without RGDS were determined using a Student’s t-test. A level of \( p < 0.05 \) was considered significant. All sGAG, collagen, histological, and confocal data is from samples collected on day 21. All gene expression data was collected on day 14.

3.3 Results

3.3.1 Influence of matrix stiffness on MSC differentiation in a permissive environment

The Young’s modulus of agarose hydrogels increased from 0.5 kPa for 1% gels to 10 kPa for 2% gels, to 25 kPa for 4% gels. In order to assess how matrix stiffness affects differentiation of bone marrow-derived MSCs cultured in a permissive environment (Media + 16% FBS), histological sections were stained for calcific deposits and sGAG accumulation as markers of osteogenesis and chondrogenesis respectively. With increasing matrix stiffness, more pronounced mineralization was observed (Fig. 3.1). However, no evidence of chondrogenesis was observed in these specific culture conditions regardless of the local matrix stiffness (Fig. 3.1).
3.3.2 Chondrogenesis of MSCs is regulated by matrix stiffness, integrin binding, and cytoskeletal organization

Cell seeded constructs were maintained in a chondrogenic media (Media + 10 ng/mL TGFβ-3) in order to assess the specific effects of matrix stiffness on the chondrogenesis of MSCs. Total sGAG and collagen production for each group was determined by summing the accumulation of specific matrix components within the hydrogels with that released into the media. While sGAG accumulation within the constructs on day 21 was greater in the 4% agarose hydrogels, overall levels of both total sGAG (1%: 45.05 ± 2.65 μg, 2%: 33.60 ± 2.58 μg, 4%: 29.39 ± 1.32 μg) and collagen (1%: 177.35 ± 24.72 μg, 2%: 84.04 ± 7.00 μg, 4%: 47.30 ± 3.00 μg) production decreased with increasing matrix stiffness (Fig. 3.2A,B). The pericellular environment was also found to depend on agarose hydrogel concentration, with a more well developed, intensely stained pericellular matrix (PCM, consisting of proteoglycans and collagens) observed in the stiffer hydrogels (Fig. 3.2E,F). To determine if interactions
between MSCs and their local pericellular environment was regulating ECM synthesis, integrin binding was blocked with the addition of RGDS to the culture media. While RGDS had no significant effect on sGAG and collagen production in the softer 1% agarose hydrogels (Fig. 3.2C,D), inhibition of integrin binding led to a significant increase in ECM synthesis in the stiffer 4% agarose hydrogels, reaching levels comparable to that in 1% hydrogels (Fig. 3.2C,D).
Figure 3.2: (A) Total sGAG and (B) total collagen retained in the construct (white) and released to the media (black). (C) Total sGAG and (D) total collagen produced when cultured either with (RGDS+, black) or without (RGDS-, white) RGDS peptide. (E) Representative Alcian blue, picro-sirius red, (F) collagen type I, and collagen type II histological and immunohistochemical images of 1% and 4% scaffolds (Scale bar = 10 μm). a: p<.05.

We next sought to explore how matrix stiffness, and associated changes in the pericellular environment, influences both focal adhesion assembly and the cytoskeletal development of MSCs undergoing chondrogenesis. Staining for vinculin, a protein found in focal adhesions, was disperse in the softer 1% hydrogels, but had a more punctate
appearance in the 4% hydrogels. This punctate structure was not evident with the addition of RGDS (Fig. 3.3). No visible changes in tubulin structure were observed with changes in matrix stiffness or the addition of RGDS, suggesting that the microtubule network is relatively insensitive to changes in the pericellular environment. The intensity of actin fluorescence increased with increasing matrix stiffness, with the addition of RGDS reducing the staining intensity. Staining for vimentin intermediate filaments became more punctate with increasing stiffness, and, as with vinculin staining, this punctate structure was less evident and staining more diffuse with the addition of RGDS (Fig. 3.3).

Figure 3.3: Representative confocal images of vinculin, actin, vimentin, and tubulin in 1%, 4%, and 4% +RGDS constructs (Scale bar = 10 µm/40 µm).
3.3.3 Influence of hydrostatic pressure on chondrogenesis and the cytoskeletal organization of MSCs

While focal adhesion assembly, cytoskeletal organization and cartilage-specific ECM synthesis are all regulated by the pericellular environment, in a developmental or regenerative context, MSCs will be additionally subjected to extrinsic mechanical forces in vivo such as hydrostatic pressure. How MSCs sense and respond to both intrinsic (i.e. those that are generated within the cell in response to the composition and stiffness of the PCM) and extrinsic (i.e. those generated from external mechanical loading) biophysical cues is poorly understood. The application of cyclic hydrostatic pressure (HP) was found to modulate ECM synthesis in a matrix stiffness dependent manner, with enhanced sGAG synthesis in response to HP (1%: 0.99 ± 0.14 fold, 2%: 1.19 ± 0.12 fold, 4%: 1.42 ± 0.23 fold) only observed in the stiffer 4% hydrogels over 21 days of culture (Fig. 3.4A). Hydrostatic pressure had no effect on total collagen synthesis (%: 0.88 ± 0.14 fold, 2%: 0.94 ± 0.11 fold, 4%: 0.94 ± 0.20 fold) in any hydrogel (Fig. 3.4B). To further explore MSC response to HP in hydrogels of differing stiffness, the expression of a number of chondrogenic genes was analyzed at day 14. Sox9, Agc, and Col2A1 gene expression all increased significantly in the stiffer 4% hydrogels exposed to HP (Sox9: 1.41 ± 0.20 fold, Agc: 1.32 ± 0.13 fold, Col2A1: 2.19 ± 0.15 fold), while HP had no positive effect on gene expression in the softer 1% hydrogels (Sox9: 0.90 ± 0.17 fold, Agc: 0.43 ± 0.46 fold, Col2A1: 0.73 ± 0.16 fold) (Fig. 3.4C-E). Hydrostatic pressure had no visible effect on the intensity of vinculin, actin, or tubulin staining or its localization within the cell; however, the punctate structure of vimentin in the stiffer 4% hydrogels was no longer present after exposure to HP (Fig. 3.4F). Vimentin is also reorganized in response to HP
regardless of cell shape and biomaterial, as vimentin appeared to collapse around the nucleus in fibrin hydrogels exposed to HP (Fig. 3.5).

Figure 3.4: (A) Total sGAG and (B) total collagen normalized to the FS condition. (C) Sox9, (D) Agc, and (E) Col2A1 relative gene expression normalized to the FS condition. (F) Representative confocal images of vinculin, actin, vimentin, and tubulin in 4% FS and HP groups (Scale bar = 10 µm/40 µm). a: p<0.05.
3.3.4 Integrin binding is necessary for mechanotransduction of hydrostatic pressure

Given that integrin binding was required for MSCs to respond to changes in the stiffness and/or composition of their pericellular environment, we explored whether a similar pathway was involved in the mechanotransduction of HP. Addition of RGDS abrogated the beneficial response of HP on sGAG synthesis in the stiffer 4% hydrogels (-RGDS: 1.42 ± 0.23 fold, +RGDS: 0.90 ± 0.11 fold) over the 21 day culture period (Fig. 3.6A). Hydrostatic pressure had no effect on collagen production whether the media was supplemented with RGDS or not (-RGDS: 0.94 ± 0.20 fold, +RGDS: 1.21 ± 0.33 fold) (Fig. 3.6B). Furthermore, the increase in the expression of Sox9, Agc, and Col2A1 due to the application of hydrostatic pressure was also abolished in the presence of RGDS (Sox9: -RGDS: 1.41 ± 0.20 fold, +RGDS: 1.26 ± 0.27 fold; Agc: -RGDS: 1.32 ± 0.13 fold, +RGDS: 1.05 ± 0.36 fold; Col2A1: -RGDS: 2.19 ± 0.15 fold, +RGDS: 0.86 ± 0.38 fold) (Fig. 3.6C-E). In the presence of RGDS, no changes were observed in vinculin, actin, vimentin, or tubulin due to the application of hydrostatic pressure (Fig. 3.6F).
Figure 3.6: (A) Total sGAG and (B) total collagen in 4% gels normalized to the FS condition. (C) Sox9, (D) Agc, and (E) Col2A1 relative gene expression in 4% gels normalized to the FS condition. (F) Representative confocal images of vinculin, actin, vimentin, and tubulin in 4% FS and HP groups cultured with RGDS peptide (Scale bar = 10 µm/40 µm). a: \( p < .05 \).

