Deciphering the Role of Caveolae in Human Meniscus Fibrochondrocytes

by

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Abstract

Menisci are fibrocartilaginous structures of the knee that function in mechanical load transmission to protect articular cartilage from forces that may initiate osteoarthritic changes. A population of cells responsible for regulating the tissues' biomechanical properties are meniscus fibrochondrocytes (MFC). MFC tailor the production of extracellular matrix (ECM) in response to mechanical stimuli and thereby regulate the functionality of the meniscus. The mechanism by which MFC sense and respond to mechanical stimuli, a process known as mechanotransduction, has yet to be determined. Caveolae are structures of the plasma membrane predominately constructed of caveolin proteins, that have been implicated in mechanotransduction in cells frequently exposed to mechanical stress. We recently identified caveolae in engineered tissues from human MFC and set out to investigate their role in MFC.

First, we confirmed the expression of caveolin proteins in human MFC at both the gene and protein level. Next, we mechanically stimulated and transcriptionally probed MFC in engineered human meniscus tissues and evaluated caveolae-related gene expression. Our data showed that caveolae respond to mechanical stimulation and perhaps are involved in mechanotransduction through Fyn-mediated signal transduction. Future studies are aimed at confirming the importance of caveolae in MFC through inhibition or suppression.

Preface

This thesis is an original work by Margaret Jenny Vyhlidal.

The literature review (Chapter 1.1-1.8) and the caveolin gene and protein expression results (Chapter 3.1) have been reproduced from the following publication: M. J. Vyhlidal and A. B. Adesida, "Mechanotransduction in meniscus fibrochondrocytes: What about caveolae?," *Journal of Cellular Physiology*, vol. 237, no. 2, pp. 1171-1181, 2022. M. J. Vyhlidal was responsible for conducting the literature review and immunofluorescence. A. Mulet-Sierra and M. Kunze assisted with *in vitro* cell culture of human MFC and RT-qPCR analysis. W. J. Cho acquired the transmission electron microscopy (TEM) image. A. B. Adesida was responsible for the supervision of the study and assisted with concept formation and statistical analysis.

Parts of the methods (Chapter 2), results (Chapter 3.2-3.4), discussion (Chapter 4.1), and conclusion (Chapter 5.1) assessing the effects of mechanical stimulation on caveolae-related gene expression, as well as the ideas proposed in the future directions (Chapter 5.2), are reproduced from the following study submitted for publication: M. J. Vyhlidal, Z. Ma, D. X. Li, M. Kunze, and A. B. Adesida, *Transcriptomics Evidence for the Role of Caveolae in Human Meniscus Fibrochondrocyte Mechanotransduction*, 2022. M. J. Vyhlidal was responsible for the data analysis, table and figure preparation, and manuscript composition. Z. Ma and D. X. Li were responsible for the study design, performed *in vitro* cell culture of human MFC, RT-qPCR analysis, RNA-sequencing data analysis with Partek Flow

bioinformatics software, statistical analysis, and contributed to table and figure preparation and manuscript edits. M. Kunze was responsible for RT-qPCR. A. B. Adesida was responsible for the supervision of the study and contributed to the study design and manuscript edits.

The use of human specimens outlined in Chapter 2 received research ethics approval from the University of Alberta's Health Research Ethics Board, Biomedical Panel, study ID: Pro00018778.

I dedicate this thesis to my family and friends.

For always believing in me and making me who I am today.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

3D = Three-dimensional

α = Alpha

ACTB = β-actin

ADAMTS = A disintegrin and metalloproteinase with thrombospondin motifs

AF = Annulus fibrosus

AFSC = Annulus fibrosus stem cell

Akt = Protein kinase B

ANOVA = Analysis of variance

AZD0530 = Saracatinib

β = Beta

B2M = Beta-2-microglobin

BSA = Bovine serum albumin

CAV = Caveolin

cDNA = Complementary deoxyribonucleic acid

CHP = Cyclic hydrostatic pressure

COL = Collagen

COX = Cyclooxygenase

CSD = Caveolin scaffolding domain

CTS = Cyclic tensile strain

DAPI = 4′,6-diamidino-2-phenylindole

DMEM = Dulbecco's modified Eagle's medium-high glucose

ECM = Extracellular matrix

EGFR = Epidermal growth factor receptor

EHD2 = EH domain containing 2

ERK = Extracellular signal-related kinase

FA = Focal adhesion

FAK = Focal adhesion kinase

FBS = Fetal bovine serum

FGF = Fibroblast growth factor

FYN = FYN proto-oncogene, Src family tyrosine kinase

GAG = Glycosaminoglycan

GSL = Glycosphingolipid

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Hh = Hedgehog

IGF = Insulin-like growth factor

IGF-R = Insulin-like growth factor receptor

IL = Interleukin

ITS = Insulin-transferrin-selenium

IVD = Intervertebral disc

KAC = Knee articular chondrocytes

KOA = Knee osteoarthritis

MAPK = Mitogen-activated protein kinase

MβCD = Methyl-β-cyclodextrin

MEK = Mitogen-activated protein kinase kinase

MFC = Meniscus fibrochondrocytes

MMP = Matrix metalloproteinase

NP = Nucleus pulposus

PBS = Phosphate buffered saline

PCA = Principal component analysis

PCM = Pericellular matrix

PI3-K = Phosphoinositide 3-kinase

PP1 = 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine

PSG = Penicillin-streptomycin-glutamine

RNA = Ribonucleic acid

ROCK = Rho-associated protein kinase

RT-qPCR = Real-time quantitative polymerase chain reaction

shRNA = Short hairpin RNA

siRNA = Small interfering RNAs

SMG = Simulated microgravity

TAZ = Transcriptional coactivator with PDZ-binding motif

TEM = Transmission electron microscopy

TGF-β = Transforming growth factor beta

TIMP = Tissue inhibitor of metalloproteinases

TLR = Toll-like receptor

TNF-α = Tumor necrosis factor alpha

TRPV = Transient receptor potential cation channel

uPAR = Urokinase-type plasminogen activator receptor

VEGF = Vascular endothelial growth factor

VEGFR = Vascular endothelial growth factor receptor

YAP = Yes associated protein 1

YWHAZ = Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta

Chapter 1

Introduction

Sections 1.1-1.8 of this chapter has been previously published in part as[1]:

M. J. Vyhlidal and A. B. Adesida, "Mechanotransduction in meniscus fibrochondrocytes: What about caveolae?," *Journal of Cellular Physiology*, vol. 237, no. 2, pp. 1171-1181, 2022 [1]. Section 1.1 has been modified from the original to include Figure 1.1.

1.1 Thesis Overview

Menisci are a pair of wedge-shaped semi-lunar fibrocartilaginous structures in the knee (Figure 1.1) that play a significant role in joint homeostasis [2], [3]. Meniscal functions including joint stabilization, compressive load transmission, joint nutrition and lubrication, and proprioception, are highly dependent on the macroscopic and microscopic anatomy of menisci [2]–[4]. The cells that are responsible for regulating these biomechanical properties are known as meniscus fibrochondrocytes (MFC) [2]. MFC function by tailoring the synthesis and assembly of the extracellular matrix (ECM) proteins in response to changes in

[1] This is the peer reviewed version of the following article: M. J. Vyhlidal and A. B. Adesida, "Mechanotransduction in meniscus fibrochondrocytes: What about caveolae?," *Journal of Cellular Physiology*, vol. 237, no. 2, pp. 1171-1181, 2022, which has been published in final form at https://doi.org/10.1002/jcp.30616. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions. This article may not be enhanced, enriched or otherwise transformed into a derivative work, without express permission from Wiley or by statutory rights under applicable legislation. Copyright notices must not be removed, obscured or modified. The article must be linked to Wiley's version of record on Wiley Online Library and any embedding, framing or otherwise making available the article or pages thereof by third parties from platforms, services and websites other than Wiley Online Library must be prohibited.

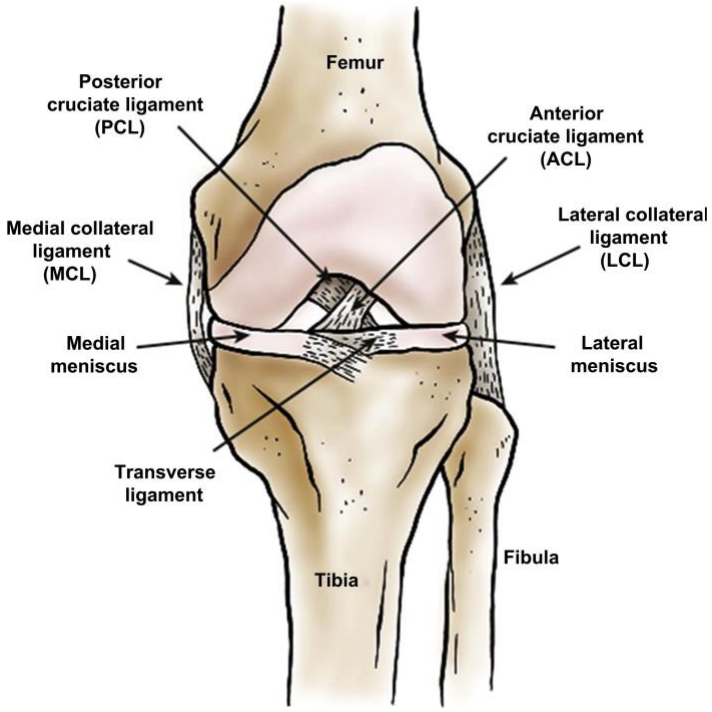

Figure 1.1 Anatomy of the human knee joint. Reproduced from Makris et al. (2011) [4][2].

the mechanical loading regimen of the joint [4]. Due to the heterogeneity and complexity of mechanical stimuli in the knee [5], MFC must, therefore, have a mechanism in place that is able to discern and interpret these environmental cues to initiate proper downstream signaling pathways to tailor ECM gene expression. This process known as mechanotransduction, has not been well characterized in MFC. To fully understand and appreciate the biomechanics of the meniscus, the underlying cellular process of MFC mechanotransduction needs to be further investigated. If this mechanism were to be identified, this information could lead to a breakthrough discovery that would have important implications for meniscus research including studies on knee osteoarthritis (KOA).

[2] Reprinted from Elsevier Biomaterials, 32(30), Eleftherios A. Makris, Pasha Hadidi, and Kyriacos A. Athanasiou, The knee meniscus: Structure—function, pathophysiology, current repair techniques, and prospects for regeneration, Page No. 7412, Copyright (2011), with permission from Elsevier.

KOA is a degenerative disease of the knee joint that poses a huge annual socioeconomic burden [6] and is characterized by progressive pain, joint immobility, and a reduced quality of life [7]. It is well established that biomechanical dysfunctions of the knee joint are contributing factors to the initiation and progression of the disease [8]. Several groups have identified a relationship between meniscal degeneration and the progression of KOA. Seitz et al. (2021) recently demonstrated that the biomechanical alterations and degeneration associated with KOA were first detectable in menisci and occurred before degeneration of the articular cartilage. Together, these findings suggest that the mechanobiology of the meniscus plays a key role in the pathogenesis of KOA. Therefore, understanding the process of mechanotransduction of MFC is valuable, as it could give us an idea of how dysregulation of joint biomechanics is coupled to changes at the cellular level in KOA. Moreover, this knowledge could potentially contribute to the development of pharmacological agents that could target specific regulators of the signaling pathway which could lead to a more effective treatment for KOA. It is therefore in our best interest to investigate mechanotransduction in MFC.

We recently discovered caveolae in menisci engineered from human MFC and the objective of this thesis was to investigate their potential role in MFC mechanotransduction. This thesis will begin with a literature review on the current knowledge on MFC mechanotransduction and mechanotransduction in related cell types including knee articular chondrocytes (KAC) and intervertebral disc (IVD) cells. Our discovery of caveolae and its relevance to MFC mechanotransduction, will then be discussed and will lead into the central research question and hypothesis of this thesis. Experiments designed to confirm the

expression and assess the function of caveolae in human MFC will be reported over subsequent chapters.

1.2 Biomechanics of the Meniscus

The meniscus is a complex tissue that exhibits regional variations at the cellular, biochemical, and tissue levels. When we examine the meniscus, we can think of dividing it into two distinct regions: the outer meniscus and the inner meniscus. The outer meniscus contains elongated fibroblast-like cells that synthesize ECM that is predominately composed of type I collagen [4]. Collagen fibrils of the outer meniscus are arranged circumferentially, which makes this region of the tissue more adapted for resisting tension [4]. The inner meniscus, on the other hand, contains round chondrocyte-like cells that synthesize ECM that is abundant in proteoglycans and collagen types I and II [4]. In addition to the tensile properties conferred by type I collagen, the abundance of electrostatic proteoglycans that draw in and sequester water allows the inner meniscus to resist compressive forces [4]. These biochemical and functional properties conferred by the inner meniscus, therefore, make it more similar to hyaline cartilage [4]. These regional differences are important as they contribute to the unique viscoelastic behaviour and function of the meniscus during loading. During knee joint loading, the force that initially acts upon the meniscus is distributed circumferentially along the periphery of the tissue due to the ligamentous attachment points of the meniscus on the tibial plateau [4]. The different regions of the meniscus have, therefore, adapted to resist and accommodate for the forces experienced in the different regions. The inner meniscus primarily resists compression, while the outer meniscus has adapted to resist the circumferential tensile ‘hoop’ stresses to prevent meniscal

extrusion [4]. As the MFC from the inner and outer menisci experience different forces, it's likely that they use different mechanotransductive mechanisms that allow them to sense and respond to changes in their unique environments. ECM gene expression is different between inner and outer menisci, evident by the variations in types I and II collagen expression, suggesting that there is a difference in how these cells respond to mechanical stimuli. Learning more about mechanotransduction in MFC is thus useful, as it will allow us to understand the cellular mechanisms governing these differences.

1.3 Current Knowledge on Mechanotransduction in Meniscus Fibrochondrocytes

MFC are exposed to a heterogeneous environment of mechanical stressors [5] that each lead to different responses in the cells. Therefore, for MFC to respond accordingly to different mechanical signals, mechanotransductive mechanisms must be in place. The current literature on MFC mechanotransduction is scare, however, there have been a few different molecules and structures that have been identified as potential mechanotransducers in MFC, including type III collagen, perlecan, primary cilia, and integrins. In this next section, we will discuss the role of these different molecules and structures in MFC to summarize our current knowledge of MFC mechanotransduction.

1.3.1 Type III collagen

Type III collagen is a major structural protein in the human body that has been identified as an important component of menisci [9]. Type III collagen is known to act as a fibral network modifier in collagen-rich tissues and has recently been thought to play a role in chondrocyte

mechanotransduction [9]. Reduction of type III collagen was shown by Wang et al. (2020) to reduce the tissue modulus of menisci, suggesting that it may have implications in MFC mechanotransduction. Despite these preliminary findings, more work needs to be done to get a better understanding of the role type III collagen in menisci.