3.4 Discussion

In agreement with previous studies seeding MSCs onto 2D substrates\(^{18}\) or embedding them into 3D hydrogels,\(^{22,23}\) we found that the stiffer 4% hydrogel supported a more osteogenic phenotype as evidenced by calcific deposits within constructs maintained in a permissive media that did not contain specific osteogenic supplements.
Irrespective of hydrogel stiffness, this permissive media did not support chondrogenesis of MSCs. As seen previously, when maintained in a media supplemented with TGF-β3, chondrogenic matrix production was inhibited in stiffer hydrogels.\textsuperscript{20,162} One potential explanation for this is that diffusivity of biomolecules (such as TGF-β3) would be lower in the stiffer, denser 4% hydrogels; however, these hydrogels are still 96% fluid and are therefore not expected to significantly inhibit biomolecule diffusivity. While MSCs cannot directly adhere to agarose, and hence initially are unlikely to be able to sense their local stiffness, they rapidly synthesize fibronectin and other extracellular matrix components in hydrogel culture,\textsuperscript{23,163} to which they can adhere, which may provide them with a mechanism through which they can sense the stiffness of the surrounding hydrogel. In agreement with previous studies,\textsuperscript{20,162} a denser, presumably stiffer, PCM also develops in the higher concentration hydrogels to which the MSCs can adhere and sense. In addition to a stiffer micro-environment for MSCs in the 4% hydrogels, it is also reasonable to assume that the more developed PCM in these constructs leads to an increase in the number of integrin binding sites per cell. It is therefore difficult to decouple whether the denser matrix exerts its effects via creating a stiffer pericellular environment, by increasing adhesion-ligand density\textsuperscript{138} or through some other feedback mechanism such as a change in the local charge density (or a combination of these factors). Previous studies have provided strong support for the hypothesis that MSCs interpret changes in the stiffness of their 3D pericellular environment as changes in adhesion-ligand presentation.\textsuperscript{22}

The observed changes in MSC phenotype in the stiffer 4% hydrogels were accompanied by a more developed actin cytoskeleton. It is well established that
development of actin stress fibers is correlated with an inhibition of chondrogenesis.\textsuperscript{164} Previous studies have also observed that conjugation of RGD-adhesion ligands to agarose hydrogels inhibited sGAG synthesis by MSCs, and this inhibition could be blocked with the addition of a pharmacological actin inhibitor, further demonstrating that cellular adhesion and subsequent actin stress fiber formation inhibits chondrogenesis.\textsuperscript{138} Based on these findings, we examined the roles of integrin binding and cytoskeletal organization in the mechanotransduction of matrix stiffness. The addition of RGDS to the media, which blocks integrin binding to the PCM, impacted actin cytoskeleton development in the stiffer 4\% gels and lead to similar levels of ECM synthesis in these constructs as observed in the softer hydrogels, further implicating integrin binding and actin as important elements in mechanotransduction. Vinculin, a component of focal adhesion complexes, exhibited a more punctate structure in the stiffer hydrogels, with the addition of RGDS leading to more diffuse staining, similar to that seen in the softer hydrogels. The more punctate organization of vinculin in the stiffer hydrogels could indicate greater focal adhesion formation and FAK signaling, which has been shown to inhibit early chondrogenesis in MSCs.\textsuperscript{65} A punctate vimentin structure, perhaps indicative of filament formation, was observed in the stiffer 4\% agarose and fibrin hydrogels, and again the addition of RGDS caused the structure to resemble that in the softer 1\% hydrogels. Vimentin is known to regulate chondrogenesis of MSCs, with siRNA-mediated knockdown of vimentin inhibiting cartilage-specific ECM production.\textsuperscript{85} These intermediate filaments also contribute to the stiffness of chondrocytes.\textsuperscript{86} Previous research has shown that vimentin can directly interact with actin, integrins $\alpha\nu\beta3$ and $\alpha2\beta1$, and their associated focal adhesions, which, together with the results of the current
study, provides a mechanism by which vimentin may play a role in the transduction of mechanical cues. The results of these and the present study demonstrate that although vimentin is critical for chondrogenesis, adaption of the intermediate filament network as the pericellular matrix becomes denser may play a role in the suppression of a chondrogenic phenotype in MSCs.

Prior studies have demonstrated that the application of HP can enhance chondrogenesis of MSCs and improve the mechanical functionality of tissue engineered cartilage. Previously, we observed that the application of HP enhances chondrogenesis of MSCs in fibrin hydrogels where cells adopt a spread morphology with clear stress fiber formation, while MSCs embedded in agarose hydrogels remained rounded and did not respond to loading. These findings implicated cell shape and cytoskeletal dynamics in modulating the response of MSCs to HP. In this study, MSCs only responded to HP (as indicated by increases in the expression of certain chondrogenic genes and increased sGAG synthesis) in the stiffer 4% hydrogels, although MSCs retained a similar rounded morphology in both constructs, suggesting cell shape alone does not determine the response of MSCs to HP. Rather, it would appear that MSCs with clear focal adhesion assemblies, intense actin staining and a specific vimentin intermediate filament organization respond anabolically to the application of HP. In such MSCs, HP would appear to disrupt the vimentin network, with more diffuse staining observed in MSCs exposed to HP, possibly implicating intermediate filaments in the mechanotransduction of HP. This diffuse staining may be indicative of depolymerization of intermediate filaments due to the application of HP, which may occur due to an increase in entropy or alterations in phosphorylation pathways due to increased
Vimentin depolymerization has been observed in certain cell types in response to HP,\textsuperscript{96} with the same study reporting that microtubules appear more resistant to high levels of pressurization. Induced swelling of cartilage explants was found to lead to vimentin disassembly, while swelling had no effect on actin organization, further implicating an important mechanosensory role for vimentin in articular chondrocytes.\textsuperscript{167}

Together, these two studies, along with the current one, also suggest that different cytoskeletal elements may be more or less sensitive to different mechanical stimuli. The results of this study also provide further support for the concept that extrinsic mechanical cues can override the influence of the local substrate in determining MSC fate.\textsuperscript{17}

Given that chondrogenesis was suppressed in the stiffer 4% hydrogels via integrin mediated binding to the pericellular matrix, and furthermore that the application of HP at least partially overcame this suppression of chondrogenesis, it seemed reasonable to assume that the response of MSCs to HP would be abrogated in the absence of integrin mediated binding to the PCM. Indeed, HP had no effect on the expression of chondrogenic genes or cartilage specific matrix production in the presence of RGDS. One interpretation of this result is that MSC-PCM integrin mediated bonds are essential for the mechanotransduction of HP. Indeed, integrins have been implicated in the mechanotransduction of multiple extrinsic mechanical cues, including tension, compression, and fluid flow.\textsuperscript{168} However the finding that HP had no influence on chondrogenesis with the addition of RGDS may not necessarily imply a direct role for integrins in the mechanosensing of HP, as blocking integrin mediated binding to the pericellular matrix also effected cytoskeletal components such as vimentin which may be the primary mechanosensors. The addition of RGDS in stiffer hydrogels led to the
development of a cytoskeleton similar to that in the softer 1% hydrogels where HP had no beneficial effect on chondrogenesis. For similar reasons it is difficult to determine if changes in vimentin organization are downstream or upstream of changes to integrin binding in the mechanotransduction pathway of HP (i.e. RGDS alters both integrin binding and vimentin organization, making it difficult to decouple their relative roles in the mechanotransduction of HP). Furthermore, the application of HP has no noticeable influence on the pattern of vinculin staining, although more quantitative analysis is required to definitively state that this mechanical cue is not influencing the composition or assembly of focal adhesions.

In conclusion, the pericellular matrix plays a crucial role in mechanotransduction of both HP and matrix stiffness through integrin binding and cytoskeletal organization. MSCs embedded in the stiffer 4% hydrogels develop a more mature PCM, leading to changes in focal adhesion formation and cytoskeletal organization and an inhibition of cartilage matrix synthesis and gene expression. The application of extrinsic mechanical cues such as HP can disrupt this process and override the influence of matrix stiffness on cytoskeletal development, promoting the maintenance of a chondrogenic phenotype as the pericellular environment matures and becomes stiffer. Integrin mediated binding to the PCM played a role in the mechanotransduction of both matrix stiffness and HP, although in the latter case downstream changes to the cytoskeleton following supplementation with RGDS make it impossible to definitively state that integrins are the primary mechanosensors of HP in MSCs. Finally, vimentin structure was also altered in the stiffer hydrogels when exposed to HP, suggesting a role for vimentin as a mechanotransductive element of HP.
CHAPTER 4:
CALCIUM SIGNALING REGULATES THE CHONDROGENIC RESPONSE OF MESENCHYMAL STEM CELLS TO HYDROSTATIC PRESSURE

4.1 Introduction

As detailed in the previous chapters, mechanical cues play a key role in mesenchymal stem cell (MSC) differentiation.\textsuperscript{14,17,19,69,154} The response to such cues strongly depends on the type (i.e. compression, fluid flow, tension, hydrostatic pressure), frequency, magnitude, and duration of loading.\textsuperscript{14,31,70,135} Fluid pressurization is the dominant load-bearing mechanism of cartilage, supporting up to 90\% of the compressive load in the \textit{in vivo} joint environment.\textsuperscript{169} In accordance with its prominence in cartilaginous tissues, hydrostatic pressure (HP) is an important regulator of chondrogenic differentiation of mesenchymal stem cells (MSCs), increasing chondrogenic gene expression and matrix production, and also suppressing markers of hypertrophy.\textsuperscript{15,16,30–38,135} Mechanotransduction of HP has been proposed to differ from other mechanical loads due to the fact that HP generates a state of stress with little deformation,\textsuperscript{133} as hydrated tissues and cells are nearly incompressible. However, surprisingly little is known about the mechanisms by which cells sense and respond to HP.

Recently, we demonstrated that integrin binding to the pericellular matrix regulates vimentin organization in MSCs and determines their response to cyclic HP (Chapter 3).\textsuperscript{16} Another proposed element of HP mechanotransduction is fluctuations in
intracellular ion concentrations, which are altered by the application of HP.\textsuperscript{46,49,108} In particular, calcium ion (Ca\textsuperscript{++}) signaling has been implicated as a critical regulator of cellular response in other mechanical loading modalities, yet no studies have examined the role of Ca\textsuperscript{++} signaling in the chondrogenic response of MSCs to HP. Ca\textsuperscript{++} signaling is complex and involves a variety of channels, receptors and secondary messengers (Fig. 4.1). Stretch activated calcium channels (SACCs) have been demonstrated to be required for mechanotransduction of a variety of loading types in chondrocytes and MSCs.\textsuperscript{108,109,170,171} Voltage gated calcium channels (VGCCs) are activated by membrane depolarization and mediate Ca\textsuperscript{++} influx.\textsuperscript{172} Ca\textsuperscript{++} influx via SACCs and/or VGCCs increases Ca\textsuperscript{++} concentrations directly and also indirectly through calcium-induced calcium release via calcium-sensitive receptors in the sarcoendoplasmic reticulum calcium stores (SERCS).\textsuperscript{172} VGCCs and SERCS were both found to be required for chondrogenesis in high density chicken MSCs culture,\textsuperscript{100} and they have both been implicated in mechanotransductive pathways.\textsuperscript{62,105,173} Finally, Ca\textsuperscript{++} utilizes secondary messengers, such as calmodulin (CaM), calmodulin kinase type II (CaMKII), and calcineurin (Cn), which subsequently initiate a variety of signaling cascades. CaM, CaMKII, and Cn have been implicated in mechanotransduction of fluid flow and compression in MSCs as well as chondrocytes.\textsuperscript{62,105}
While intracellular Ca\(^{++}\) concentrations are known to increase in chondrogenic cells after application of HP,\(^ {108}\) the effects of this increase on the chondrogenesis of MSCs and the specific channels and downstream effectors utilized by MSCs to sense and respond to HP are unknown. Further, integrins, the cytoskeleton, and Ca\(^{++}\) channels have previously been proposed to interact with one another;\(^ {170,173–176}\) thus, recent findings that integrin binding and cytoskeletal organization regulate the mechanotransduction of HP implicate a possible role for Ca\(^{++}\) signaling in mediating this process. Therefore, in order to elucidate the role of Ca\(^{++}\) signaling in the mechanotransduction of HP, chondrogenic matrix accumulation, focal adhesion formation and cytoskeletal organization were
examined in MSCs exposed to HP in the presence of pharmacological inhibitors of Ca\(^{++}\) mobility and downstream signaling molecules.

4.2 Materials and Methods

4.2.1 Materials

Porcine femurs were procured from a local abattoir, Martin’s Custom Butchering, Wakarusa, IN. Dulbecco’s modified Eagle’s Medium and insulin-transferrin-selenium was purchased from Gibco, Life Technologies, Grand Island, NY. Fetal bovine serum was purchased from Omega Scientific, Tarzana, CA. Penicillin-streptomycin was purchased from Corning, VWR, USA. Agarose, media supplements, calcium inhibitors, the secondary antibody for immunohistochemistry, and all other chemicals (unless otherwise noted) were purchased from Sigma-Aldrich, St. Louis, MO. Transforming growth factor-\(\beta\)3 was purchased from PeproTech, Rocky Hill, NJ. Sodium pyruvate was purchased from Lonza, VWR. Ethanol was purchased from Acros Organics, VWR. Guanadine-HCl was purchased from Calbiochem, VWR. Bovine serum albumin was purchased from Fisher Scientific, Pittsburgh, PA. The pressure vessel was purchased from Parr Instrument Company, Moline, IL. Isopropanol was purchased from Alfa Aesar, VWR. Paraformaldehyde was purchased from Fisher Scientific, USA. The primary antibody was purchased from Abcam, Cambridge, MA. The ABC reagent and DAB peroxidase were purchased from Thermo Scientific, VWR.
4.2.2 Cell isolation, expansion, and encapsulation

MSCs were isolated, expanded, and encapsulated in 4% type VII agarose hydrogels as described in Section 2.2.2. In order to inhibit Ca\(^{++}\) mobility, constructs were incubated with either 5 \(\mu\)M BAPTA-AM (an intracellular calcium chelator, -Ca\(^{++}\)), 10 \(\mu\)M GdCl\(_3\) (inhibits stretch-activated calcium channels, -SACC), 10 \(\mu\)M verapamil (inhibits voltage-gated calcium channels, -VGCC), 50 nM thapsigargin (depletes sarco/endoplasmic reticulum calcium stores, -SERCS), or without any pharmacological inhibitors as a control (Ctrl). In a second experiment, the role of downstream targets of Ca\(^{++}\) signaling was investigated by incubating constructs with either 5 \(\mu\)M W-7 (inhibits calmodulin, -CaM), 10 \(\mu\)M KN-62 (inhibits calmodulin kinase type II, -CaMKII), or 3.5 \(\mu\)M cyclosporine A (inhibits calcineurin, -Cn). Constructs were allowed to equilibrate overnight before the initiation of hydrostatic pressure.

4.2.3 Application of hydrostatic pressure

Hydrostatic pressure was applied as described in section 2.2.3. The only difference was that a computer controlled Instron 88215 materials testing machine was used in the current experiment.

4.2.4 Biochemical analysis

Constructs were digested as described in section 2.2.4. Sulphated glycosaminoglycan (sGAG) content was quantified using a modified dimethylmethylen blue (DMMB) dye-binding assay with a chondroitin sulphate standard. Briefly, aliquots of the papain digest were mixed with a dye solution consisting of 80 \(\mu\)M DMMB, 1%
ethanol, 40 mM guanidine-HCl, 315 µM formic acid, and 25 µM sodium hydroxide at a pH of 3.5 for 30 minutes and then centrifuged. The supernatant was removed and the remaining pellet was resuspended in a dissociation buffer of 10% isopropanol and 4 M guanidine-HCl. The resultant solution was measured colorimetrically at 600 nm. Media samples were also analyzed using the modified DMBB assay, and subsequently added to that accumulated within constructs to yield the total sGAG produced. Total sGAG values from the HP groups were normalized to the FS groups when applicable. All assays were performed in triplicate.

4.2.5 Confocal microscopy and immunohistochemistry

Constructs were fixed and imaged using confocal microscopy as described in Section 2.2.5; however, in the current study, the samples were imaged using a Nikon A1R confocal microscope at 40x magnification.

A custom code (MATLAB R2013a, The Mathworks, Inc., Natick, MA) was developed in order to analyze the confocal images semi-quantitatively (Fig. 4.2). All cells in the plane of view of at least five representative images were selected in MATLAB and converted to an image of pixel intensity. Then, the intensities of 2 x 2 pixel squares (0.6 x 0.6 µm) were averaged in order to smooth the image. Next, all non-zero intensity values were compiled and the intensity of each square was normalized to the mean intensity of the given image. Finally, the standard deviation of the intensities was found as a measure of the homogeneity of the cytoskeletal architecture. These standard deviations were then averaged across multiple cells from each group (27≤n≤48).
Figure 4.2: A custom MATLAB code converts a raw confocal image of vimentin structure to an RGB image and subsequently averages over a 2x2 grid of pixels to smooth the image. Finally, a distribution of intensities within each cell are plotted and the standard deviation is used as a measure of homogeneity within each cell.

The remaining samples were embedded and analyzed for collagen type II content via immunohistochemistry as described in Section 2.2.5.

4.2.6 Statistical analysis

Statistical analysis was performed using Prism (version 4.03, GraphPad Software, La Jolla, CA). Biochemical results, both numerical and graphical, are expressed in the form of mean ± standard deviation. Differences between HP and FS samples were determined using a Student’s t-test, while differences between groups cultured with or without the various inhibitors tested were determined using a one-way ANOVA with Bonferroni post-test. A level of $p < 0.05$ was considered significant.
4.3 Results

4.3.1 Ca\(^{++}\) mobility is a key regulator of the mechanotransduction of HP

In the first experiment, in which the effect of Ca\(^{++}\) mobility was assessed, it was first determined whether the pharmacological inhibitors had any influence on proliferation or differentiation of MSCs. To do so, DNA and sGAG/DNA levels in the free-swelling Ctrl, -Ca\(^{++}\), -SACC, -VGCC and -SERCS constructs were assessed. None of the inhibitors had a significant effect on either the DNA or sGAG/DNA levels (Fig. 4.3).

![Figure 4.3: A) DNA and B) sGAG/DNA in constructs exposed to various calcium signaling inhibitors. *p≤0.05, relative to control.](image)

In the control specimens, HP significantly enhanced sGAG synthesis by 23% (Fig. 4.4A), and also increased collagen type II immunostaining (Fig. 4.4B). Interestingly, inhibition of Ca\(^{++}\) signaling with an intracellular calcium chelator (-Ca\(^{++}\)) suppressed the beneficial effect of HP on chondrogenesis in sGAG production and collagen type II accumulation (Fig. 4.4A,B). Next, SACCs, VGCCs, and SERCS were suppressed in order to determine how Ca\(^{++}\) signaling was being initiated by HP. Hindering SACC activity did not negate the beneficial effects of HP on sGAG
accumulation or collagen type II deposition; however, inhibition of both VGCCs and SERCS abrogated the mechanoresponse of MSCs to HP (Fig. 4.4A,B).