1.3.2 Perlecan

Perlecan is a large negatively charged heparan sulphate proteoglycan that is abundant in the pericellular matrix (PCM) of many tissues, including the meniscus [10]. Perlecan binds and interacts with collagen VI in the PCM, to form a mechanosensory network that relays changes in the ECM environment to the cell [10]. The electrostatic nature of perlecan allows it to regulate the flux of ions in and around the PCM during mechanical compression of the tissue [10]. During compression of the meniscus, water is extruded from the meniscus, which allows negatively charged proteoglycans, like perlecan, to attract positively charged ions (e.g., calcium ions) into the PCM [10]. This, in turn, changes the osmolarity of the PCM and influences the physiological behavior of cells [10]. One of the key ion channels involved in interpreting these changes is the transient receptor potential cation channel subfamily V member 4 (TRPV4) [10], [11]. TRPV4 is a non-selective cation channel that is highly expressed in chondrocytes that has previously been found to play an important role in mechano-osmotic calcium signaling [10], [11]. Calcium is an important cation and second messenger that has been implicated in mechanotransduction in meniscus cells. Increases in intracellular calcium levels in response to oscillatory fluid flow shear stress were previously shown by Eifler et al. (2006), to correlate with increases of glycosaminoglycan (GAG) production in rabbit meniscal cells [12]. When calcium signaling was blocked with

thapsigargin, these changes were inhibited. Together, this demonstrates that perlecan has an important role in mechano-osmotic signal transduction in chondrocytes, including MFC.

In addition to its role in mechano-osmotic signal transduction, perlecan has also been shown to mediate growth factor signaling in chondrocytes. In a study by Vincent et al. (2007), fibroblast growth factor 2 (FGF-2) was found to be bound and sequestered by perlecan in the collagen VI rich PCM of KAC [13]. Upon compressive loading, perlecan permitted the release and binding of FGF-2 to FGF receptors on the surface of KAC, which lead to initiation of extracellular signal-related kinase (ERK) signaling and the downstream production of matrix modifying enzymes including tissue inhibitor of metalloproteinases 1 (TIMP-1), matrix metalloproteinase 1 (MMP-1), and matrix metalloproteinase 3 (MMP-3) from KAC. Despite the fact that these effects were shown in KAC, the information is still relevant and translatable to MFC, as the meniscus is abundant in perlecan. Perlecan therefore is likely to play an important role in matrix metabolism in the meniscus, however more investigation is required. Additionally, as perlecan has been shown to act as both a mechanosensor and regulator of matrix metabolism, it's likely that it could be involved in the load-induced catabolism of cartilage tissue in KOA. It is therefore useful to further study the function of perlecan in MFC, as it may shed some insight on the pathophysiological processes governing KOA.

1.3.3 Primary cilium

Primary cilium are nonmotile cytoplasmic projections found on most eukaryotic cells that have been shown to play a role in signal transduction [14]. These sensory organelles have previously been identified in rabbit menisci and were shown to be inhomogenously

expressed across the different regions of the meniscus [15]. The number of cytoplasmic projections was shown to correspond to the amount of tension each of the regions experience, with cilia being the most numerous in outer margins of the meniscus and least numerous deeper in the tissue [15]. These observations suggest that cilia may be important in the sensation of tension in the meniscus. In a review by Spasic & Jacobs (2017), the mechanotransductive role of cilia in different tissues was discussed [16]. Primary cilia were summarized to act as shear sensors in most tissues including bone, however, their role in cartilage was thought to be more distinct. In chondrocytes, it was proposed that cilia, instead, act as tissue compression sensors, as both integrins and ECM binding proteins have been shown to localize to cilia. As the meniscus is frequently exposed to compression, it is therefore reasonable to predict that cilia may also act as compression sensors in the meniscus. Taken together, the work by Hellio Le Graverand et al. (2001) and the review by Spasic & Jacobs (2017) suggest that cilia may therefore play an important role in MFC mechanotransduction, however, more investigation is necessary.

1.3.4 Integrins

Integrins are cell adhesion receptors located in focal adhesion (FA) sites that play a major role in mechanotransduction in many cells [17] including chondrocytes [18]. Integrins act as mechanotransducers that physically connect ECM fibers to cytoskeletal proteins and signaling molecules in FAs, which allows them to transduce mechanical stimuli from the external environment into intracellular responses [17], [19]. Previous work by Zhang et al. (2019) suggested that integrins also play an important role in mechanotransduction in MFC [20]. Following 12 hours of cyclic hydrostatic pressure, the expression of integrins $\alpha 5$ and

$\beta 1$ was found to be significantly increased at both the messenger RNA and protein level in rat MFC. Integrins $\alpha 5\beta 1$ are major fibronectin receptors in chondrocytes that have been implicated in matrix degradation [19]. Previously, it's been shown that fragments of fibronectin can signal through integrin $\alpha 5\beta 1$ receptors to initiate proinflammatory and catabolic signaling in chondrocytes, suggesting that they may be involved in KOA pathogenesis [19]. Zhang et al. (2019) additionally showed that the increased integrin expression was accompanied with a significant increase in the phosphorylated downstream signaling molecule focal adhesion kinase (FAK). FAK is a tyrosine kinase that is a key mediator of integrin signaling [19]. FAK couples the activation of integrins with changes in the signaling of kinases including ERK, c-Jun N-terminal kinases, and p38 mitogen-activated protein kinases, which, in turn, leads to alterations in downstream gene expression [19]. Together, these results imply that integrins, particularly $\alpha 5\beta 1$, play a role in MFC mechanotransduction.

In addition to $\alpha 5\beta 1$, there are other integrins present on the surface of MFC that interact with collagens that may also be involved in mechanotransduction. Currently, the MFC expression profile of integrins is not well understood so more research is necessary to get a better picture [21], however, we can predict the integrins that are likely to be expressed based off our knowledge on KAC. As KAC and MFC reside in biochemical environments with similar integrin ligands, it's likely that the types of integrins expressed will be the same. The integrins that interact with collagens in KAC include $\alpha 1\beta 1$, $\alpha 2\beta 1$, and $\alpha 10\beta 1$ [19]. Of these, integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ have been the most studied [22]. Both have been shown to interact with collagen types II and VI in KAC [19], however, they have also been implicated in type I collagen binding in other cell types [22]. Interestingly, integrins $\alpha 1\beta 1$ and $\alpha 10\beta 1$

are mainly expressed by healthy KAC, while $\alpha 2\beta 1$ integrins seem to be expressed by chondrocytes in KOA [19]. This suggests that the ECM environment changes in KOA and causes KAC to adapt by altering their integrin expression profile. By altering integrin expression, this changes the intracellular signaling in response to external stimuli and thus contributes to different phenotypes and behaviours of KAC in KOA. Taken together, integrins seem to be essential mechanotransducers that are likely to play important roles in MFC. Characterizing the specific integrins and signaling cascades involved in MFC mechanotransduction is, therefore, useful and merits more investigation.

1.4 Mechanotransduction in Related Cell Types

Most of our current knowledge on MFC mechanotransduction is rooted in our understanding of mechanotransduction in other cartilaginous cell types [5], therefore, in order for us to gain some insight, it is useful for our discussion to look at mechanotransduction in related cells. In this next section, we will briefly highlight mechanotransduction in KAC and IVD cells to bring together knowledge from different fields that will support and enhance our understanding of MFC.

1.4.1 Knee articular chondrocytes

Knee articular cartilage is a hyaline cartilage structure situated on the femoral condyles that function to create a smooth and lubricated surface for joint articulation [23]. During locomotion, articular cartilage experiences a significant degree of mechanical loading which translates to shear and tensile forces in the periphery and compression in deeper tissue regions [23]. The integrity of articular cartilage is maintained by KAC which synthesis and

remodel the ECM in response to mechanical stimuli [23]. Given that KAC and MFC are both a part of load-bearing tissues in the knee joint that experience similar mechanical stimuli and share similar physiological environments, it's likely that the mechanotransductive mechanisms used by these cells are the same. Therefore, highlighting mechanotransduction in KAC is important, as it will provide some context for our understanding of MFC.

In KAC, the key player in mechanotransduction is the PCM. The PCM is a heterogenous structure of proteoglycans and proteins like perlecan and collagen VI, that give the PCM unique viscoelastic properties which allow it to mechanically regulate KAC [24]. The PCM is coupled to a number of mechanosensitive structures on the surface of KAC including primary cilium, calcium channels, and integrins, which all can be activated during mechanical stimulation [24]. The PCM additionally sequesters a number of growth factors and signaling molecules whose receptors are found on the plasma membrane of KAC, including FGF-2, connective tissue growth factor, bone morphogenetic protein, and transforming growth factor beta (TGF-β) that are released upon deformation of the PCM [24]. Either through the physical coupling of mechanosensitive structures or growth factor release, these changes are then able to initiate biochemical responses in KAC, which downstream, regulate cartilage metabolism. Some of the different signaling pathways that have been shown to be mechanically regulated in KAC include the hedgehog (Hh), Wnt, TGF-β, and yes associated protein 1 (YAP)/Transcriptional coactivator with PDZ-binding motif (TAZ) pathways, which all, in turn, regulate mitogen-activated protein kinase/extracellular signal-related kinase (MAPK-ERK) activity [24]. The downstream effects of these changes have been shown to influence the expression of genes including

matrix metalloproteinase 13 (*MMP-13*), a disintegrin and metalloproteinase with thrombospondin motif 5 (*ADAMTS5*), and RUNX Family Transcription Factor 2 (*RUNX2*), which all have been tied to KOA pathogenesis [24]. Together, these effects demonstrate that mechanical stimulation of KAC appears to play an important role in cartilage homeostasis. Thus, better understanding mechanotransduction in KAC may not only further our knowledge on MFC mechanotransduction, but it may also provide some insight on the disease state of KOA.

1.4.2 Intervertebral disc cells

IVDs are fibrocartilaginous structures situated between spinal vertebrae that serve an important role in spinal load transmission, support, and flexibility [25]. IVDs are composed of two distinct regions: the fibrous outer annulus fibrosus (AF) and the gelatinous inner nucleus pulposus (NP). The anatomical region, load type, magnitude, and duration of mechanical stimuli all influence the response of IVDs to mechanical loading [25]. Cells in the NP region tend to experience axial compression and high osmotic and hydrostatic pressures due to an abundance of negatively charged GAGs in the interior of the tissue, while cells in the AF predominately experience tension [25]. IVDs are therefore structurally and functionally similar to menisci as they experience similar forces over the different regions of the tissue. It is thus useful for us to highlight mechanotransduction in IVDs as it may provide some insight into the region-specific mechanotransduction in MFC.

Currently, the mechanotransductive mechanisms of IVDs have not been well characterized, however, there are some ideas that have been suggested in the literature. The main factor that is believed to regulate the region-specific responses to mechanical stimuli is

the cytoskeletal differences between anatomical regions [25]. Both cell-matrix and cell-cell interactions that mediate actin dynamics are thought to contribute to the mechanosensing properties of cells in IVDs [25]. In regions where the matrix is 'stiff' (i.e., peripheral AF) and polymeric actin and the number of FAs tend to be high, cells tend to take on a more fibrotic phenotype [25]. On the other hand, cells in the more gelatinous regions of the tissue (i.e., NP) where both polymeric actin and the number of FAs are lower, appear more chondrocytic [25]. These differences in cell morphology and FA formation are thought to be regulated by a few different proteins including integrins, cadherins, Rho-associated protein kinase (ROCK)/RhoA, and YAP/TAZ [25]. Integrins, in particular, are thought to be important sensors in both the NP and AF, that sense changes in matrix stiffness and translate it to changes in gene expression via ROCK/RhoA and YAP/TAZ regulated signaling cascades [25]. This influences the actin dynamics at the periphery of the cells and tailors the cell morphology to the specific environment. Mechanosensitive ion channels, such as TRPV4, have also been implicated in osmotic pressure sensing in NP cells [25], however, more investigation is required in this area.

1.5 Caveolae and MFC

Caveolae are mechanosensitive plasma membrane invaginations approximately 60-80 nm in diameter that are predominantly constructed of cholesterol, phosphatidylserine, and phosphatidylinositol (4,5)-bisphosphate [17], [26]. These cell elements are known to regulate lipid homeostasis, organize cell signaling, and couple with stress fibers to adapt to changes in membrane tension [17]. Caveolae have been previously shown to act as mechanosensors in other cell types, hence their expression tends to be the highest in cells that are frequently

exposed to mechanical stress, such as endothelial cells, adipocytes, muscle cells, and fibroblasts [26]. The predominant structural proteins of caveolae are caveolins-1, 2 and 3, with caveolin-1 believed to be the most critical for caveolar biosynthesis [26].

Caveolae and caveolins were previously identified *in vivo* in 1999 by Schwab and colleagues in the knees of both newborn and adult rats [27]. Caveolin proteins were shown by immunofluorescence to be expressed in the different structures of the knee, including the articular cartilage, meniscus, patellar tendon, and femur. The cells that had the highest expression of caveolin proteins were located in tissue regions that experience higher degrees of tension. These include the superficial layers of articular cartilage, the outer fibrous portion of the meniscus, and the transition zone of the patellar tendon. To determine whether caveolin protein expression corresponded to caveolar structures, transmission electron microscopy (TEM) was additionally performed on the articular cartilage and meniscus fibrocartilage samples. Both articular cartilage and meniscal samples were shown to have caveolar structures which confirmed their original findings. Interestingly, the caveolae in menisci were situated close to the rough endoplasmic reticulum and expressed in clusters, unlike articular cartilage.

We recently identified the presence of caveolae in a pellet culture of human MFC by TEM, which coincided with the findings from Schwab et al. (1999) (Figure 1.2). Taken together with these previous findings, it suggests that caveolae may likely have an important and conserved role in tension sensing in the knee. Since menisci have been identified as critical regulators of knee health and homeostasis, it is, therefore, useful for us to investigate the function of caveolae in meniscal mechanobiology and their effects on joint biomechanics.