Figure 4.4: A) Total sGAG/DNA normalized to the FS condition. B) Representative collagen type II immunohistological images. Scale Bars = 50 µm. *p<0.05, relative to FS condition.

4.3.2 Ca$^{++}$ mobility is required for changes in vimentin architecture in response to HP

Focal adhesion formation was previously determined to be necessary for mechanotransduction of HP (Chapter 3). Therefore, focal adhesions were next examined with confocal microscopy in order to determine if inhibition of Ca$^{++}$ mobility was regulating the mechanoresponse of MSCs to HP via alterations in focal adhesion formation. Neither the pharmacological inhibitors nor application of HP affected focal adhesion formation, with all groups demonstrating a punctate structure (Fig. 4.5).
Figure 4.5: Representative confocal images of vinculin, actin, and tubulin staining of constructs in either the HP or FS groups. Scale Bars = 10 µm.

The cytoskeleton has long been implicated in mechanotransduction, yet no changes were observed in actin or tubulin structure with either inhibition of Ca^{++} mobility or application of HP (Fig. 4.5). However, exposure to HP led to a more diffuse vimentin
structure in the mechanosensitive groups (Control and –SACCs), while retaining a punctate structure in the other groups (Fig. 4.6A). Semi-quantitative analysis of vimentin architecture verified this observation, as there was only a significant difference in vimentin architecture with loading in the mechanosensitive groups (Fig. 4.6B).

Figure 4.6: A) Representative confocal images of vimentin in constructs in either the HP or FS groups. B) Semi-quantitative analysis using custom MATLAB code to determine differences in vimentin structures in FS constructs normalized to those exposed to HP.

Scale Bars = 10 µm. *p≤0.05, relative to HP condition.

4.3.3 CaM, CaMKII, and Cn are all key regulators of the mechanotransduction of HP

In the second experiment, DNA and sGAG/DNA values were also assessed in free-swelling constructs exposed to inhibitors of the downstream calcium targets CaM, CaMKII, and Cn. While inhibiting CaMKII led to a significant increase in DNA, none of
the inhibitors had a significant effect on the chondrogenic state of the MSCs (Fig. 4.7). Inhibition of CaM, CaMKII, and Cn all led to a suppression of the beneficial effect of HP on sGAG accumulation (Fig. 4.8A). Similarly to above, collagen type II accumulation was increased in the control group, but inhibition of CaM, CaMKII, and Cn abrogated this response (Fig. 4.8B).

Figure 4.7: A) DNA and B) sGAG/DNA in constructs exposed to various inhibitors of downstream calcium targets. *p≤0.05, relative to control.
4.3.4 CaM, CaMKII, and Cn are required for changes in vimentin architecture in response to HP

No changes in focal adhesion formation or tubulin architecture were observed with application of either HP or downstream calcium target inhibitors, with all groups retaining punctate focal adhesion formations and diffuse tubulin staining. However, all three inhibitors led to a punctate actin structure that was not affected by application of HP (Fig. 4.9). As above, MSCs demonstrated a punctate vimentin structure that became more
diffuse in response to loading in the control group. However, inhibition of CaM, CaMKII and Cn all abrogated the affect of HP on vimentin architecture (Fig. 4.10A). Semi-quantitative analysis of the vimentin structure further verified that inhibition of CaM, CaMKII, and Cn block the change in vimentin structure in response to HP (Fig. 4.10B).
Figure 4.9: Representative confocal images of vinculin, actin, and tubulin staining of constructs in either the HP or FS groups. Scale Bars = 10 µm.
Figure 4.10: A) Representative confocal images of vimentin in constructs in either the HP or FS groups. B) Semi-quantitative analysis using custom MATLAB code to determine differences in vimentin structures in FS constructs normalized to those exposed to HP. Scale Bars = 10 µm. *$p \leq 0.05$, relative to HP condition.

4.4 Discussion

Similar to previous studies examining other loading modalities such as tension, compression, fluid flow, and osmotic pressure, \textsuperscript{62,103–105,109,115,118,124,170,177} Ca++ signaling was found to be necessary for the mechanotransduction of HP by MSCs. This was evidenced by the lack of a response to the mechanical load when intracellular calcium
was chelated (-Ca\textsuperscript{++}). Mechatransduction of HP has been proposed to differ from other mechanical loads due to the fact that HP generates a state of stress with little deformation\textsuperscript{133} and indeed this was observed in the mechanism by which Ca\textsuperscript{++} signaling was initiated. Stretch-activated calcium channels (SACCs) play a key role in the mechanoresponse of MSCs to tension, compression, and fluid flow\textsuperscript{109,115,170,177} yet, in the current study, their inhibition did not suppress the mechatanoresponse of the MSCs to cyclic HP. These results suggest that the mechatransductive pathways utilized by MSCs in response to HP are distinct from those used to sense and respond to other loading modalities.

Although inhibition of SACCs did not alter the mechatanoresponse of MSCs to cyclic HP in the current study, calcium flux through these channels has been observed in response to static HP loading. Mizuno utilized X-rhod-1 AM and a live-cell imaging bioreactor to measure Ca\textsuperscript{++} concentrations in chondrocytes exposed to 0.5 MPa static HP. Blocking SACCs decreased Ca\textsuperscript{++} mobility and inhibition of VGCCs had no effect on Ca\textsuperscript{++} mobility in response to this static load\textsuperscript{108}. In a separate study, cyclic HP had been shown to increase proteoglycan synthesis, while static HP had no effect\textsuperscript{43}. Therefore, the differences between Mizuno’s observations and those of the current study may be explained by a differential response of MSCs to static versus cyclic HP. Similarly, inhibition of SACCs inhibited Ca\textsuperscript{++} mobility and early osteogenic markers in response to static fluid flow\textsuperscript{177–179} however, inhibition of SACCs did not affect Ca\textsuperscript{++} mobility or osteopontin gene expression in response to oscillatory fluid flow\textsuperscript{180}. Together, these data suggest that in some cases, SACCs may be more sensitive to static stimuli than oscillatory ones.
In the current study, inhibition of VGCCs and SERCS both inhibited the chondrogenic response of MSCs to HP. These results suggest that Ca\(^{++}\) could potentially enter through VGCCs and subsequently activate calcium-induced calcium release from SERCS. Both VGCCs and SERCS have been shown to be involved in the mechanotransduction of extrinsic mechanical loads such as fluid flow and compression as well.\(^{62,105,173}\) Therefore, although the mechanotransductive pathways involved in the response to HP are distinct from other loading modalities, there appear to be some overlapping principles. Compression of cartilage in the joint pressurizes the fluid phase of cartilage and generates HP. Therefore, the fact that studies utilizing compression and HP appear to have overlapping mechanotransductive principles could be due to the fact that compressive loading generates a state of HP as well.

Once Ca\(^{++}\) enters the cell, second messengers are commonly utilized to translate the signal into changes in gene expression. Ca\(^{++}\) binds to CaM, inducing a conformational change that allows it to bind to CaMKII and Cn. CaM, CaMKII, and Cn are all proposed to regulate the mechanotransduction of extrinsic loads.\(^{105}\) Compressive loading of bovine articular cartilage explants led to a 2-4 fold increase in aggrecan expression that was abrogated with the additions of inhibitors of CaM, CaMKII, and Cn.\(^{105}\) Similarly, in the current study, CaM, CaMKII, and Cn all suppressed the beneficial effects of HP on the chondrogenesis of MSCs. Together, this indicates that Ca\(^{++}\)/CaM binding, and its subsequent interaction with CaMKII and Cn, is a ubiquitous pathway utilized in response to a variety of mechanical signals.

Cell-matrix interactions regulate chondrogenesis (Chapter 2),\(^{69}\) and previously, we have demonstrated that integrin binding is required for the mechanotransduction of
Integrin binding has been correlated with Ca$$^{++}$$ signaling in response to several mechanical stimuli. Integrins have been found to co-localize with VGCCs, and inhibition of integrin binding has been found to decrease Ca$$^{++}$$ mobility. Vinculin staining was not altered with either the application of HP or pharmacological inhibitors, suggesting that any differences observed in response to loading were not due to alterations in integrin binding or focal adhesion formation following the application of such stimuli. The cytoskeleton has been demonstrated to be involved in the mechanotransduction of many different mechanical stimuli and has also been associated with Ca$$^{++}$$ signaling. None of the inhibitors of Ca$$^{++}$$ mobility effected actin, vimentin, or tubulin organization; however, the inhibitors of CaM, CaMKII, and Cn all led to a more punctate actin structure. While this could potentially alter the mechanoresponse of the MSCs to HP, a previous study indicated that complete disruption of the actin structure did not influence the mechanotransduction of HP. Therefore, it seems unlikely that the alterations in actin structure observed in the presence of inhibitors of CaM, CaMKII, and Cn influenced the chondrogenic response of MSCs to HP.

Previously, we demonstrated a novel role for vimentin in the chondrogenic response of MSCs to HP, as vimentin staining transitioned from a punctate appearance in free-swelling conditions to a more diffuse organization when exposed to HP (Chapter 3). In the current experiment, vimentin organization was punctate in all of the free-swelling conditions, including in the controls and in the specimens exposed to the pharmacological inhibitors. Similarly to the previous experiment, vimentin staining became more diffuse when the MSCs were exposed to HP in the mechanoresponsive
groups (Ctrl and -SACCs). However, vimentin remained punctate with loading in the -Ca++, -VGCCs, -SERCS, -CaM, -CaMKII, and -Cn groups. Overall, this might suggest that Ca++ mobility and associated downstream targets act upstream of changes to vimentin structure. On the other hand, Ca++ signaling may inhibit the chondrogenic mechanoresponse to HP via a different mechanism that in turn affects the vimentin structure. Overall, this suggests that vimentin reorganization is integrally correlated to the chondrogenic response of MSCs to HP, and that this reorganization is calcium-dependent.

While VGCCs were found to be critical for a chondrogenic response to HP, the mechanism triggering these channels is unclear. One possibility is that purinergic signaling is acting to trigger the VGCCs. In the purinergic mechanotransduction pathway, MSCs release ATP in response to mechanical loading, and this extracellular ATP in turn induces P-receptors to increase the intracellular Ca++ concentration via either direct or indirect methods. The increased intracellular Ca++ depolarizes the membrane, leading to activation of VGCCs.° Purinergic signaling has been implicated in the mechanotransduction of fluid flow and compression, yet no studies have investigated its potential role in the mechanotransduction of HP. Alternatively, HP has been shown to inhibit the Na/K pump, Na/K/2Cl pump, and enhance the Na/H pump. By altering the concentrations of these other ions, VGCCs could potentially be activated. Finally, although HP generates a state of little deformation, it has been proposed that ion channels may contain compressible void spaces that, when exposed to HP, may lead to conformational changes in these proteins and subsequently alter ion mobility.
Overall, we have demonstrated that Ca\(^{++}\) signaling plays a critical role in the chondrogenic response of MSCs exposed to HP. The signaling pathway utilized by MSCs involves VGCCs, SERCS, CaM, CaMKII, and Cn. SACCs do not appear to play a role in the mechanotransduction of HP, suggesting that the mechanotransductive pathways utilized in response to cyclic HP are distinct from other loading modalities. Vimentin reorganization in response to HP appears to be Ca\(^{++}\) signaling dependent and possibly plays a key role in the chondrogenic response of MSCs to HP. Finally, further research is needed to fully understand the mechanisms by which HP alters Ca\(^{++}\) mobility.
CHAPTER 5:

PURINERGIC SIGNALING REGulates THE CHONDROGENIC RESPONSE OF MESENCHYmal STEM CELLS TO HYDROSTATIC PRESSURE

5.1 Introduction

As stated in Chapters 1-4, both intrinsic and extrinsic mechanical cues play key roles in the differentiation of mesenchymal stem cells (MSCs). Hydrostatic pressure (HP) specifically regulates the chondrogenic differentiation of MSCs. Exposure to HP increases chondrogenic gene expression and matrix production, and suppresses hypertrophy, yet the underlying mechanisms involved are not fully understood.

Previously, we demonstrated that cell-matrix interactions (Chapter 2), matrix stiffness and/or density (Chapter 3), and calcium ion (Ca\(^{++}\)) mobility (Chapter 4) are crucial for mechanotransduction of HP. However, the mechanisms by which these Ca\(^{++}\) signaling pathways are initiated remains unclear. In the purinergic mechanotransduction pathway, ATP is released from the cell in response to mechanical signals and then acts as a paracrine/autocrine factor to increase intracellular Ca\(^{++}\) concentrations (Fig. 5.1). Compression of articular chondrocytes was found to release ATP into the pericellular environment, and hemichannels have been proposed as the conduit through which ATP exits the cell. Hemichannels are part of gap junctions and are composed of connexin subunits that allow non-selective permeability to molecules smaller than 1
kDa.\textsuperscript{188} Once ATP is released, it binds to purine receptors (P-receptors) which, depending on the specific P-receptor type activated, triggers a Ca\textsuperscript{++} signaling cascade via either direct or indirect methods. P2X receptors act as ATP-gated ion channels, while P2Y receptors are coupled to G-proteins and elicit a Ca\textsuperscript{++} response via IP3 mediated release from the sarcoendoplasmic reticulum calcium stores (SERCS) (Fig. 5.1).\textsuperscript{121} Once Ca\textsuperscript{++} enters the cell, it can initiate a variety of signaling pathways, including an amplification of the Ca\textsuperscript{++} signal through activation of voltage-gated calcium channels (VGCCs) and through Ca\textsuperscript{++} release from intracellular calcium stores.

As described in Chapter 4, both VGCCs and SERCS were found to play a key role in the chondrogenic response of MSCs to HP; however, the mechanism by which these channels are activated is unknown. Therefore, the first objective of this study was to investigate the role of purinergic signaling in the response of MSCs to HP by utilizing pharmacological inhibitors to block various components of the purinergic pathway. Matrix stiffness has previously been demonstrated to play a role in the mechanotransduction of HP via changes in integrin binding and cytoskeletal organization (Chapter 3).\textsuperscript{16} The current study aimed to examine whether matrix stiffness acts upstream or downstream of purinergic signaling by investigating the release of ATP in soft and stiff hydrogels exposed to HP. Finally, focal adhesion formation has been found to be required for the mechanotransduction and exposure to HP was also found to alter vimentin organization (Chapters 3,4). Further, Ca\textsuperscript{++} signaling was found to suppress the effect of HP on vimentin architecture. Therefore, since purinergic signaling is thought to act upstream of Ca\textsuperscript{++} signaling, the current study aimed to investigate whether purinergic signaling altered focal adhesion formation and/or vimentin organization.
Figure 5.1: Diagram displaying the purinergic signaling pathways examined and the inhibitors utilized in the current study.
5.2 Materials and Methods

5.2.1 Materials

All supplies were obtained from the same companies listed in Section 4.2.1. Apyrase, PPADS, flufenamic acid, and the luciferin-luciferase assay kit were all obtained from Sigma-Aldrich, St. Louis, MO.

5.2.2 Cell isolation, expansion, and encapsulation

MSCs were isolated, expanded, and encapsulated in either 1% (soft) or 4% (stiff) type VII agarose hydrogels as described in Section 2.2.2. In order to inhibit purinergic signaling, stiff constructs were incubated with either 10 U/ml apyrase (catalyzes the hydrolysis of extracellular ATP, -ATP), 100 µM PPADS (a P2 receptor antagonist, -PRec), or 100 µM flufenamic acid (blocks hemichannels, -Hemi), or without any pharmacological inhibitors as a control (Ctrl). Constructs were allowed to equilibrate overnight before the initiation of hydrostatic pressure.

5.2.3 Application of hydrostatic pressure

Hydrostatic pressure was applied as described in section 4.2.3.

5.2.4 Biochemical analysis

Constructs were digested as described in section 2.2.4. Sulphated glycosaminoglycan (sGAG) content was quantified using the DMMB assay detailed in section 4.2.4.
5.2.5 ATP release assay

On days 7, 14, and 21, the stiff gels (both the Ctrl and –Hemi groups) and soft gels were loaded for 15 minutes. Media samples were then obtained and immediately boiled for 1 minute to inactivate any ATPases. The samples were then analyzed with a commercially available luciferin-luciferase assay kit and quantified using a luminescence reader. The samples were then loaded for an additional 4 hours and the assay was repeated as described.

5.2.6 Statistical analysis

Statistical analysis was performed using Prism (version 4.03, GraphPad Software, La Jolla, CA). Biochemical results, both numerical and graphical, are expressed in the form of mean ± standard deviation. Differences between HP and FS samples were determined using a Student’s t-test. A level of $p < 0.05$ was considered significant.

5.3 Results

5.3.1 ATP is released by MSCs in response to HP via hemichannels

The first step in determining whether purinergic signaling regulates the mechanotransduction of HP was to assess whether HP increases the amount of ATP released into the media. After 15 minutes of loading, no significant increase in ATP in the media was found. After 4 hours of exposure to HP, extracellular ATP tended to increase in the control hydrogels on days 7 and 14 and increased significantly on day 21; however, flufenamic acid abrogated this response at all three time points (Fig. 5.2).
5.3.2 Purinergic signaling regulates the chondrogenic response of MSCs to HP

Next, in order to determine the effect of purinergic signaling on the response of MSCs to HP, pharmacological inhibitors of various components of the purinergic signaling pathway were utilized. In the control specimens, HP significantly enhanced sGAG synthesis by 15% (Fig. 5.3A). Not only was ATP found to be released from MSCs via hemichannels (Section 5.3.1), but inhibition of hemichannels was also found to suppress the chondrogenic response of MSCs to HP. Next, extracellular ATP was hydrolyzed and P-receptors were blocked to investigate the downstream effects of ATP release on the mechanotransduction of HP. Hindering both ATP and P-receptors abrogated the beneficial effect of HP on sGAG production (Fig. 5.3A). HP also enhanced collagen type II accumulation, but inhibiting hemichannels, extracellular ATP and P-receptors suppressed this response (Fig. 5.3B).
5.3.3 Purinergic signaling is required for changes in vimentin architecture in response to HP

Focal adhesion formation was previously determined to be necessary for mechanotransduction of HP (Chapter 3). Therefore, focal adhesions were next examined with confocal microscopy in order to determine if inhibition of purinergic signaling regulates the chondrogenic response of MSCs to HP via alterations in focal adhesion formation. Neither the pharmacological inhibitors nor application of HP
affected focal adhesion formation, with all groups demonstrating a punctate structure (Fig. 5.4).