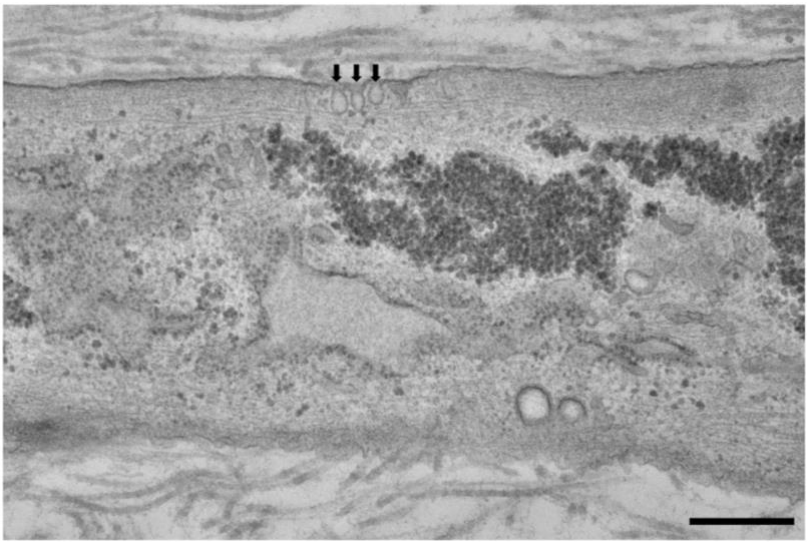

Figure 1.2 Caveolae located in human MFC. TEM image of a pellet culture of human MFC at ×15,000 magnification. Black arrows denote the location of caveolae at the plasma membrane. The expression and localization of caveolae coincide with the findings presented by Schwab et al. (1999) in rat meniscus fibrocartilage. Voltage of the beam was 60.0kV, 500 nm scale bar.

1.6 Caveolae and Caveolin-1 in Mechanotransduction

Caveolae act as cell signaling hubs that sequester receptors and signaling molecules and regulate their activation. Some of the different receptors and signaling molecules that have been identified to interact with caveolae, either directly or indirectly, include endothelial nitric oxide synthase, tyrosine kinase receptors, G-protein coupled receptors, TGF-β type I and II receptors, TRPV4 [28], insulin receptors, epidermal growth factor receptors (EGFR), integrins, Src kinases including Fyn, small GTPases, MAPK-ERK, YAP/TAZ, and some proteins in Wnt signaling [26]. Caveolae have also been shown to associate with stress fibers, through interactions with linker proteins like Filamin A, which are known regulators of cell morphology and membrane tension [17]. By coupling mechanical cues to receptors, signaling proteins, and stress fibers at the periphery of cells, this makes caveolae liaisons for

mechanotransduction. One of the downstream effects of caveolae-mediated mechanotransduction is ECM remodeling, which is mediated by changes in YAP/TAZ [29] and RhoA signaling [17]. In response to mechanical cues such as high tension, caveolae have additionally been shown to flatten and disassemble [17]. Disassembly of caveolae can lead to the release of proteins like cavin-1 and EH Domain Containing 2 (EHD2) that are able to translocate to the nucleus and influence gene expression [30]. EHD2, for example, has previously been shown to modulate genes coding for proteins involved in ECM interactions as well as those involved in K-Ras and tumor necrosis factor alpha (TNF-α) signaling [30]. A summary of these different mechanotransductive events can be found in Figure 1.3.

Caveolin-1 is the predominant caveolar protein that is thought to be the key regulator of caveolae-mediated mechanotransduction. Most of our current knowledge lies in our understanding of caveolin-1 in caveolar domains, however, the role of caveolin-1 in noncaveolar domains is another area that is beginning to expand [17]. Recently, Rangel et al. (2019) identified an interaction between the isoform caveolin-1α and the length of primary cilia. Caveolin-1α in this study was shown to regulate the length of primary cilia through its actions on RhoA and effector proteins ROCK and mDia. Deletion of caveolin-1α led to the increased immobilization and transportation of globular actin to the base of primary cilia, which promoted the polymerization of actin filaments and lengthening of cilia. This study supports the fact that caveolin-1 is a key regulator of actin dynamics and hence may be a critical component of mechanotransduction in cells. Moving forward, it will be important to further investigate the role of caveolin-1 in noncaveolar domains, as its function may be equally relevant to cell mechanotransduction.

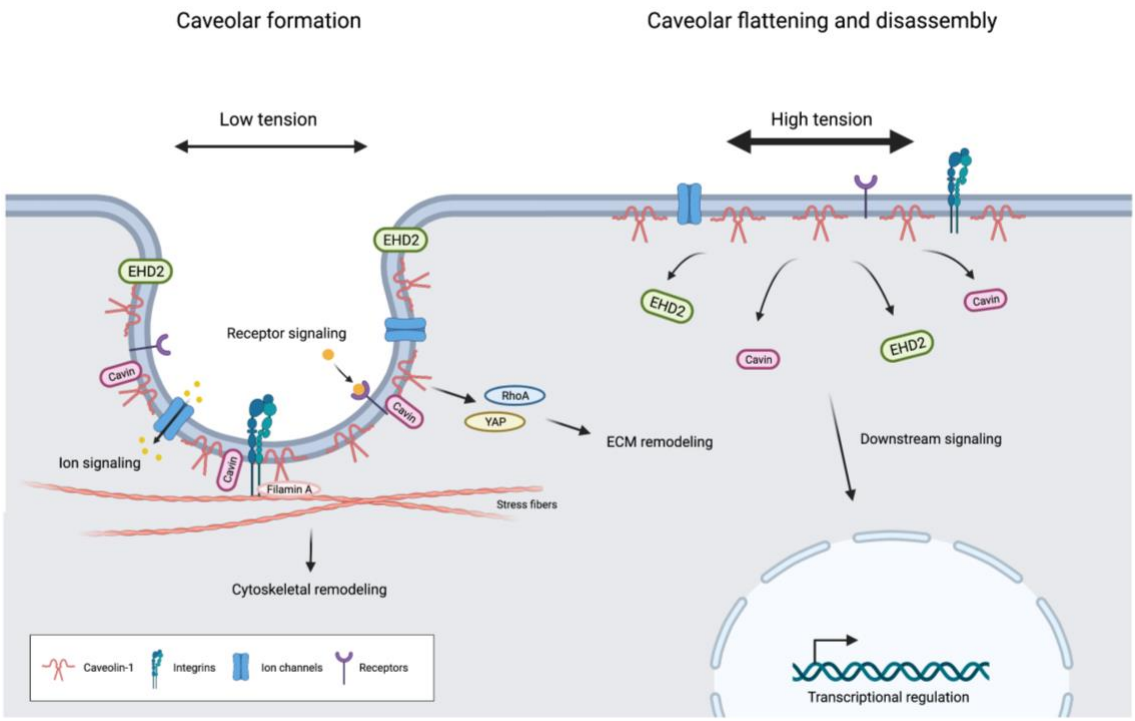

Figure 1.3 Summary of the different events in caveolae-mediated mechanotransduction. Adapted from Buwa et al. (2020) and Echarri and Del Pozo (2015). Image was created with BioRender.com. EHD2; EH domain containing 2, YAP; yes associated protein 1.

1.7 Caveolae-Mediated Mechanotransduction in Related Cell Types

Due to the lack of knowledge of caveolae in the meniscus, it is important for us to address their role in related cell types, such as KAC and IVD cells. Caveolin-1, which is the predominant structural protein implicated in caveolae-mediated mechanotransduction, has been shown to regulate mechanotransductive mechanisms in both KAC and the cells of the AF. Therefore, highlighting these findings may provide us with some insight into the signaling mechanisms that may be occurring in MFC. In the next section, we will discuss caveolae-mediated mechanotransduction in KAC and AF cells to create a platform of

knowledge from where we can interpret and apply the information to our discussion on MFC mechanotransduction.

1.7.1 Knee articular chondrocytes

The current knowledge on caveolae-mediated mechanotransduction in KAC is scare, however, there are a few groups that have identified some interactions. The study done by Ren and colleagues in 2018 is probably the most insightful as they looked at the direct effects of caveolar inhibition on KAC exposed to compression [31]. In this study, rat KAC were exposed or not to 1 hour of periodic hydraulic pressure (i.e., compression) in either the absence or presence of caveolar disruptors, methyl-β-cyclodextrin (MβCD) and caveolin-1 short hairpin RNA (shRNA). This allowed for the testing and comparison of two different variables: effects of compression and effects of caveolar disruption. Exposing KAC to compression was shown to increase cell proliferation and increase the phosphorylation of caveolin-1, insulin-like growth factor type 1 receptor (IGF-1R), and ERK1/2, in comparison to the static control group. However, when the cells were treated with MβCD and shRNA, these pressure-induced changes were inhibited. These results indicate that caveolae may be important for the mechanotransduction of compressive stimuli in KAC, as without intact caveolae, ERK1/2 and IGF-1R-mediated intracellular signaling events (i.e., phosphorylation) appear to be disrupted. ERK1/2 is part of the mitogen-activated protein kinase kinase (MEK) signaling cascade which has been implicated in cartilage tissue metabolism [32]. Inhibition of MEK-ERK1/2 signaling has previously been shown to decrease matrix degradation and enhance hyaline-cartilage-like matrix deposition [32]. Insulin-like growth factor 1 (IGF-1), the ligand of IGF-1R, has also been shown to be an important regulator of cartilage

metabolism in KAC [33]. Reduced levels of IGF-1 have been shown to correlate with increased catabolism of tissues, associated with increased damage of cartilage and loss of proteoglycans and chondrocytes [33]. Therefore, if caveolae can regulate ERK1/2 and IGF-1R signaling in response to mechanical cues like compression, as shown by Ren et al. (2018), this suggests that caveolae may play an important role in matrix homeostasis and potentially KOA pathogenesis.

Another study by Wang et al. (2011) looked at the effects of shear stress on caveolin-1 signaling in primary human KAC [34]. KAC were grown on glass slides and then subjected to 20 dyn/cm^2 of shear stress for varying periods of time. Shear stress was shown to temporally regulate the expression of caveolin-1 and transmembrane protein toll-like receptor 4 (TLR-4) in a manner dependent on cyclooxygenase 2 (COX-2). Short term application of shear stress led to the rapid and transient expression of TLR-4 while, prolonged exposure decreased TLR-4 and increased caveolin-1 expression. Additionally, this group identified a binding interaction between TLR-4 and caveolin-1 that was believed to contribute to the opposing regulation of phosphoinositide 3-kinase, protein kinase A, and ERK1/2 signaling. TLR-4 acted as an initiator of signaling which promoted the downstream expression and production of interleukin 6 (IL-6). Caveolin-1, however, served as a signaling inhibitor which downstream reduced the expression and synthesis of IL-6. IL-6 is a proinflammatory cytokine whose involvement in cartilage metabolism has been debated. Elevated serum levels of IL-6 have been associated with enhanced cartilage loss and knee radiographic osteoarthritis [35]. However, IL-6 has also been shown to have modest anabolic effects on cartilage repair, as application of IL-6 was found to promote matrix production by healthy chondrocytes and reduce matrix catabolism by osteoarthritic chondrocytes [36]. As

caveolin-1 was found to inhibit the production of IL-6 in response to shear stress, these findings suggest that caveolin-1 may be involved in shear mechanotransduction and the downstream regulation of cartilage metabolism. Targeting caveolin-1 may, therefore, be a potential future therapeutic approach to alter the metabolic state of KAC and treat KOA.

The effect of glycosphingolipid (GSL) depletion on KAC mechanotransduction was also investigated by Matsubara et al. (2019) [37]. Even though caveolae were not specifically investigated in this study, the findings are still relevant to our discussion because caveolae are rich in GSLs [38]. Global GSL depletion would disrupt the formation and function of caveolae, therefore the effects seen in this study are quite relevant to our discussion. In this study, KAC from *Ugcg* knockout ($Ugcg^{-/-}$) and control mice were isolated and cultured on type I collagen gels to generate three-dimensional (3D) tissue models. *Ugcg* is a chondrocyte-specific gene that encodes the enzyme glucosylceramide synthase which functions in the first step of GSL synthesis [37]. Therefore, knockout of *Ugcg* essentially depletes chondrocytes of GSLs and theoretically disrupts caveolar formation. Engineered tissues were exposed to varying amplitudes and durations of cyclic tensile strain (CTS) and gene expression was then subsequently assessed by real-time quantitative polymerase chain reaction. At 5% CTS, the expression of anabolic genes collagen type II alpha 1 (*COL2A1)* and aggrecan (*ACAN*), as well as the gene coding the proteinase *MMP-13*, were significantly decreased at 3 hours in $Ugcg^{-/-}$ KAC in comparison to control KAC. At 10% CTS however, the gene expression of proteinases *MMP-13* and *ADAMTS5* were significantly higher in the $Ugcg^{-/-}$ KAC at 3 hours and 24 hours, respectively. These findings imply that the metabolic profiles of KAC are regulated by both stimulus intensity and the presence of GSLs. To further investigate the role of GSLs in KAC

responsiveness to CTS, live KAC from $Ugcg^{-/-}$ and control mice were labelled with the green, fluorescent dye, Fluo4-AM, and visualized with a confocal laser scanning microscope to determine real-time intracellular calcium dynamics in response to different levels of CTS. The number of KAC with transient calcium oscillations was found to be significantly higher in the $Ugcg^{-/-}$ group than the control group following 10% and 16% CTS. In other words, GSL depletion increased the number of KAC responsive to high levels of tension. Taken together, the findings from this study suggest GSLs are involved in the mechanotransduction of tensile stimuli in KAC, as the absence of GSLs alters the metabolic profile and intracellular calcium dynamics in KAC exposed to tension. Therefore, if we interpret these results in context of caveolae, we see that disrupting GSL synthesis, and thus caveolar formation, would potentially have a significant effect on chondrocyte adaption to mechanical stimuli like tension.

Schwab and colleagues continued to build from their initial discovery of caveolae in rat knee structures over subsequent years and proceeded to identify some additional caveolar interactions in KAC. Even though they did not specifically investigate mechanotransduction, the information obtained is still relevant to our discussion. The following year in 2000, Schwab and colleagues identified a colocalization of $\beta 1$ integrins and caveolins in caveolae of human KAC [39]. They believed that the caveolins functioned to enhance integrin-mediated mechanotransduction by clustering the integrins at the plasma membrane. As caveolae are enriched with various signaling proteins, such as kinases, it's thought that caveolae could facilitate the coupling of mechanical stimuli sensed by integrins with the activation of intracellular signaling cascades. In 2001, this group identified an additional interaction between urokinase-type plasminogen activator receptors (uPAR) and $\beta 1$

integrin/caveolin complexes [40]. uPAR is a glycosyl-phosphatidylinositol-linked glycoprotein that has been shown to play a crucial role in cartilage catabolism in KOA [40]. When activated, uPAR facilitates the conversion of plasminogen to its active form plasmin, which is an enzyme that is able to degrade the proteins and proteoglycans of the PCM [40]. In this study, arthritic KAC and KAC stimulated with phorbol 12-myristate 13-acetate were found to have an increased expression of uPAR that was shown to colocalize in caveolae at the plasma membrane with $\beta 1$ integrins and caveolins. This suggests that activated uPAR may interact with $\beta 1$ integrins to regulate PCM metabolism in response to external stimuli. Matrix degradation is a hallmark of KOA, therefore, the fact that arthritic KAC showed an increased expression of uPAR is consistent with what would be expected. Over the next two years, this group proceeded to investigate the effects of interleukin 1 beta (IL-1β), a proinflammatory cytokine implicated in KOA [41], on the organization and signaling of this protein complex. IL-1β simulation was shown to promote the colocalization of matrix metalloproteinase 9 (MMP-9), an enzyme implicated in KOA, with uPAR [41] and vascular endothelial growth factor receptor 3 (VEGFR-3) with $\beta 1$ integrins [42]. IL-1β was additionally found to regulate the expression of both uPAR [41] and VEGFR-3 in a dose-dependent manner [42].