Figure 5.4: Representative confocal images of vinculin, actin, and tubulin staining of constructs in either the HP or FS groups. Scale Bars = 10 µm.
Exposure to HP had no effect on actin or tubulin architecture with either inhibition of purinergic signaling or application of HP (Fig. 5.4). However, the application of HP led to a more diffuse vimentin structure in the control group, while retaining a punctate structure in the other groups (Fig. 5.5).

5.3.4 Purinergic signaling acts upstream of matrix stiffness in the mechanotransduction of HP

As stated previously (Chapter 3), matrix stiffness mediates the mechanotransduction of HP; however, whether this differential response is regulated by purinergic signaling had yet to be investigated. Application of HP had no effect on ATP release in either the soft or stiff hydrogels after 15 minutes of loading. However, ATP release was stimulated in both after 4 hours of loading on days 7 and 14, and significantly increased on day 21 (Fig. 5.6A). While ATP release was upregulated in both groups, sGAG production was only enhanced in the stiff hydrogels in response to HP (Fig. 5.6B).
Figure 5.6: A) ATP released by MSCs cultured in either soft or stiff hydrogels after 4 hours of exposure to HP on days 7, 14, and 21. B) Total sGAG/DNA of constructs cultured with various pharmacological inhibitors or in control conditions. *p<0.05, relative to FS condition.

5.4 Discussion

Previously, Ca$^{++}$ signaling was found to play a key role in the mechanotransduction of HP (Chapter 4); however, the underlying mechanisms initiating the Ca$^{++}$ signaling pathway is unclear. Purinergic signaling is activated in response to other mechanical loads such as compression and fluid flow and initiates Ca$^{++}$ signaling.$^{119,124,130,131}$ Similarly, HP was found to increase extracellular ATP via hemichannels in the current study. Further, the application of HP enhanced the chondrogenesis of MSCs, while inhibition of hemichannels, extracellular ATP, and P-receptors decreased the beneficial response to HP. Together, this data suggests that purinergic signaling regulates the chondrogenic response of MSCs to HP.

Matrix stiffness also regulates the mechanotransduction of HP (Chapter 3); however, it was unclear whether purinergic signaling acts upstream or downstream of the effects of matrix stiffness. HP was found to release ATP from MSCs embedded in both soft and stiff hydrogels, yet chondrogenesis was only enhanced by HP in the stiff gels. Therefore, purinergic signaling appears to act upstream of the effects of matrix stiffness on the mechanotransduction of HP; however, the specific pathway regulated by matrix
stiffness that alters the effects of purinergic signaling is unclear. P-receptors have been found to contain arginine-glycine-aspartic acid (RGD) sequences that interact with integrins. Inhibition of this linkage was found to decrease the signaling capabilities of both the P-receptors and focal adhesions. Altogether, matrix stiffness is known to modulate integrin binding (Chapter 3), which in turn is known to influence P-receptor sensitivity. Therefore, although ATP was released in the soft hydrogels in response to HP, the P-receptors may not be “primed” to initiate a signaling cascade in the soft pericellular environment.

Previously, we demonstrated a novel role for vimentin in the chondrogenic response of MSCs to HP, as vimentin staining transitioned from a punctate appearance in free-swelling conditions to a more diffuse organization when exposed to HP (Chapter 3). Vimentin reorganization in response to HP was also found to be dependent on Ca++ signaling. Since purinergic signaling is proposed to act upstream of Ca++ signaling, vimentin organization was investigated in the presence of inhibitors of purinergic signaling. Vimentin organization was punctate in all of the free-swelling conditions, including in the controls and in the specimens exposed to the pharmacological inhibitors. Similarly to the previous experiments, vimentin staining became more diffuse when the MSCs were exposed to HP in the control group. However, vimentin remained punctate with loading in the presence of all inhibitors of purinergic signaling utilized in this study, further suggesting that purinergic signaling acts upstream of matrix stiffness and Ca++ signaling. Overall, this suggests that vimentin reorganization is integrally correlated to the chondrogenic response of MSCs to HP, and that this reorganization is dependent on purinergic signaling.
Understanding the mechanotransductive pathways involved in the chondrogenic response of MSCs to HP could have important implications in tissue engineering and regenerative medicine. While mechanical stimulation with HP clearly increases the chondrogenesis of MSCs, long-term mechanical loading is very costly. Since purinergic signaling appears to act upstream of both Ca$$^{++}$$ signaling and matrix stiffness in the chondrogenic response of MSCs to HP, stimulating purinergic signaling, via pharmaceuticals or supplementation with exogenous ATP, could simulate the beneficial effect of HP on chondrogenesis in the stiff hydrogels. Addition of exogenous ATP to MSCs seeded in agarose hydrogels was found to increase the mechanical properties of the constructs.\textsuperscript{129} P-receptors are potential pharmaceutical targets of interest as well, as an increase in P-receptor quantity or sensitivity could potentially enhance the chondrogenic response.

Overall, purinergic signaling could potentially be the primary pathway by which the mechanotransduction of HP is initiated, as it appears that ATP is released via hemicannels and subsequently activates P-receptors. The activated P-receptors then allow an influx of Ca$$^{++}$$ which depolarizes the membrane and activates VGCCs. Once activated, the VGCCs increase intracellular Ca$$^{++}$$ concentration directly and also indirectly via calcium-induced calcium release from SERCS. The increased intracellular Ca$$^{++}$$ concentration could then initiate further Ca$$^{++}$$ signaling cascades, eventually altering gene expression and the differentiation of MSCs. Matrix stiffness also regulates this pathway, possibly by altering P-receptor sensitivity. Finally, a deeper understanding of the mechanotransductive pathways used by MSCs in response to HP may elucidate
pharmaceutical targets to reap the benefits of HP without the costly application of mechanical loading.
CHAPTER 6: CONCLUSIONS

Together, the studies presented in this dissertation aimed to elucidate the complex interplay between several signaling pathways involved in the chondrogenic response of MSCs to HP. In Chapter 2, cell-matrix interactions were found to regulate the differentiation of MSCs, as MSCs in fibrin hydrogels were less chondrogenic yet elicited a stronger response to HP than those encapsulated in agarose. In Chapter 3, matrix stiffness and/or density alone was found to regulate the lineage commitment of MSCs. Similarly to the first study, although chondrogenic matrix synthesis was highest in the soft scaffolds, the chondrogenic response of MSCs to HP was only enhanced in the stiff scaffolds. Inhibition of integrin binding abrogated the beneficial effects of HP on chondrogenesis. Vimentin was also found to reorganize in response to HP in the stiff hydrogels. Together, these studies uncovered a complex interplay between MSCs’ response to both intrinsic and extrinsic mechanical signals via integrin binding and vimentin remodeling. As detailed in Chapter 4, Ca\(^{++}\) mobility (via VGCCs and SERCS) and signaling (via CaM, CaMKII, and Cn) were found to be necessary for MSCs to respond to HP. Further, inhibition of Ca\(^{++}\) signaling suppressed the vimentin remodeling seen previously, implicating a link between Ca\(^{++}\) signaling and the mechanotransduction pathways examined in Chapters 2 and 3. Purinergic signaling has been proposed to initiate Ca\(^{++}\) signaling, but the role of purinergic signaling in the mechanotransduction of...
HP had yet to be investigated. As detailed in Chapter 5, HP enhanced chondrogenesis in stiff gels only, but ATP release was found to increase with loading regardless of matrix stiffness, indicating that purinergic signaling acts upstream of matrix stiffness. Inhibition of the purinergic signaling pathway also suppressed the beneficial effect of HP on chondrogenesis, suggesting a key role for purinergic signaling in the mechanotransduction of HP. Together, the results found in Chapters 4 and 5 suggest that purinergic signaling initiates Ca\(^{++}\) mobility, signaling, and subsequent changes in MSC differentiation (Fig. 6.1).
Figure 6.1: Diagram displaying the purinergic and calcium signaling pathways proposed to act in the mechanotransduction of HP. 1) HP initiates release of ATP via hemichannels. 2) ATP activates P-receptors to allow Ca\(^{++}\) to enter the cell directly or via IP3-mediated release from SERCS. 3) The increase in intracellular Ca\(^{++}\) concentration triggers VGCCs which in turn 4) induce calcium-induced calcium release from SERCS. 5) Ca\(^{++}\) then binds to the downstream targets CaM, CaMKII, and Cn, which are known to trigger a variety of signaling cascades that 6) alter gene expression and differentiation.