1.7.2 Intervertebral discs

Despite the fact that caveolae have been previously identified in IVDs [43], there is not much knowledge on their function, let alone their role in mechanotransduction in IVDs. There have, however, been a couple groups that have identified a mechanotransductive role of caveolin-1 in IVDs. One of them was Zhang et al. (2021), which demonstrated a

proinflammatory role of caveolin-1 in IVD degeneration in response to adverse mechanical loading. In this study, different levels of CTS were applied to AF cells and the effects on inflammatory signaling were monitored. 5% CTS was shown to be beneficial and promote antiinflammatory gene expression, while 12% CTS led to more proinflammatory effects. Caveolin-1, together with $\beta 1$ integrins, were identified as coregulators of inflammatory signaling in AF cells exposed to CTS. At 12% CTS, activation of caveolin-1/$\beta 1$ integrin complex was shown to promote the nuclear translocation of p65, an important regulator of nuclear factor kappa B signaling, and enhance the expression of proinflammatory genes including *COX-2*, interleukin 8 (*IL-8)*, *IL-6*, and *TNF-*α. Knockdown of caveolin-1 with small interfering RNAs (siRNA) suppressed proinflammatory signaling at 12% CTS, while overexpression of caveolin-1 with caveolin-1 encoded plasmids suppressed the antiinflammatory effects observed at 5% CTS. *In vivo* studies additionally performed in mice with mildly degenerated IVDs demonstrated that moderate mechanical loading suppressed caveolin-1 expression and led to better IVD repair and regeneration. Together, these results suggest that caveolin-1 sways integrin-mediated mechanotransduction towards more proinflammatory and catabolic signaling in IVDs. Suppressing caveolar signaling in IVDs may, therefore, be a potential therapeutic approach to treat IVD degeneration that could even potentially be applied to KOA.

The other study by Chu et al. (2021) instead identified a role of caveolin-1 in AF stem cell (AFSC) differentiation [44]. In this study, AFSCs were seeded on scaffolds with different fiber diameters aimed to mimic the different regions of IVDs, and the corresponding cell morphologies and expression of phenotypic marker genes were assessed. AFSCs seeded on scaffolds with larger fiber diameters were shown to take on a more

ligament/tendon-like phenotype with higher type I collagen expression, while AFSCs on smaller fiber diameter scaffolds appeared more chondrocyte-like and expressed more type II collagen and aggrecan. Large fiber diameter scaffolds, that mechanically are stiffer than their smaller counterparts, correlated to a higher expression of caveolin-1 and increased nuclear translocation of YAP in AFSCs. It was proposed that these stiff scaffolds enhanced the expression of caveolin-1, which in turn influenced the downstream nuclear translocation of YAP and subsequent expression of genes supporting FA formation. With an enhanced number of FAs, it promoted AFSC spreading and contributed to AFSC differentiation towards a more ligament/tendon-like phenotype. Additionally, when AFSCs were treated with verteporfin, a YAP inhibitor, type I collagen expression was suppressed, and the expression of type II collagen and aggrecan was enhanced. Together, these results suggest that caveolin-1 plays an important role in AFSC differentiation. This knowledge is useful as it may aid in the understanding and development of more biomimetic biomaterials for tissue engineering. In this case, engineering a scaffold with a gradient of fiber diameters could promote region-specific differentiation of AFSCs, which would allow the tissue formed to closer resemble IVDs [44]. Applying this knowledge to other areas, such as meniscus tissue engineering, could lead to some important advancements and therefore is worth further exploration.

1.8 Study Rationale

Currently, the precise mechanisms of MFC mechanotransduction are not well understood. For us to truly understand how the meniscus functions in a mechanical environment, we need to get a better understanding of how mechanotransduction is played out in MFC. As

caveolae and caveolins have previously been identified in both human and rat MFC and have been shown to be important regulators of mechanotransduction in both KAC and AF cells, it is likely that they may be equally relevant in MFC mechanotransduction. Recent evidence from a transcriptome profiling study of human MFC in a dynamic compression loaded tissue-engineered meniscus, revealed that caveolin-1 (*CAV1*) was significantly upregulated [45]. This evidence suggests that caveolin-1, a key component of caveolae, is responsive to mechanical loading in MFC, but because the data to support these claims is limited, more research is necessary. The central research question outlining this thesis is, do caveolae function in mechanotransduction in human MFC?

1.9 Research Hypothesis and Objectives

Based off the supporting literature and our recent finding of caveolae in human MFC, we hypothesize that caveolae do function in human MFC mechanotransduction. To address this hypothesis we proposed two objectives. First, we aimed to confirm the expression of caveolin proteins in human MFC at both the gene and protein level. Second, we aimed to mechanically stimulate and transcriptionally probe MFC in engineered human meniscus and evaluate caveolae-related gene expression.

Chapter 2

Materials and Methods

The materials and methods, results, discussion and conclusion addressing the second objective of this study over the subsequent chapters are a part of the following manuscript submitted for publication[3]: M. J. Vyhlidal, Z. Ma, D. X. Li, M. Kunze, and A. B. Adesida, *Transcriptomics Evidence for the Role of Caveolae in Human Meniscus Fibrochondrocyte Mechanotransduction*, 2022.

2.1 Donor Information

Different donors were used to explore the first and second objectives and their non-identifying information is presented in Table 2.1. All tissue specimens were collected from individuals undergoing partial meniscectomy for acute sport-related injuries.

2.2 Cell Isolation and Culture

Fresh human meniscus specimens from independent donors were fragmented and digested with 0.15% w/v of collagenase type II (300 units/mg; Worthington, United States) to isolate meniscus fibrochondrocytes (MFC). Cells were then given 48 hours to recover. Following recovery, the cells were collected and replated at a fixed density of 10^4 cells/cm^2 and

[3] This study uses the same RNA sequencing dataset as the following published study from our group: Z. Ma, D. X. Li, M. Kunze, A. Mulet-Sierra, L. Westover, and A. B. Adesida, "Engineered Human Meniscus in Modeling Sex Differences of Knee Osteoarthritis in Vitro," *Frontiers in Bioengineering and Biotechnology*, vol. 10, 2022, https://www.frontiersin.org/article/10.3389/fbioe.2022.823679, [46]. The cell culture methods pertaining to objective two were, therefore, performed identically as described before.

Table 2.1 Donor information

Objective #1				Objective #2			
Donor	**Age (years)**	**Height (cm)**	**Weight (kg)**	**Donor**	**Age (years)**	**Height (cm)**	**Weight (kg)**
Female (F1)	28	170	88.5	Female (F1)	33	167	114.7
Female (F2)	42	168	107.0	Female (F2)	44	162	99.7
Female (F3)	39	165	92.2	Female (F3)	30	161	62.7
Female (F4)	42	178	82.6	Female (F4)	28	162	73.7
Male (M1)	28	185	108.8	Male (M1)	19	174	72.0
Male (M2)	45	183	88.9	Male (M2)	45	179	75.4
Male (M3)	27	173	70.5	Male (M3)	22	186	79.9
				Male (M4)	35	189	112.2

expanded in Dulbecco's modified Eagle's medium-high glucose (DMEM) supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (all from Sigma-Aldrich, United States), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (PSG) (Life Technologies, United States), and growth factors at 37°C. Between the objectives, MFC were expanded under slightly different conditions. For the first objective, MFC were expanded under hypoxic conditions (3% O_2, 5% CO_2) with 5 ng/mL of FGF-2 (ORF Genetics, Iceland, #01-A01110) to reduce MFC dedifferentiation [47]. For the second objective, MFC were cultured under normoxic conditions (20% O_2, 5% CO_2) with both 5 ng/mL of FGF-2 and 1 ng/mL of TGF-β1 (ProSpec, Israel, #CYT-716), which was informed by previous work by our group [48], [49].

2.3 Tissue Formation

To address the second objective, 3D tissues were engineered from human MFC for mechanical stimulation as follows. Following expansion, MFC were collected and seeded onto bovine type I collagen scaffolds (diameter= 6 mm, height= 3.5 mm, Integra LifeSciences, United States) at a density of 5×10^6 cells/cm^3 and cultured statically for 2 weeks in a defined serum-free chondrogenic media (DMEM supplemented with 100 nM dexamethasone, 365 μg/mL ascorbic acid 2-phosphate, 40 μg/mL L-proline [all from Sigma-Aldrich], 125 μg/mL human serum albumin [Lee Biosolutions Inc., United States], ITS+1 premix [Corning Inc., United States], HEPES, PSG, and 10 ng/mL of TGF-β3 [ProSpec, #CYT-113 and Proteintech, United States, #HZ-1090]) to promote meniscus' matrix formation. Media was changed once per week.

2.4 Mechanical Stimulation

Following the 2-week preculture period, tissues were randomly assigned to different mechanical treatment groups, which consisted of cyclic hydrostatic pressure (CHP), simulated microgravity (SMG), or static control. We chose CHP to mimic physiological loading of the meniscus and SMG to mimic tissue unloading. For the mechanical loading group, tissues were subjected to 0.9 MPa of CHP for 1 hour per day at a frequency of 1 Hz using the MechanoCulture TR (CellScale, Canada) bioreactor. This loading regime was chosen as it considers both the approximated hydrostatic pressure range of human meniscal tissue [4], [50] and fundamental frequency of human gait [51]. For the mechanical unloading group, tissues were suspended in SMG using the RCCS-4 bioreactor by Synthecon Inc., United States. Static controls remained in a 100 mL tissue culture tube. Tissues were

cultured in serum-free chondrogenic media as described above and stimulated for 3 weeks with media changes performed once a week. The volume of medium per tissue (approximately 6.5 mL per tissue construct per week) was equivalent among the different treatment groups. At the end of 3 weeks, tissues from the CHP group were harvested following 30 minutes of rest after the last loading event to allow for gene expression changes to occur. Tissues from the SMG and static groups were harvested immediately after mechanical stimulation at approximately the same time. The experimental outline for the second objective is presented in Figure 2.1.

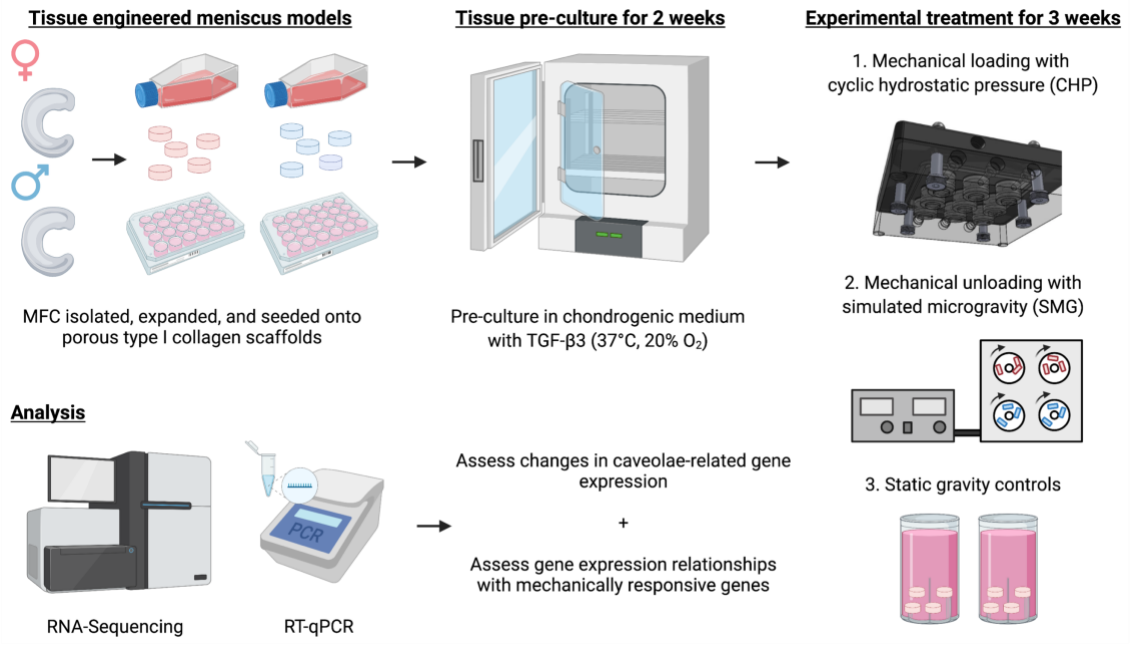

Figure 2.1 Objective two experimental outline. Image has been adapted from Ma et al. (2022) [46].

2.5 RNA Extraction and Gene Expression Analysis

Tissues were placed in TRIzol™ reagent (Life Technologies) immediately upon harvesting to prevent changes in gene expression and were stored at -80°C until RNA extraction. Total RNA was extracted via two different methods including traditional extraction with TRIzol™ reagent (objective one) and using the PuroSPIN Total RNA Purification Kit (objective two; Luna Nanotech, Canada) following the manufacturer's instructions. 100 ng of total RNA was reverse transcribed to cDNA for real-time quantitative polymerase chain reaction (RT-qPCR) analysis by GoScript™ reverse transcriptase with oligo (dT) primers (both obtained from Promega, United States). RT-qPCR was performed using Takyon™ SYBR® Green detection (Eurogentec, Belgium) using the primers listed in Table 2.2. Gene expression levels were normalized to housekeeping genes *β-actin* (*ACTB*), Beta-2-microglobin (*B2M*), and Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta (*YWHAZ*) and presented using the $2^{-\Delta CT}$ and $2^{-\Delta\Delta CT}$ methods [52]–[54] for objectives one and two, respectively. Briefly, normalization was performed by subtracting the average threshold cycle (C_T) value of the housekeeping genes from the C_T values of the genes of interest. Next-generation RNA-sequencing[4] was performed on RNA samples from objective two on the Illumina NextSeq 500 platform, and FastQ files were obtained for further analysis[5].

[4] RNA-sequencing was performed by Ms. Tara Stach and the team at the Biomedical Research Centre from the University of British Columbia.
[5] The RNA-sequencing dataset used for this study is openly available in Gene Expression Omnibus (GEO) at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE192982, reference number GSE192982.

Table 2.2 Primer sequences used in real-time quantitative polymerase chain reaction

Gene	Primer sequences		NCBI Reference
ACTB	5'-AAGCCACCCCACTTCTCTCTAA-3'	Forward	NM_001101.4
	5'-AATGCTATCACCTCCCCTGTGT-3'	Reverse	
ACAN	5'-AGGGCGAGTGGAATGATGTT-3'	Forward	NM_001135.3
	5'-GGTGGCTGTGCCCTTTTTAC-3'	Reverse	
ADAMTS5	5'-TGTAGCCTGCATTCCACAACA-3'	Forward	NM_007038.5
	5'-CCCAAACGGCTCAGTTCAA-3'	Reverse	
B2M	5'-TGCTGTCTCCATGTTTGATGTATCT-3'	Forward	NM_004048.4
	5'-TCTCTGCTCCCCACCTCTAAGT-3'	Reverse	
CAV1	5'-AGGCCAGCTTCACCACCTT-3'	Forward	NM_001753.5
	5'-GCAGACAGCAAGCGGTAAAAC-3'	Reverse	
CAV2	5'-ACCGGCTCAACTCGCATCT-3'	Forward	NM_001233.5
	5'-CCGGCTCTGCGATCACAT-3'	Reverse	
CAV3	5'-AGGTGGATTTTGAAGACGTGATC-3'	Forward	NM_033337.3
	5'-CACACGCCGTCAAAGCTGTA-3'	Reverse	
COL1A2	5'-GCTACCCAACTTGCCTTCATG-3'	Forward	NM_000089.3
	5'-GCAGTGGTAGGTGATGTTCTGAGA-3'	Reverse	
COL2A1	5'-CTGCAAAATAAAATCTCGGTGTTCT-3'	Forward	NM_033150
	5'-GGGCATTTGACTCACACCAGT-3'	Reverse	
COL10A1	5'-GAAGTTATAATTTACACTGAGGGTTTCAAA-3'	Forward	NM_000493.3
	5'-GAGGCACAGCTTAAAAGTTTTAAACA-3'	Reverse	
FYN	5'-AAGACTCTTAAACCAGGCACAATGT-3'	Forward	NM_002037.5
	5'-CATGATCTGCGCTTCCTCAA-3'	Reverse	
IHH	5'-CCTTGTCAGCCGTGAGGCCG-3'	Forward	NM 002181.4
	5'-GCTGCCGGCTCCGTGTGATT-3'	Reverse	
MMP13	5'-CATCCAAAAACGCCAGACAA-3'	Forward	NM_002427.4
	5'-CGGAGACTGGTAATGGCATCA-3'	Reverse	
SOX9	5'-CTTTGGTTTGTGTTCGTGTTTTG-3'	Forward	NM_000346.3
	5'-AGAGAAAGAAAAAGGGAAAGGTAAGTTT-3'	Reverse	
YWHAZ	5'-TCTGTCTTGTCACCAACCATTCTT-3'	Forward	NM_003406
	5'-TCATGCGGCCTTTTTCCA-3'	Reverse	

2.6 Bioinformatics

Preliminary bioinformatics analyses were performed with Partek® Flow® software (Version 10.0.21.0302, Copyright © 2021, Partek Inc., United States) as previously described [46]. Briefly, raw input reads were trimmed to achieve a quality score beyond 20 and aligned to the reference human genome hg38 using the STAR 2.7.3a aligner. Aligned reads were quantified to a transcript model (hg38-RefSeq Transcripts 94 - 2020-05-01) using the Partek E/M algorithm. Genes were filtered using a gene count cut-off of at least 50 to reduce noise. Filtered reads were normalized in sequential order using the Add: 1.0, TMM, and Log 2.0 methods. Statistical analysis was performed using an analysis of variance (ANOVA) for treatment and biological sex; donors were assigned as a random variable. Differentially expressed genes for each comparison were determined by p-values, adjusted p-values (q-values), and fold changes. Principal component analysis (PCA) was additionally performed to assess sources of variability, including sex as a possible factor in gene expression.

2.7 Immunofluorescence

To address the first objective, caveolin-1 protein expression was examined by immunofluorescence with a modified protocol from Gilbert et al. (2016) [55]. Monolayer cultured MFC on chamber glass slides were first formalin fixed and permeabilized with 0.4% Triton-X-100. Cells were then blocked with 5% bovine serum albumin (BSA) (Cell Signaling Technology, United States) and labelled overnight with a 1:200 dilution of mouse anti-caveolin-1 primary antibodies (Novus Biologicals, United States, #NB100-615) at 4°C on an orbital shaker (150 rpm). Primary control chambers were not treated with primary antibodies. Secondary antibodies (goat anti-mouse, Abcam, United Kingdom, #ab150117)

were applied to each chamber in a 1:200 dilution for 45 minutes at room temperature, followed by 10 minutes staining with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) to visualize cell nuclei. Slides were then mounted with 1:1 glycerol to phosphate buffered saline (PBS) and visualized with an Eclipse Ti-S microscope (Nikon, Canada).

2.8 Statistical Analysis

Gene expression results from objective one were analyzed with a student's t-test on SPSS version 26. RNA sequencing data from objective two was analyzed with the Partek® Flow® software. For analysis in Table 3.1, ANOVA was used to compare the RNA-sequencing gene expression between mechanical treatment and static control groups independently for each sex as well as the values from combining both sexes. Adjusted p-values (q-values; adjusting for the false discovery rate) of < 0.05 was used as a threshold for statistical significance. Correlation analysis was conducted on the normalized expression values of genes from the RNA sequencing dataset. Heatmaps were generated by calculating the pairwise Pearson correlation coefficients between genes and statistical significance was determined by paired t-tests with Prism 9 (GraphPad). RT-qPCR data from objective two was analyzed on Prism 9 including a logistic regression to validate RNA sequencing results, paired t-tests to compare gene expressions between CHP and SMG groups within each sex, and unpaired t-tests to compare gene expressions between female and male groups within each mechanical treatment. A p-value of $p<0.05$ was considered statistically significant.

Chapter 3

Results

3.1 Caveolin Gene and Protein Expression in Human MFC[6]

To support our original TEM findings, we assessed the gene expression of caveolin-1 (*CAV1*), caveolin-2 (*CAV2*), and caveolin-3 (*CAV3*) in human MFC by RT-qPCR. Both *CAV1* and *CAV2* were found to be expressed in MFC, with *CAV1* expressed to the greatest degree (Figure 3.1a). The expression of *CAV1* was also found to be significantly higher than both *CAV2* ($p<0.001$) and *CAV3* ($p<0.001$). To further visualize the protein expression of caveolin-1, we additionally performed immunofluorescence on a monolayer culture of human MFC. Caveolin-1 was found localized at the plasma membrane of MFC, supporting its expression at the protein level (Figure 3.1b).

3.2 Caveolae-Related Gene Expression in Response to Mechanical Stimulation

To investigate whether caveolae play a role in MFC mechanotransduction, we assessed the gene expression of *CAV1*, *CAV2*, and Fyn (*FYN*) —a gene encoding one of the key downstream signaling factors of caveolin-1 [56], [57]—of engineered human meniscus in response to CHP, SMG, and static culture conditions with RNA sequencing. The expression

[6] These results are part of the previously published study: M. J. Vyhlidal and A. B. Adesida, "Mechanotransduction in meniscus fibrochondrocytes: What about caveolae?," *Journal of Cellular Physiology*, vol. 237, no. 2, pp. 1171-1181, 2022 [1].

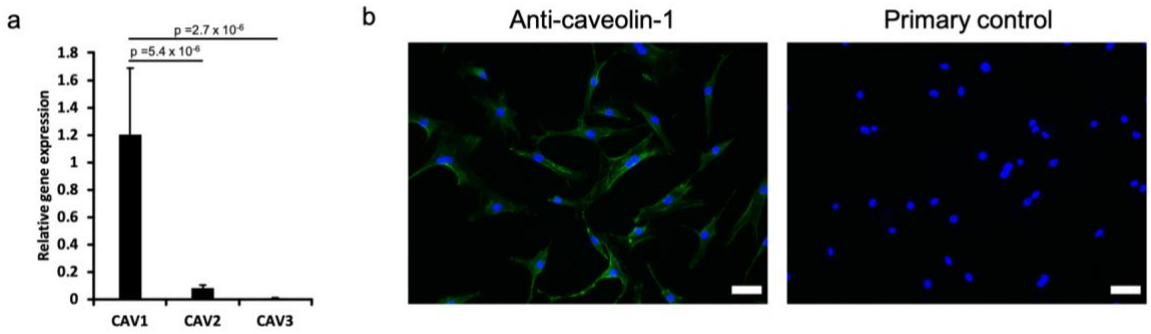

Figure 3.1 Gene and protein expression of caveolins in human MFC. (a) Relative gene expression of caveolins-1, 2, and 3. Values shown are $2^{-\Delta CT}$ values from RT-qPCR. Data were analyzed with a student's t-test. n=6 donors (in triplicates: M1, M2, F1-F4). (b) Immunofluorescent localization of caveolin-1 in monolayer cultured MFC (M3). Caveolin-1 (green) is shown to be localized at the plasma membrane. Similar findings were demonstrated by Schwab et al. (1999) in an *in vivo* culture of both young and adult rat meniscus fibrocartilage. Primary control shows no evidence of nonspecific binding of the primary antibody. Nuclei are labelled by DAPI as blue. Magnification ×200, 50 μm scale bar.

of *CAV1*, *CAV2*, and *FYN* for each treatment group was normalized to static controls and are presented as fold changes in Table 3.1. The expression of *CAV1* was found to be significantly higher in both CHP (1.68-fold, $q<0.01$) and SMG (1.40-fold, $q<0.05$) in comparison to static controls. *CAV2* expression was also significantly higher in CHP (1.34-fold, $q<0.01$) but remained the same in SMG (-1.04-fold, $q=0.81$). *FYN* expression, when compared to the control group, was significantly higher in SMG (1.34-fold, $q<0.01$) and slightly lower in CHP (-1.14-fold, $q=0.16$). To explore whether *CAV1*, *CAV2*, and *FYN* expressions were different between males and females, the data was subsequently sorted by sex and fold changes were calculated respectively and are presented in Table 3.1. For the most part, no biological sex differences were observed. A few differences in statistical significance were observed between the male and female groups, which was most likely due

to inter-donor variability affecting the analysis. To assess this, we performed a PCA analysis to assess sources of variability and did not identify sex as a dominant factor, as a large overlap between the male and female donors was present (Figure 3.2).

Table 3.1 RNA-sequencing fold changes of caveolae-related genes across treatment groups and by biological sex. Statistical analysis using ANOVA was used to compare gene expression between treatment and static control groups independently for each sex and for the combined values. *q < 0.05, **q < 0.01, non-significant differences labelled as 'ns'.

Gene	**Fold change: CHP/Static**			**Fold change: SMG/Static**		
	Combined	**Females**	**Males**	**Combined**	**Females**	**Males**
CAV1	1.68^{**}	1.92^{*}	1.51^{ns}	1.40^{*}	1.40^{ns}	1.40^{ns}
CAV2	1.34^{**}	1.42^{*}	1.28^{ns}	-1.04^{ns}	1.07^{ns}	-1.15^{ns}
FYN	-1.14^{ns}	1.00^{ns}	-1.30^{ns}	1.34^{**}	1.49^{*}	1.21^{ns}

3.3 Caveolae-Related Gene Expression Correlations

To determine the potential molecular interactions underlying these gene expression changes observed, *CAV1*, *CAV2*, and *FYN* expressions were correlated with the expression of mechanically responsive genes listed in Table 3.2. Pearson correlation coefficients and heatmaps for the pairwise comparisons are presented in Table 3.2 and Figure 3.3, respectively. Majority of the correlations were weak in both treatment groups, however, there were a number of genes that significantly correlated with the expression of *CAV2*. When correlations were sub-divided by sex, some correlations were shown to be directionally different between males and females, which was likely again due to inter-donor variability (Figure 3.4). When assessing the gene expression correlations between the caveolae-related genes themselves, all of the correlations were not significant.

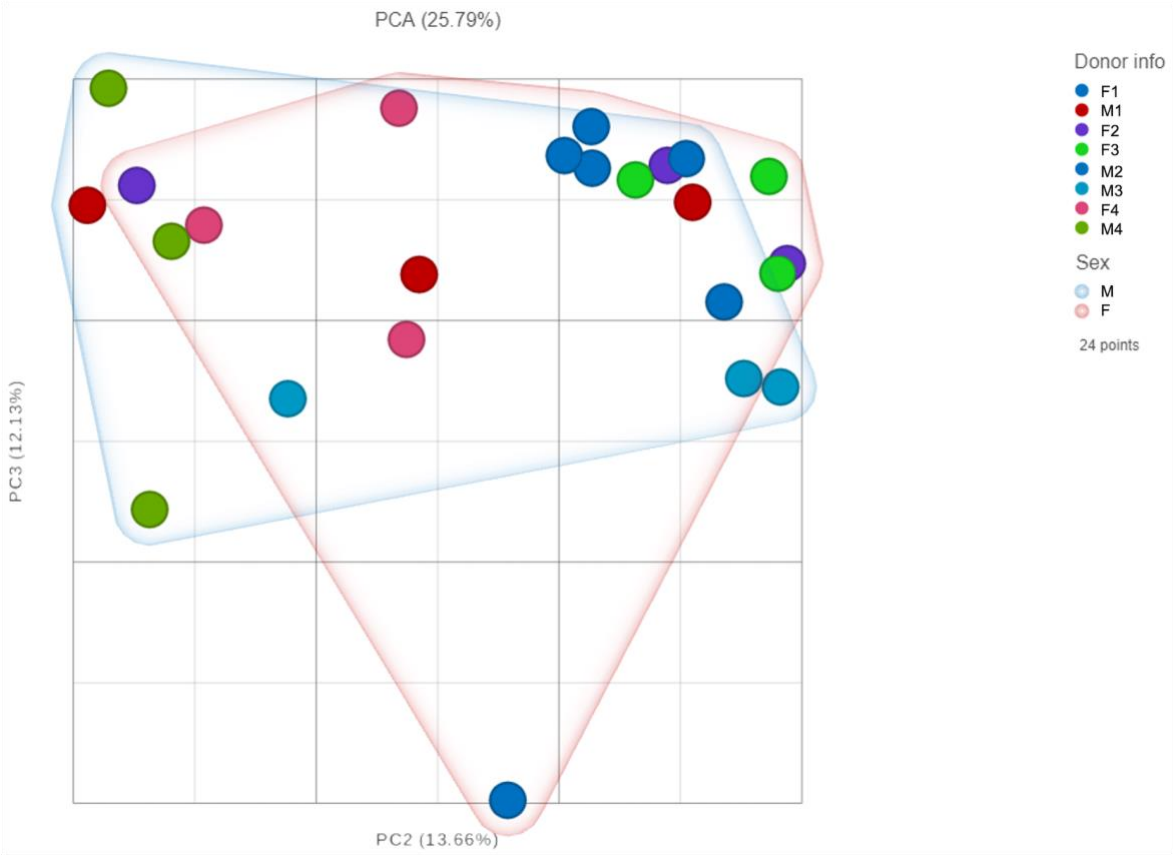

Figure 3.2 Principal component analysis (PCA) plot. Clustering of RNA-sequencing samples are by donor (color) and sex (highlight). PC2 and PC3 explains for 13.66% and 12.31% of the total variance, respectively.