Overall, there appears to be a complex interplay between HP, integrin binding, cytoskeletal remodeling, Ca\(^{++}\) signaling, and purinergic signaling (Fig. 6.2). The results in this dissertation determined which components are involved and began to explain their interactions, but further research is needed. First, although differences in cytoskeletal organization were found to influence the response of HP (Chapter 2) and HP has been found to alter vimentin architecture (Chapter 3), whether the cytoskeleton is required for mechanotransduction of HP to occur is still unclear. Further, the effect of vimentin...
inhibition on integrin binding and Ca++ signaling is currently unknown. Second, although Ca++ signaling was found to play a key role in the mechanotransduction of HP (Chapter 4), the roles of cytoskeletal organization and integrin binding in Ca++ signaling are unclear. Future work in this field should aim to elucidate the effects of inhibiting cytoskeletal organization via pharmacological inhibitors and shRNA knockdown on chondrogenesis, Ca++ signaling, and integrin binding. Further, a live-cell imaging pressure vessel has been developed that will allow real-time monitoring of Ca++ signaling which will aid in determining the interplay between the various mechanotransductive pathways utilized by MSCs. Lastly, the purinergic pathway was found to play an important role in the mechanotransduction of HP (Chapter 5), and future research should investigate if biochemical activation of the purinergic pathway mimics the application of HP. If so, biochemical activation of purinergic signaling could serve as a method for improving current cartilage tissue engineering strategies and act as a less labor intensive model for mechanobiological research.
Figure 6.2: Diagram displaying the known and proposed interactions between HP induced chondrogenesis, integrin/focal adhesion formation, cytoskeletal organization, Ca\(^{++}\) mobility and signaling, and purinergic signaling. Solid arrows indicate relationships that have been investigated, while dashed arrows indicate relationships that require further research to fully understand.
APPENDIX A:

EXPLORING THE ROLES OF INTEGRIN BINDING AND CYTOSKELETAL REORGANIZATION DURING MESENCHYMAL STEM CELL MECHANOTRANSDUCTION IN SOFT AND STIFF HYDROGELS SUBJECTED TO DYNAMIC COMPRESSION

A.1 Introduction

Both extrinsic and intrinsic mechanical signals (i.e. those generated outside the cell and transmitted inwards via the pericellular matrix (PCM) and those generated within the cell in response to its substrate) have been well characterized as key regulators of mesenchymal stem/stromal cell (MSC) differentiation.\(^1^{4-18,69,190}\) Dynamic compression (DC) has been shown to enhance chondrogenesis of MSCs depending on the timing and duration of its application;\(^1^{43,191-196}\) however, the underlying mechanisms responsible for this temporal mechanosensitivity are not well understood. The application of DC from the onset of TGF-β3 stimulation has been shown to suppress markers of chondrogenesis, myogenesis and endochondral ossification; however, the application of this mechanical cue following 3 weeks of TGF-β3 induced differentiation has been shown to promote chondrogenesis and suppress markers of both myogenic differentiation\(^1^{7}\) and progression along the endochondral pathway.\(^1^{97}\) Both adaption of a chondrogenic phenotype (by long term exposure to chondrogenic growth factors) and development of a mature PCM have been proposed as possible mechanisms to explain the observed temporal
mechanosensitivity of MSCs.\textsuperscript{192} Chondrocytes seeded in agarose hydrogels synthesize a more mature cartilaginous matrix at later time points than at earlier ones, and this more developed matrix may be necessary for the mechanotransduction of DC.\textsuperscript{198} Furthermore, blocking integrin mediated binding to the PCM has been shown to alter chondrocyte response to DC.\textsuperscript{199} A similar temporal development of the PCM has been observed for MSCs embedded into agarose hydrogels,\textsuperscript{192} however it is still unclear whether this is merely correlating with a more anabolic response to DC or if it is indeed causative.

As the PCM develops not only does its biochemical composition change but it also stiffens.\textsuperscript{200} The intrinsic stiffness of the local matrix is known to modulate MSC differentiation in both 2D\textsuperscript{18,21} and 3D.\textsuperscript{22–24,201} Recently, studies have begun investigating the interplay between extrinsic and intrinsic mechanical cues and their subsequent effects on cellular differentiation. Denser and stiffer hydrogels were found to alter PCM organization, cytoskeletal organization, and integrin binding relative to less dense and softer hydrogels (Chapter 3).\textsuperscript{16} These changes led to a more robust response to the application of extrinsic mechanical signals in the stiffer hydrogels, demonstrating the importance of the PCM for determining the response of MSCs to such cues (Chapter 3).\textsuperscript{16} These changes to the PCM occurred in parallel with changes to the internal cytoskeleton, raising the possibility that the temporal response of MSCs to extrinsic mechanical cues could be related to changes in either the PCM or to the cytoskeleton during chondrogenesis.

The objective of this study was to first characterize changes in both the pericellular environment and cytoskeletal organization during TGF-\(\beta\)\textsubscript{3} mediated chondrogenesis of MSCs in hydrogel culture, with the aim of potentially correlating such
alterations with the temporal response of the cells to DC. Since both the pericellular environment and cytoskeleton may adapt as differentiation proceeds, decoupling the relative importance of such changes in determining the temporal response of MSCs to DC from other phenotypic changes that may occur in response to increasing durations of exposure to TGF-β3 becomes difficult. Therefore we next aimed to determine how altering the pericellular environment of MSCs, by changing the stiffness/concentration of the hydrogel within which they were encapsulated, would impact their response to DC. Furthermore, we explored how blocking integrin mediated binding of MSCs to their local pericellular environment would impact their response to DC. The final aim of the study was to elucidate whether cytoskeletal adaptation (i.e. changes in actin, intermediate filaments and microtubules post DC) is a feature of the response of MSCs to extrinsic mechanical loading.

A.2 Materials and Methods

A.2.1 Materials

All materials were acquired from the same companies as in Section 2.2.1 unless otherwise noted.

A.2.2 Cell isolation, expansion, and encapsulation

Cell isolation, expansion and encapsulation methods were performed as described in Section 3.2.2. The only exception is that in the current experiment, only 1% and 4% agarose hydrogels were utilized.
A.2.3 Application of dynamic compression

Constructs were placed in a custom pneumatic based compressive loading bioreactor\textsuperscript{143} inside an incubator (37°C, 5% CO\textsubscript{2}) and exposed to cyclic dynamic compression (DC) on days 7, 14, or 21 only. Constructs were not loaded on any other day. 1% constructs were only loaded on day 7. DC was applied with ~10% strain superimposed on a 0.01 N preload at a frequency of 1 Hz for 1 hour only. Free swelling (FS) controls were kept in the incubator adjacent to the bioreactor. Half-medium exchanges were performed every 3-4 days.

A.2.4 Confocal microscopy and histology

Confocal microscopy and histology were performed as in Section 3.2.5.

A.2.5 RNA isolation and quantitative real-time polymerase chain reaction

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed as described in Section 3.2.6. However, in addition to the genes examined in Chapter 3, transforming growth factor beta receptor 1 (TGFBR1, Ss03392141\_m1), transforming growth factor beta 3 (TGB3, Ss03394351\_m1), bone morphogenetic protein 2 (BMP2, Ss03373798\_g1), and bone morphogenetic protein 7 (BMP7, Ss03389523\_m1) were also used in this study.

A.2.6 Statistical Analysis

Statistical analysis was performed using GraphPad Prism (version 5.00, GraphPad Software) with a minimum of 3 samples in triplicate analyzed for each experimental
group for each assay. qRT-PCR results, both numerical and graphical, are expressed in the form of mean ± standard deviation. Differences between DC and FS samples or between samples cultured with or without RGDS were determined using a Student’s t-test. A level of $p < 0.05$ was considered significant.

A.3 Results

A.3.1 MSCs display a temporal response to dynamic compression despite minimal changes to their gross actin, vimentin and tubulin networks during chondrogenesis in 3D hydrogels

MSCs embedded into stiff 3% agarose hydrogels were subjected to 1 hour (total duration) of dynamic compression (DC) following either 7, 14 or 21 days of free swelling (FS) culture in chondrogenic media. Similar to previous results,\textsuperscript{192} MSCs displayed a temporal gene expression pattern in response to DC (Fig. A.1A), with a more anabolic response observed on day 21 compared to day 14. Sox9 expression was significantly increased by DC (relative to FS) on day 7 (1.63 ± 0.40), decreased on day 14 (0.21 ± 0.59), with no significant change on day 21 (0.74 ± 1.33). DC had no significant effect on Agc expression on day 7 (1.27 ± 0.37), but significantly enhanced Agc gene expression on days 14 and 21 (2.39 ± 1.03 and 1.85 ± 0.39, respectively). DC significantly increased Col2A1 expression on days 7 and 21 (6.58 ± 1.36 and 2.97 ± 0.86, respectively), while it significantly decreased its expression on day 14 (0.35 ± 0.51). Next, the PCM and cytoskeleton were analyzed in order to determine if temporal changes in their development and organization correlated with the observed temporal effects of DC on
MSC chondrogenic gene expression. The PCM of MSCs stained intensely for Alcian blue and picro-sirius red, but no dramatic changes were observed with time in culture (Fig. A.1B), suggesting the PCM develops rapidly in the stiff/dense 3% agarose hydrogels. All of the cytoskeletal elements (actin, vimentin, and tubulin) generally exhibited a diffuse staining within spherical cells, with minimal changes observed in the gross appearance of these elements with time in culture (Fig. A.1C). Vinculin (a membrane-cytoskeletal protein in focal adhesions) staining appeared more intense by day 21 (Fig. A.1C).
Figure A.1: (A) Sox9, Agc, and Col2A1 gene expression in stiff hydrogels normalized to FS condition. (B) Representative Alcian blue and picro-sirius red images, and (C) representative vinculin, actin, vimentin and tubulin confocal images of stiff hydrogels on days 7, 14, and 21 (Scale bar = 50 µm; 12.5 µm for inset images). *p < 0.05 relative to FS.
A.3.2 A well developed PCM appears important for dynamic compression to promote chondrogenesis of MSCs

Given that relatively minor changes in both the gross cytoskeleton and PCM were observed with time in culture, we next explored how modulating the development of the PCM (by reducing the agarose hydrogel concentration from 3% to 1%) would impact the response of MSCs to DC. The total duration of exposure to TGF-β3 was kept constant (7 days) in order to minimize other changes in phenotype that will occur within the hydrogels with increasing duration of exposure to this growth factor. The application of DC on day 7 elicited a significantly greater relative increase in Sox9 (soft: 0.70 ± 0.15, stiff: 1.63 ± 0.40), Agc (soft: 0.47 ± 0.28, stiff: 1.26 ± 0.37) and Col2A1 (soft: 1.77 ± 0.13, stiff: 6.58 ± 1.36) gene expression in MSCs embedded in stiff hydrogels compared to soft hydrogels (Fig. A.2A). The PCM was less developed in the soft 1% agarose hydrogels compared to the stiffer 3% gels (Fig. A.2B), implicating a well developed PCM in the mechanotransduction of DC into an anabolic change in chondrogenic gene expression. Again, all cytoskeletal elements exhibited a generally diffuse staining, with little differences observed in the gross cytoskeletal structure between the soft and stiff hydrogels (Fig. A.2C).
A.3.3 Integrin mediated binding by MSCs to their PCM is involved in mechanotransduction of dynamic compression

Next, to determine if integrin-mediated binding of MSCs to their local PCM was necessary for mechanotransduction of DC, integrin binding was inhibited in the stiffer 3% gels, where an anabolic response to DC was observed, with addition of the soluble
RGDS peptide for the first 7 days of culture. The significant increase in Sox9 and Col2A1 gene expression in response to DC was not observed with the addition of RGDS (Sox9: $1.63 \pm 0.40, 0.56 \pm 0.20$; Col2A1: $6.58 \pm 1.36, 1.49 \pm 0.11$). The addition of RGDS had no significant effect on Agc expression after application of DC (Fig. A.3A). No differences in PCM development or gross cytoskeletal organization were observed with the addition of RGDS (Fig. A.3B,C).
Figure A.3: (A) Sox9, Agc, and Col2A1 gene expression in stiff hydrogels on day 7, with or without the RGDS peptide, normalized to FS condition. (B) Representative Alcian blue and picro-sirius red images, and (C) representative vinculin, actin, vimentin and tubulin confocal images of stiff hydrogels on day 7, with or without the RGDS peptide (Scale bar = 50 µm; 12.5 µm for inset images). *p < 0.05 relative to groups cultured with RGDS peptide.

A.3.4 Tubulin rapidly reorganizes within the cytoskeleton of MSCs in response to DC

Although there were no temporal changes observed in cytoskeletal structure, we next examined if either the actin, vimentin or tubulin networks in MSCs were altered by the application of DC in the stiff 3% hydrogels, where an anabolic response to loading
was observed. DC induced no observable changes in gross actin or vimentin structure in MSCs seeded in stiff 3% hydrogels (data not shown). However, tubulin displayed an altered ring morphology after the application of DC, appearing to localize around the cell membrane and nucleus following loading (Fig. A.4). Interestingly, this effect was not negated by the addition of RGDS, as microtubule organization still adapted to DC even in the presence of the RGDS peptide (Fig. A.4).

Figure A.4: Representative tubulin confocal images on days 7, 14, and 21 in either the FS, DC, or DC with RGDS conditions (Scale bar = 50 µm; 12.5 µm for inset images).
A.4 Discussion

Numerous studies have demonstrated that MSCs display a temporal response to the application of dynamic compression. A similar result was observed in the present study. The cytoskeleton has often been proposed to play a key role in mechanotransduction and MSC differentiation. Therefore we initially hypothesized that cytoskeletal development may explain the temporal gene expression patterns observed in MSCs in response to DC. However, no dramatic differences were observed in the gross cytoskeletal organization over time or in how the cytoskeleton adapted to loading over time, suggesting that changes to the cytoskeleton (that can be detected using the experimental approach adopted in this study) during chondrogenesis are not responsible for the temporal gene expression profile observed in MSCs in response to DC.

Next, the possible role of PCM development in determining the response of MSCs to DC was examined. No dramatic differences in PCM maturation were observed between day 7 and day 21 of culture in the stiff/dense 3% agarose hydrogels (where the PCM develops relatively quickly), initially suggesting that PCM maturation does not play a role in determining the temporal response of MSCs to loading. Previous work in our lab has shown that the PCM develops differently in agarose gels of different concentrations, with the PCM becoming more developed and compact in stiffer hydrogels (Chapter 3). Therefore, to further probe the role of the pericellular environment in MSC mechanotransduction, ‘soft’ and ‘stiff’ MSC seeded agarose gels were subjected to DC after 7 days in chondrogenic media. A less well developed PCM was observed in the softer 1% gels, which correlated with a less anabolic response to DC than that observed
in the stiffer 3% gels. It is well established that the PCM plays an important role in chondrocyte biomechanics and mechanotransduction. Finite element models have been used to demonstrate that the presence of a PCM dramatically alters the mechanical environment of cells that are embedded in hydrogels subjected to DC, decreasing the deformation and deviatoric strains experienced by the cell but increasing the volumetric strain, which may be important for appropriate mechanotransduction. Taken together, these findings suggest that a well developed PCM is important to ensure an anabolic response to DC, although changes to the PCM may not completely explain the temporal gene expression patterns observed in response to extrinsic loading in this and other studies.

While it is well established that both intrinsic and extrinsic mechanical signals drive differentiation of MSCs, the mechanotransductive pathways with which MSCs respond to these signals are not fully understood. Integrin binding to the extracellular matrix (ECM) and cytoskeletal organization have long been implicated in many mechanotransductive pathways. The integrin-cytoskeletal linkage becomes stronger with increasing matrix stiffness, suggesting a potential downstream role for cytoskeletal organization in mechanotransduction. Given that PCM maturation was critical for mechanotransduction of DC, and that previous work has demonstrated that integrin binding to the PCM was needed for mechanotransduction of cyclic hydrostatic pressure to occur (Chapter 3), we next explored whether integrin binding was also necessary for proper mechanotransduction of DC by abrogating integrin mediated binding with a soluble RGDS peptide. Similar to MSC response to hydrostatic pressure in the presence of RGDS, no significant increase in Sox9 and Col2A1 gene expression in
response to DC was observed in the presence of RGDS. This suggests that integrin mediated binding to the PCM plays a key role in the mechanotransduction of DC into a pro-chondrogenic signal to the cell.

Previously, the cytoskeleton was found to remodel in MSCs exposed to hydrostatic pressure, and this remodeling was inhibited in the presence of RGDS (Chapter 3). In the current study, it was found that the tubulin network in MSCs remodeled in response to DC. Microtubules have previously been demonstrated to play a role in resisting cell deformation, and together with this study, suggests that microtubule reorganization plays a key role in the response and adaption of chondrogenically primed MSCs to DC. Interestingly, the addition of RGDS did not inhibit tubulin reorganization, suggesting that integrin mediated binding to the PCM is not involved in tubulin reorganization in response to DC. Since microtubules appear to adapt in both the presence and absence of RGDS, but that DC only enhanced chondrogenic gene expression in the absence of RGDS, tubulin reorganization in of itself may not be involved in the mechanotransduction of DC into an anabolic change in chondrogenic gene expression.

In conclusion, MSCs embedded in agarose hydrogels were found to display a temporal chondrogenic gene expression pattern in response to DC. Neither changes in gross cytoskeletal organization nor PCM development could explain these temporal gene expression patterns, although it is acknowledged that certain changes may be occurring that are not detectable using the experimental methods adopted in this study. We did however observe greater increases in the relative expression of chondrogenic marker genes in response to DC in the presence of a well developed PCM. Integrin-mediated
binding to the PCM was also observed to play a key role in mechanotransduction of DC. Microtubules were found to reorganize in MSCs exposed to DC, demonstrating that the microtubule network in chondrogenically primed MSCs is mechanosensitive; however, this network does not appear to be involved in integrin binding-mediated mechanotransduction of DC into increases in Sox9 and Col2A1 gene expression as microtubule reorganization in response to DC was not inhibited by abrogating integrin binding. Therefore, although the PCM, integrin binding, and cytoskeletal organization are all involved in MSC mechanotransduction, none of these factors in isolation can provide a clear explanation for the temporal response of MSCs to DC.


12. Bosetti, M., Boccafoschi, F., Leigheb, M., Bianchi, A. E. & Cannas, M. Chondrogenic induction of human mesenchymal stem cells using combined growth


121. Pingguan-Murphy, B. & Knight, M. M. in *Mechanosensitive Ion Channels* (Kamkin, A. & Kiseleva, I.) **1**, 235–251 (Springer Netherlands, 2008).