3.3.1 Correlations with FA signaling factor expression

The expression of *CAV1*, *CAV2*, and *FYN* was correlated with the expression of several genes involved in FA signaling. The genes determined were based off previous findings from Sedding et al. (2005) and Wary et al. (1998) and relationships observed in the FA-KEGG pathway. Firstly, *CAV1*, *CAV2*, and *FYN* expressions were correlated with the gene expression of integrins previously identified in caveolae-mediated FA signaling, including *ITGA1*, *ITGA5*, and *ITGB1*. For the most part, correlations were similar between mechanical treatment groups and not significant. A notable difference between treatment groups was that

Table 3.2 Gene expression correlations. Pearson correlation coefficients comparing the expression of *CAV1*, *CAV2*, and *FYN* to the expression of a select panel of genes. Analysis was performed on normalized expression values. Correlations were calculated independently for both treatment groups and assessed for significance with paired t-tests. *p<0.05, **p<0.01, ***p<0.001.

Gene	**Description**	**Cyclic Hydrostatic Pressure (CHP)**			**Simulated Microgravity (SMG)**		
		CAV1	***CAV2***	***FYN***	***CAV1***	***CAV2***	***FYN***
I: Caveolae-related genes							
CAV1	Caveolin-1	–	0.31	-0.29	–	-0.06	< 0.01
CAV2	Caveolin-2	0.31	–	0.02	-0.06	–	< 0.01
FYN	FYN Proto-Oncogene, Src Family Tyrosine Kinase	-0.29	0.02	–	< 0.01	< 0.01	–
II: Integrin subunit genes							
ITGA1	Integrin Subunit Alpha 1	0.40	-0.30	-0.30	0.45	-0.47	-0.35
ITGA5	Integrin Subunit Alpha 5	0.45	0.32	-0.06	0.25	-0.54*	0.31
ITGB1	Integrin Subunit Beta 1	-0.24	-0.16	-0.22	-0.21	-0.18	-0.33
III: PI3-K/Akt signaling genes							
AKT1	AKT Serine/Threonine Kinase 1	-0.13	0.31	0.05	-0.26	0.29	< 0.01
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha	0.40	-0.09	0.06	0.53*	-0.10	-0.10
PTK2	Protein Tyrosine Kinase 2	-0.21	0.66**	0.47	-0.4	0.64**	-0.17
ROCK1	Rho Associated Protein Kinase 1	0.50**	0.45	-0.10	0.29	0.50	0.03
IV: Ras-ERK signaling genes							
ELK1	ETS Transcription Factor ELK1	0.40	0.28	0.25	0.13	-0.35	0.37
GRB2	Growth Factor Receptor Bound Protein 2	-0.16	-0.46	0.04	-0.16	-0.55*	0.09
HRAS	HRas Proto-Oncogene, GTPase	0.17	-0.30	-0.24	0.15	0.18	0.29
MAP2K1	Mitogen-Activated Protein Kinase Kinase 1	-0.06	0.75***	0.37	-0.21	0.77***	0.14
MAPK1	Mitogen-Activated Protein Kinase 1	-0.24	-0.80***	-0.07	0.10	-0.61*	< -0.01
MAPK3	Mitogen-Activated Protein Kinase 3	0.41	0.02	-0.47	0.01	-0.26	-0.73**
RAF1	Raf-1 Proto-Oncogene, Serine/Threonine Kinase	0.59*	0.58*	-0.37	0.40	0.16	-0.16
SHC1	SHC Adaptor Protein 1	0.04	-0.19	0.40	0.03	-0.43	0.47
SOS1	SOS Ras/Rac Guanine Nucleotide Exchange Factor 1	0.06	-0.49	0.27	0.22	-0.39	-0.25
V: Mechanosensitive genes							
EHD2	EH Domain Containing 2	0.06	-0.29	0.01	-0.12	-0.67**	< -0.01
FOS	Fos Proto-Oncogene, AP-1 Transcription Factor Subunit	0.27	0.06	0.29	-0.01	0.74***	0.12
FOSB	FosB Proto-Oncogene, AP-1 Transcription Factor Subunit	0.33	-0.1	0.16	0.48	0.11	0.32
JUN	Jun Proto-Oncogene, AP-1 Transcription Factor Subunit	-0.03	-0.82***	0.08	0.14	-0.81***	-0.17
JUNB	JunB Proto-Oncogene, AP-1 Transcription Factor Subunit	< 0.01	0.65**	0.43	-0.23	0.79***	0.10

Table 3.2 Gene expression correlations (continued)

Gene	Description	Cyclic Hydrostatic Pressure (CHP)			Simulated Microgravity (SMG)		
		CAV1	***CAV2***	***FYN***	***CAV1***	***CAV2***	***FYN***
V: Mechanosensitive genes							
TRPV4	Transient Receptor Potential Cation Channel Subfamily V Member 4	-0.03	0.73**	0.42	< 0.01	0.72**	0.16
YAP1	Yes Associated Protein 1	0.35	0.33	0.18	0.21	-0.11	0.18
VI: ECM-related genes							
ACAN	Aggrecan	-0.06	0.71**	0.39	0.13	0.54*	0.18
COL1A2	Collagen Type I Alpha 2	-0.61*	0.07	0.27	-0.76***	0.12	-0.22
COL2A1	Collagen Type II Alpha 1	0.02	0.69**	0.42	0.03	0.74**	0.13
SOX9	SRY-Box Transcription Factor 9	-0.28	0.46	0.56*	-0.14	0.78***	0.10
VII: Osteoarthritis-related genes							
ADAMTS5	A Disintegrin and Metalloproteinase With Thrombospondin Motif 5	-0.46	-0.59*	-0.14	-0.51*	-0.24	-0.48
CD36	CD36 Molecule	-0.45	-0.13	0.60*	-0.11	0.04	0.67**
COL10A1	Collagen Type X Alpha 1	-0.17	0.51*	0.41	0.09	0.71**	0.21
IBSP	Integrin Binding Sialoprotein	-0.34	0.44	0.53*	-0.08	0.83***	0.3
IHH	Indian Hedgehog Signaling Molecule	-0.24	0.58*	0.54*	-0.09	0.82***	0.14
MMP13	Matrix Metalloproteinase 13	0.02	-0.26	-0.15	-0.47	-0.35	0.36
PTCH1	Patched 1	-0.28	0.62**	0.56*	-0.25	0.81***	0.14
RUNX2	RUNX Family Transcription Factor 2	-0.38	-0.33	0.31	-0.60*	-0.37	0.03
SPP1	Secreted Phosphoprotein 1	0.04	0.65**	0.38	0.19	0.48	0.28
VEGFA	Vascular Endothelial Growth Factor A	-0.25	0.52*	0.47	-0.4	0.59*	-0.18

the correlations between *CAV2* and *ITGA5* expressions were directionally different, slightly positive in CHP (r=0.32; p=0.23) and significantly negative in SMG (r=-0.54; p<0.05).

Gene expression correlations were further calculated for the expression of *CAV1*, *CAV2*, and *FYN* with the expression of genes encoding proteins involved in phosphoinositide 3-kinase (PI3-K)/protein kinase B (Akt) signaling, including *AKT1*, *PIK3CA*, *PTK2*, and *ROCK1*. Gene correlations were again shown to be similar between CHP and SMG, with a few of the correlations identified as significant. *CAV1* was found to correlate strongly and positively with the expression of *ROCK1* (r=0.50; p<0.01) in CHP, and *PIK3CA* (r=0.53; p<0.05) in SMG. *CAV2* correlated strongly and positively with *PTK2* in both CHP (r=0.66; p<0.01) and SMG (r=0.64; p<0.01). The expression of *FYN* was not shown to significantly

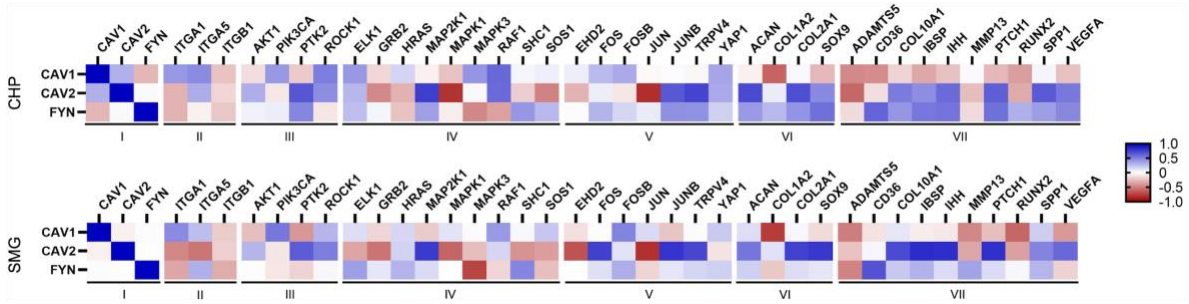

Figure 3.3 Gene expression correlation heatmaps. *CAV1*, *CAV2*, and *FYN* expressions were correlated with the expression of a panel genes including I) the genes themselves, genes encoding II) integrin subunits, III) PI3-K/Akt proteins, and IV) Ras-ERK proteins involved in FA signaling, V) mechanosensitive genes, VI) ECM-related genes, and VII) osteoarthritis-related genes. Pearson correlation coefficients were calculated independently for both treatment groups.

correlate with any genes' expression. There was, however, a noticeable difference in the relationship between *FYN* and *PTK2* expressions across treatment groups, as the expression of these genes was positively correlated in CHP (r=0.47, p=0.06) and weakly negatively correlated in SMG (r=-0.17, p=0.53).

The expression of *CAV1*, *CAV2* and *FYN* was also correlated with the expression of genes encoding proteins in the Ras-ERK signaling cascade, including *ELK1*, *GRB2*, *HRAS*, *MAP2K1*, *MAPK1*, *MAPK3*, *RAF1*, *SHC1*, and *SOS1*. *CAV1*, *CAV2*, and *FYN* expressions were both positively and negatively correlated with the expression of these genes with no apparent patterns. When comparing between CHP and SMG, correlational directions were similar for most gene combinations, with mainly the magnitude of the correlations differing between the treatment groups. For instance, the gene expression correlations between *FYN* and *MAPK3* were moderately negative in CHP (r=-0.47, p=0.067) and strongly negative in SMG (r=-0.73, p<0.01). *CAV2* was found to correlate significantly with a few of these genes

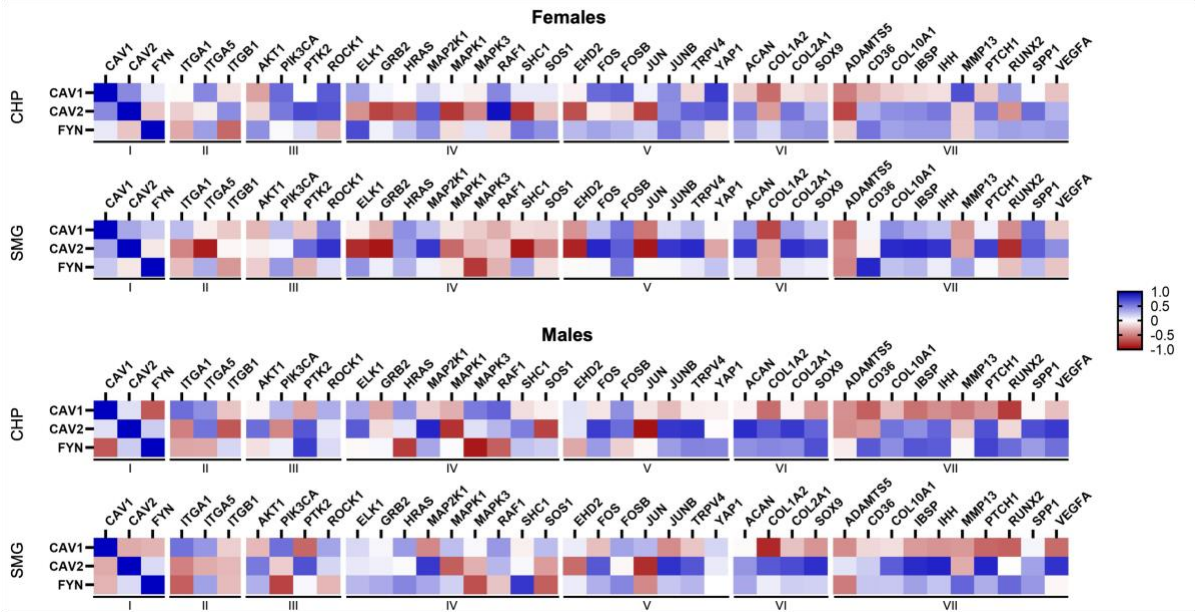

Figure 3.4 Gene expression correlation heatmaps by sex. *CAV1*, *CAV2*, and *FYN* expressions were correlated with the expression of a panel genes including I) the genes themselves, genes encoding II) integrin subunits, III) PI3-K/Akt proteins, and IV) Ras-ERK proteins involved in FA signaling, V) mechanosensitive genes, VI) ECM-related genes, and VII) osteoarthritis-related genes. Pearson correlation coefficients were calculated independently for both sexes and treatment groups.

including *MAP2K1* (r=0.75; p<0.001), *MAPK1* (r=-0.80; p<0.001), and *RAF1* (r=0.58; p<0.05) in CHP and *GRB2* (r=-0.55; p<0.05), *MAP2K1* (r=0.77; p<0.001), and *MAPK1* (r=-0.61; p<0.05) in SMG.

3.3.2 Correlations with mechanosensitive gene expression

CAV1, *CAV2*, and *FYN* expressions were correlated with the expression of mechanosensitive genes, including *FOS*, *FOSB*, *JUN*, *JUNB*, and *TRPV4* [45], [58], as well as genes that encode proteins that function in caveolae-mediated mechanotransductive events such as *EHD2* and *YAP1* [26]. For the most part, the gene expression correlations were positive in

both treatment groups. Correlational directions remained the same for most gene combinations but varied in magnitude between treatment groups. *CAV2* expression showed a number of significant correlations with these mechanosensitive genes, including *JUN* ($r=-0.81$, $p<0.001$), *JUNB* ($r=0.65$, $p<0.01$), and *TRPV4* ($r=0.73$, $p<0.01$) in CHP, and *EHD2* ($r=-0.67$, $p<0.01$), *FOS* ($r=0.74$, $p<0.001$), *JUN* ($r=-0.81$, $p<0.001$), *JUNB* ($r=0.79$, $p<0.001$), and *TRPV4* ($r=0.72$, $p<0.01$) in SMG. Correlations with *CAV1* and *FYN*, however, were all not significant.

3.3.3 Correlations with ECM-related gene expression

CAV1, *CAV2*, and *FYN* expressions were also correlated with the expression of key genes involved in ECM synthesis in MFC, including *ACAN*, *COL2A1*, and *SOX9*, and fibrocartilage marker *COL1A2* [45]. In both treatment groups, *CAV2* and *FYN* expressions, for the most part, correlated positively with all four genes. A number of these correlations were significant, including the *CAV2* correlations with *ACAN* ($r=0.71$, $p<0.01$) and *COL2A1* ($r=0.69$, $p<0.01$) in CHP, and *ACAN* ($r=0.54$, $p<0.05$), *COL2A1* ($r=0.74$, $p<0.01$), and *SOX9* ($r=0.78$, $p<0.001$) in SMG. The expression of *CAV1,* on the other hand, was significantly negatively correlated with the expression of *COL1A1* in both CHP ($r=-0.61$, $p<0.05$) and SMG ($r=-0.76$, $p<0.001$).

3.3.4 Correlations with osteoarthritis-related gene expression

CAV1, *CAV2*, and *FYN* expressions were correlated with the expression of genes known to be highly expressed in osteoarthritic cartilage, including *ADAMTS5*, *CD36*, *COL10A1*, *IBSP*, *IHH*, *MMP13*, *PTCH1*, *RUNX2*, *SPP1*, and *VEGFA* [59]–[62]. The expression of *CAV1*

correlated negatively with these genes whereas, the expression of *CAV2* and *FYN* was mostly positively correlated for both treatment groups. *FYN* expression correlated more strongly with osteoarthritis-related gene expression in CHP than SMG, with significant positive correlations with *CD36* ($r=0.60$; $p<0.05$), *IBSP* ($r=0.53$; $p<0.05$), *IHH* ($r=0.54$; $p<0.05$), and *PTCH1* ($r=0.56$; $p<0.05$) in CHP and only *CD36* ($r=0.67$; $p<0.01$) in SMG. The opposite was true for *CAV2*, as correlations for the most part, were stronger in SMG than CHP. In SMG, the expression of *CAV2* correlated significantly with *COL10A1* ($r=0.71$, $p<0.01$), *IBSP* ($r=0.83$, $p<0.001$), *IHH* ($r=0.82$, $p<0.001$), *PTCH1* ($r=0.81$, $p<0.001$) and *VEGFA* ($r=0.59$, $p<0.05$), whereas in CHP, all of these correlations were weaker.

3.4 RT-qPCR Validation and Analysis

To validate the RNA-sequencing results, the expression of *ACAN*, *ADAMTS5*, *CAV1*, *CAV2*, *COL1A2*, *COL2A1*, *COL10A1*, *FYN*, *IHH*, *MMP13*, and *SOX9* was further quantified with RT-qPCR and correlated with the RNA sequencing values. RNA-sequencing and RT-qPCR expressions were shown to be strongly correlated ($r^2= 0.946$), thus validating our results (Figure 3.5).

To gain a more comprehensive look at the expression of specific genes, fold changes were further calculated for the expression of caveolae-related genes, *CAV1*, *CAV2*, *FYN*, and osteoarthritis-related genes, *ADAMTS5*, *COL10A1*, and *MMP13*, for each sex and mechanical treatment group by normalizing to static controls (Figure 3.6). Gene expressions were additionally compared between mechanical treatment groups for each sex to determine any significant differences. *CAV1*, *CAV2*, and *FYN* expressions were all shown to have similar trends to what was observed with RNA-sequencing. There were some slight

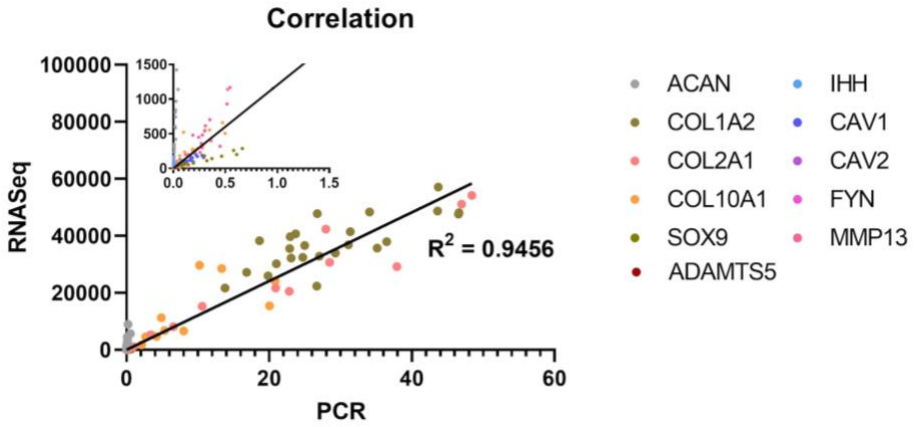

Figure 3.5 RNA-sequencing validation by RT-qPCR analysis. A panel of eleven genes were selected for further RT-qPCR validation analysis. Normalized RNA-sequencing expression values were plotted against the $2^{-\Delta\Delta Ct}$ values from RT-qPCR for each respective gene.

differences in the fold change values when compared between the two analytic methods, however, these differences were negligible as they were identified in groups with no significant differences between conditions. When comparing between mechanical treatment groups, *CAV1* and *CAV2* expressions were higher in CHP than SMG, and differences approached significance ($p=0.069$) and significance ($p<0.05$) for females, respectively. Male groups showed a similar trend, but the differences were not significant. *FYN* expression was similar between sexes and was shown to increase following SMG exposure only, with no significant differences between the treatment groups. The expression of matrix modulating enzymes *ADAMTS5, MMP13*, and chondrocyte hypertrophy marker *COL10A1*, were affected by mechanical treatment, however, there were no significant differences between treatment groups or sexes. *ADAMTS5* expression was slightly decreased in females and remained like the control group for males, whereas the expression of *COL10A1* and *MMP13*

increased in both males and females (approx. 2 to 3-fold), with females and males showing slightly higher expressions of *COL10A1* and *MMP13*, respectively.

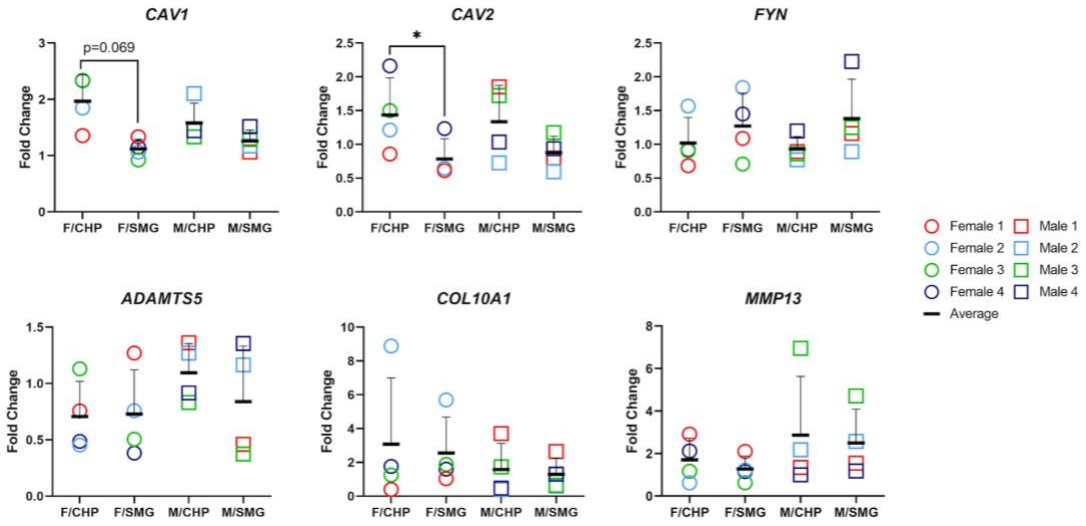

Figure 3.6 RT-qPCR analysis of caveolae and osteoarthritis-related gene expression. Fold changes were calculated independently by mechanical treatment group for both males and females. Circles and squares correspond to female and male donors, respectively. Fold changes were assessed for significance using paired t-tests to compare gene expressions between CHP and SMG groups within each sex, and unpaired t-tests to compare gene expressions between female and male groups. All comparisons not marked with '*' were not statistically significant. *$p < 0.05$.

Chapter 4

Discussion

4.1 Discussion of Results

To explore the first objective of this thesis, we began by assessing the gene expression of *CAV1*, *CAV2*, and *CAV3* in human MFC. Both *CAV1* and *CAV2* were shown to be expressed, with the expression of *CAV1* significantly higher than *CAV2* ($p<0.001$). *CAV3* was not expressed as we had predicted, as *CAV3* is exclusively expressed in muscle cells [26]. The expression of caveolin-1 was also identified at the protein level with immunofluorescence, where it was localized at the plasma membrane of the cells. Together, these results supported our initial TEM findings, as they demonstrated both gene and protein expression of caveolin-1, a protein critical for caveolar formation [26].

To next assess whether these structures function in mechanotransduction, we engineered 3D meniscal tissue models from human MFC and exposed them to either CHP, SMG, or static control conditions for 3 weeks. CHP and SMG were used as *in vitro* models to mimic physiological mechanical loading and unloading of the meniscus, respectively [63]. Following 3 weeks of mechanical stimulation, RNA-sequencing was then used to determine gene expression changes between the conditions. To investigate whether caveolae and one of their key mechanotransductive mediators, Fyn, were responsive to these mechanical treatments, the data was probed to assess changes in *CAV1*, *CAV2*, and *FYN* expressions. *CAV1* was found to be significantly increased in expression following both CHP (1.68-fold, $q<0.01$) and SMG (1.40-fold, $q<0.05$) treatments, whereas *CAV2* was increased significantly

only in CHP (1.34-fold, q<0.01). Increased expression of both *CAV1* and *CAV2* suggests that exposure to CHP, which mimics physiological loading, may have stimulated caveolar synthesis by MFC.

Caveolae are known to function as membrane reservoirs that help buffer mechanical stressors, like tension, at the plasma membrane both in the short and long term [64]. Short-term stimuli (< 5 minutes) are thought to be buffered through a reversible process involving the immediate flattening and disassembly of caveolae [65], whereas long term exposure to mechanical stress (> 5 minutes) has been shown to promote caveolar synthesis [66], [67]. This is achieved by increasing the expression of caveolin scaffolding proteins like caveolins-1 and 2 and is regulated by the release of cavins from caveolae upon flattening, which translocate to the nucleus and influence caveolar gene expression [64]. The increase of *CAV1* and *CAV2* observed in response to CHP in our study reflects these changes. The increase of *CAV1* (1.40-fold, q<0.05) and stable expression of *CAV2* (-1.04-fold, q=0.81) observed in response to mechanical unloading with SMG, on the other hand, may rather suggest an increase in non-caveolar associated caveolin-1 opposed to caveolar synthesis.

Caveolin-1 usually exists in two major forms at the plasma membrane, caveolar-associated and non-caveolar associated caveolin-1 [64]. The main difference between these two forms is the activity imparted by the catalytic site. Caveolin-1 is a regulatory protein that contains a caveolin scaffolding domain (CSD) within its catalytic site, that interacts differently with a number of signaling proteins depending on the localization of caveolin-1 [64], [68]. When caveolin-1 is 'bound' to caveolae, the CSD has been shown to impart inhibitory effects on several proteins, including Fyn [68], whereas the CSD of non-caveolar associated caveolin-1 has been shown to have stimulatory effects [64]. This phenomenon

may be due to interactions with caveolin-2, which, when assembled as hetero-oligomeric structures in caveolae, may block or alter the catalytic site on caveolin-1 and interfere with signaling. The differential expression of *FYN* observed between CHP and SMG treatments in our study may provide some further support for our theory. *FYN* expression (-1.14-fold, q=0.16) remained relatively unchanged following physiological mechanical loading, however, upon mechanical unloading with SMG, the expression of *FYN* increased significantly (1.34-fold, q<0.01). This suggests that Fyn signaling was likely enhanced upon mechanical unloading and may reflect a change in the activity of the CSD site and localization of caveolin-1 at the plasma membrane. This molecular event might be an important step in mechanotransduction in MFC that has not been previously discussed. We have summarized our findings and proposed a model underlying these events in Figure 4.1. We did not observe any major differences in caveolae-related gene expression between males and females, suggesting that caveolar response mechanisms are likely similar between sexes. However, due to the small sample size of males (n=4) and females (n=4) in this study, larger sample sizes would be needed to further investigate this.

To gain a better look at the relationships between *CAV1*, *CAV2*, and *FYN* expressions with different signaling events, the expression of these genes was correlated with the expression of FA signaling, mechanosensitive, ECM-related, and osteoarthritis-related genes. Overall, most of the correlations were weak. There were, however, a number of significant correlations with *CAV2* expression, suggesting that caveolin-2 may be an important mediator of mechanotransduction. Caveolin-2 is a phospho-protein that has previously been thought to function alongside caveolin-1 to fine-tune various signaling events [68], [70]. It is therefore possible that through the actions of kinases, caveolin-2

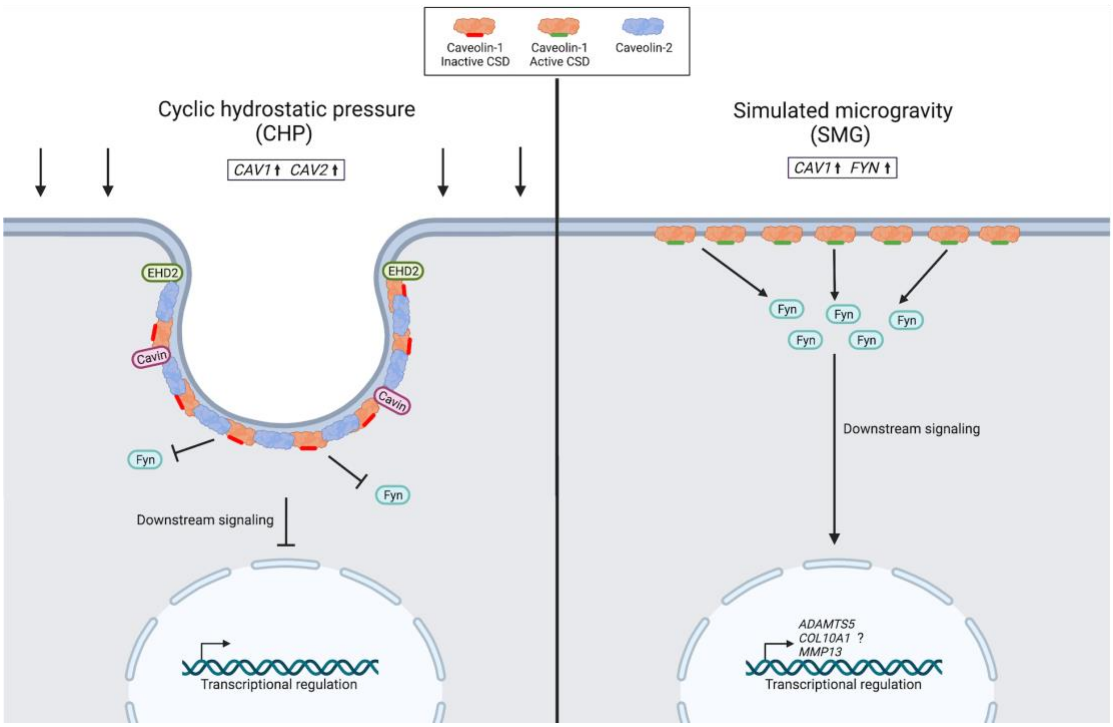

Figure 4.1 Proposed model of caveolar signaling in response to the mechanical treatments[7]**.** Caveolin structure has been adapted to reflect cryo-electron microscopy findings that unveiled the proteins' structure that has been recently summarized by Parton and Collins, 2022 [69]. Image was created with BioRender.com. ADAMTS5; a disintegrin and metalloproteinase with thrombospondin motif 5, CAV1; caveolin-1, CAV2; caveolin-2, COL10A1; collagen type X alpha 1, CSD; caveolin scaffolding domain, EHD2; EH domain containing 2, MMP13; matrix metalloproteinase 13.

phosphorylation might act as a 'switch' either promoting or inhibiting caveolin-1-mediated signal transduction in response to mechanical stimuli. Thus, this might explain the strong correlations observed between the *CAV2* and the genes investigated, as it suggests that perhaps, caveolin-2 might be a key regulator of downstream signaling. However, given the little focus on the functions of caveolin-2 in the literature, more investigation is further

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required to validate this idea. In addition to this, some correlational differences were observed between males and females. However, these changes were most likely a result of inter-donor variability and larger sample sizes would be needed to further investigate this.

Firstly, we assessed gene expression correlations between *CAV1*, *CAV2*, and *FYN* to better understand the relationships amongst caveolae-related gene expression. No significant correlations were noted, suggesting that caveolae-related genes likely do not influence the direct expression of one another. Next, *CAV1*, *CAV2*, and *FYN* expressions were correlated with the expression of several genes involved in FA signaling, including genes encoding integrin subunits and downstream PI3-K/Akt and Ras-ERK signaling proteins, to decipher possible relationships between caveolae-related gene expression and the expression of FA-signaling factors. Correlations showed that the expression of the caveolae-related genes correlated positively and negatively with a number of these signaling factors, with a few differences between mechanical treatment groups. A number of these gene expression correlations were statistically significant, including the correlations between *CAV2* with *PTK2* ($p<0.01$), *MAP2K1* ($p<0.001$), and *MAPK1* ($p<0.001$ and $p<0.05$ for CHP and SMG, respectively) in both CHP and SMG. FAs are the main structural components that link cells with the ECM and play a critical role in mechanotransduction in several cell types [71]. Given that caveolae have previously been implicated in FA-mediated mechanotransduction in other cells types [17], [26], the correlational evidence shown here may also suggest the same for MFC. Regarding the correlational differences observed between treatment groups, this may suggest that caveolae could also interact with FAs to facilitate mechanical stimulus distinction, however, most of these correlations were weak and would require more investigation to draw stronger conclusions.

To further examine the relationship between caveolae-related gene expression and mechanotransductive processes, *CAV1*, *CAV2*, and *FYN* expressions were correlated with mechanosensitive genes and ECM-related genes whose expressions are known to be modulated by mechanical stimuli. The expression of *CAV1*, *CAV2*, and *FYN* was mainly positively correlated with the expression of mechanosensitive genes with some similarities between treatment groups. The expression of *CAV2* correlated the strongest with the mechanosensitive genes and demonstrated highly significant correlations with *JUN* ($p<0.001$), *JUNB* ($p<0.001$), and *TRPV4* ($p<0.01$) for both treatment groups. Similarly, the expression of *CAV2* was also the most strongly correlated with ECM-related gene expression. The expression of *CAV2* showed strong positive correlations with the expression of *ACAN*, *COL2A1*, and *SOX9* in both conditions, which were all significant ($p<0.05$) except for the correlation with *SOX9* expression in CHP ($r=0.46$, $p=0.077$). These results, in particular, imply that caveolin-2 might be an important player in MFC mechanotransduction, as there seems to be a strong relationship between its expression with the expression of genes known to be affected by mechanical stimulation. *CAV1* expression was also found to strongly and negatively correlate with the expression of fibrocartilage marker *COL1A2* in both CHP ($r=-0.61$; $p<0.05$) and SMG ($r=-0.76$; $p<0.001$). Previous groups have identified a similar relationship between caveolin-1 and type I collagen expression and have shown caveolin-1 to have inhibitory effects on fibrosis in multiple tissues [72], [73]. Based off the correlations we observed, it is possible that caveolin-1 may similarly function in MFC to regulate fibrocartilage development of the meniscus. This deserves further exploration as this may have potential applications for meniscus tissue engineering.

To take this idea a step further, *CAV1*, *CAV2*, and *FYN* expressions were further correlated with the expression of osteoarthritis-related genes to assess possible molecular relationships between caveolar signaling and osteoarthritis. Osteoarthritis is a degenerative disease characterized by cartilage deterioration that affects the major load-bearing joints of the body, with the knee being the most affected. As mentioned earlier, abnormalities in meniscal biomechanics are thought to be one of the contributing factors in the progression and development of KOA [6]. Mechanotransductive processes in MFC play a crucial role in the pathogenesis of KOA because how these cells interpret and respond to mechanical stimuli in their microenvironment has a direct impact on the resulting biomechanics of the tissue. It is, therefore, important to incorporate osteoarthritis-related gene expression and assess the relationship with caveolae-related gene expression to build a stronger and more comprehensive discussion on MFC mechanotransduction.

Mechanical unloading of the knee joint, through limb immobilization or spaceflight microgravity, has been shown to lead to degradative changes associated with KOA in both articular cartilage [74], [75] and the meniscus [76]. SMG has been developed as an *in vitro* model of spaceflight microgravity which we showed, could promote osteoarthritis-like gene expression in our engineered tissues from human MFC [46]. Building on our findings, here, we investigated whether the osteoarthritis-like gene expression observed in SMG showed any interactions with caveolae-related gene expression. In SMG, we found the expression of *CAV2* and *FYN* to be positively correlated with the expression of a number of osteoarthritis-related genes. *CAV2* expression was found to correlate significantly with *COL10A1* ($r=0.71$, $p<0.01$), *IBSP* ($r=0.83$, $p<0.001$), *IHH* ($r=0.82$, $p<0.001$), *PTCH1* ($r=0.81$, $p<0.001$) and *VEGFA* ($r=0.59$, $p<0.05$) in SMG, whose expressions are known to be increased in

osteoarthritic articular chondrocytes [59], [60], [62]. Interestingly, *FYN* expression correlated significantly with *CD36* ($r=0.67$, $p<0.01$), a glycoprotein surface receptor associated with more severe forms of KOA [77]. *CAV1* expression on the other hand, correlated weakly with most osteoarthritis-related genes in SMG, except for *ADAMTS5* ($r=-0.51$, $p<0.05$) and *RUNX2* ($r=-0.60$, $p<0.05$) whose correlations were significantly negative. In CHP, correlational directions between the genes were similar, however the correlational strengths were weaker and stronger for *CAV2* and *FYN*, respectively, suggesting that the molecular mechanisms underlying these interactions are likely mechanically sensitive. Previous studies have shown caveolin-1 and Fyn expression to be significantly increased in osteoarthritic articular chondrocytes at both the gene and protein level [78]–[80]. Fyn in particular, has been proposed as a potential mediator of KOA pathogenesis through its alternative actions on the canonical Wnt/β-catenin signaling pathway, whereby Fyn works to phosphorylate β-catenin and promote its nuclear translocation and downstream expression of osteoarthritis-related genes [79], [80]. Knockdown or inhibition of Fyn, was shown to significantly reduce the expression of osteoarthritis-related genes *ADAMTS5*, *COL10A1*, and *MMP13* both *in vitro* and *in vivo* and corresponded to reduced cartilage degradation *in vivo* [79], [80]. Given these previous findings, and the correlations between caveolae-related and osteoarthritis-related genes herein, it is possible that caveolar signaling might have an important function in osteoarthritic-like gene expression. In particular, caveolin-2 may be a key mediator in this process whose role has not been previously described and thus should be further investigated.

To validate the gene expression evidence and further investigate the relationship with osteoarthritis-related gene expression, RT-qPCR was used to quantify the expression of

CAV1, *CAV2*, *FYN*, *ADAMTS5*, *COL10A1*, and *MMP13*. RT-qPCR results were shown to correlate strongly with our RNA-sequencing outputs (r^2= 0.946) and thus validated our initial findings. *CAV1*, *CAV2*, and *FYN* expressions showed a similar trend between treatment groups as determined by RNA-sequencing, with the expression of *CAV1* and *CAV2* upregulated in CHP and the expression of *FYN* upregulated in SMG. The expression of these genes were also similar between males and females. Gene expressions of *COL10A1* and *MMP13*, but not *ADAMTS5*, were also shown to increase following both CHP and SMG treatments for both males and females, with no significant differences observed. The increase in *FYN* expression in SMG, a model previously shown to promote osteoarthritic-like gene expression, was interesting, as it resembled gene expression changes observed in osteoarthritic articular chondrocytes [80]. Besides this change, the RT-qPCR findings were rather inconclusive with respect to a molecular relationship between caveolae-related and osteoarthritis-related gene expression. When taken together with our correlation results, and in context of previous literature, it's possible that caveolar scaffoldings proteins and their key signaling factor Fyn may play a molecular role in KOA development, however more investigation is required.

4.2 Limitations

There are a few limitations that should be considered with respect to the studies performed. Firstly, the characterization and expression of caveolae was assessed in *in vitro* cultured MFC, which may not translate to the native meniscus. It is possible that the expression of caveolae may be influenced by *in vitro* culture, thus to assess this, future work should include TEM imaging of native menisci and an assessment of caveolin gene and protein

expression in native MFC. Another limitation with respect to the second objective was the lack of protein expression data. Despite seeing interesting changes in caveolae-related gene expression in response to mechanical stimulation, this may not translate to changes at the protein level. In order to have a more complete picture, protein expression of caveolin-1, caveolin-2, and Fyn, should be assessed following CHP and SMG exposure using methods such as western blotting and immunofluorescence. Immunofluorescence in particular, would be useful technique to investigate the localization of Fyn in response to mechanical stimulation and could provide some more insight with respect to its role in signal transduction. Additionally, it would also be beneficial to assess the protein expression of ADAMTS5, COL10A1, and MMP13 to supplement the RT-qPCR results. Lastly, the limitation of sample size is something that cannot be overlooked. In both studies, the sample size was relatively small; n=6 for objective one and n=8 for objective 2. To increase the statistical power and better control for donor variability, that is inherent when working with human specimens, it would be useful to increase sample size. This however is limited by tissue availably, which is dependent on tissue procurement from surgical procedures.

Chapter 5

Conclusion and Future Directions

5.1 Conclusion

The scope of this thesis was to investigate whether caveolae play a role in mechanotransduction in human MFC. We hypothesized that caveolae do function in MFC mechanotransduction and proposed two objectives to explore the hypothesis. First, we aimed to confirm the expression of caveolins in human MFC at the gene and protein level. Next, we aimed to assess the effects of mechanical stimulation on caveolae-related gene expression. Exploring our first objective, we demonstrated both gene and protein expression of the predominant caveolar protein, caveolin-1, in human MFC. These results supplemented our initial TEM findings in engineered human meniscus tissue, and supported the presence of caveolae in human MFC. To explore the second objective, we engineered meniscus tissues from human MFC and utilized CHP and SMG bioreactors and global transcriptome analyses to assess the role of caveolae in mechanotransduction. Expression of caveolae-related genes *CAV1*, *CAV2*, and *FYN*, were found to be modulated by mechanical stimuli and was thought to reflect changes in the organization of caveolins at the plasma membrane. The expression of these genes, in particular *CAV2*, was also shown to strongly correlate with the expression of several genes related to cell signaling and mechanotransduction, including a number of osteoarthritis-related genes. When taken together, the work presented herein, suggests a plausible and novel role of caveolae in human MFC mechanotransduction, however, further investigation is required to draw stronger conclusions, as the findings are

rather preliminary. Nonetheless, this work is still valuable and provides support for our initial hypothesis.

Our current understanding of MFC and how they respond to mechanical loading in the knee joint is relatively limited. Without this knowledge, we will not be able to fully understand, nor appreciate, the intricate cellular mechanisms underlying meniscus function. Here, we presented the potential role of caveolae in MFC mechanotransduction. This discovery is valuable, as it presents new information that may address a significant gap in the field of meniscus mechanobiology. If in fact caveolae do function as mechanotransducers in MFC, not only will this knowledge further our understanding of the meniscus, but it may also be useful in the development of new therapeutic interventions for knee pathologies like KOA. Given that caveolae-related and osteoarthritis-related genes were shown to be strongly correlated, this suggests that caveolae may be involved in KOA development. Therefore, further exploring the role of caveolae in MFC is important for meniscus research and deserves more attention in the scientific community.

5.2 Future Directions

To further assess the functional role of caveolae in human MFC mechanotransduction, addition studies are needed. One approach would be to employ the use of caveolae-disrupting agents like MβCD or siRNAs against caveolins-1 and 2 [31], or the use of Fyn inhibitors like 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine (PP1) or Saracatinib (AZD0530) [79], [80] to block downstream signaling. When coupled together with mechanical stimulation, this would give us a better assessment of the functionality of caveolae in MFC and whether they are dispensable for mechanotransduction. *In vivo* studies

utilizing caveolin-1, caveolin-2, or Fyn knockout mice should also be considered. Comparing gene expression outputs of MFC between control and knockout mice subjected to weight bearing exercises, could also provide more in depth information on the role of caveolae.

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