Investigating the Effect of Exogenously Supplemented FGF-18 on Expanded Meniscus Fibrochondrocytes Using a Cell Aggregate Model

by

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A thesis submitted in partial fulfillment of the requirements of the degree of

Master of Science

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Abstract

Osteoarthritis (OA) is a disease affecting >10% of Canadians and is associated with a huge economic burden on the Canadian economy and healthcare system. The disease is characterized by a variety of symptoms including chronic pain, inflammation, and decreased joint mobility. Therefore, the development of effective treatments is being investigated. Within the knee joint, the state of meniscus health is a direct correlate of healthy joint physiology. Meniscal trauma is frequently corrected through surgical debridement in the form of partial meniscectomies; however this treatment predisposes the patient for OA development due to increased stress on surrounding articular cartilage.

Meniscus tissue engineering aims to develop new repair or replacement strategies to maintain proper joint physiology in cases of meniscal injury. Because the menisci's functional properties are derived from its extracellular matrix (ECM), tissue engineering focuses on creating biomimetic constructs that maintain biomechanical functionality by promoting meniscus-like ECM formation. This may be achieved by combining candidate cell types, experimental models, and growth factor supplementation. Fibroblast growth factors have demonstrated potent anabolic effects in a number of cell types, however they are relatively understudied in cells isolated from healthy, adult meniscal tissue.

Here we examined the effects of supplementing fibroblast growth factor 18 on differentiated, primary MFCs and expanded MFCs to assess the capacity for FGF-18 to drive the redifferentiation of MFCs to produce meniscus-like ECM formation. Our findings suggest that FGF-18 is insufficient to restore a differentiated ECM-forming phenotype in expanded MFCs.

Dedication

To my mother, who believed in me even when I didn't believe in myself

Acknowledgements

I'd like to firstly thank my supervisor, committee members, and colleagues for their expertise and mentorship; without their guidance this thesis would not have been possible. Thank you Dr. Adetola Adesida for highlighting my strengths and weaknesses and helping me become a more competent scientist. The lab environment you provided was both challenging and rewarding and forced me to become a better man – and for that I am forever grateful. In addition, the meaningful lessons you imparted on me have made a substantial impact on my life and I cannot express my gratitude enough. I would like to extend my thanks to the members of the Adesida lab for their unconditional support and providing an enjoyable work environment, including: Aillette Mulet-Sierra, Enaam Idrees, Melanie Kunze, Yan Liang, and Alexander Szojka. Thank you Aillette and Melanie for your expertise and technical support with every experiment. I'd like to thank my good friend Dr. Yan Liang for the mentorship and teaching me how to perform experiments skillfully. Thank you Drs Nadr Johma and Gregory Korbutt for their thoughtful input and allocated time for manuscript review. I'd also like to also thank Alexander Szojka for being so selfless, his constant advocacy in my behalf, and providing me with valuable insights and perspectives I may have overlooked. I'd also like to express my gratitude to Cameron Lindsey for always bringing positive energy into the laboratory and making research that much more enjoyable. I'd like to thank Kuzho Wu for his interesting demeanour and teaching me how to use chopsticks. I also like to extend my thanks to Tracey Zawalusky for being the most amazing program administrator and always going above and beyond for every student, myself included. Lastly, I'd like to express my gratitude and appreciation for the unequivocal support from my friends and family. This has been an experience of a lifetime. Thank you to everyone who played a role – I cannot thank you enough.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

AC = articular chondrocyte
ACAN = aggrecan
ACL = anterior cruciate ligament
AD-MSC = adipose-derived MSC
B2M = Beta-2-Microglobulin
BMI = body mass index
BM-MSC = bone marrow MSC
BMPs = bone morphogenic proteins
CTGF = connective tissue growth factor
DAPI = 4',6-Diamidino-2-Phenylindole, Dihydrochloride
DMEM = Dulbecco's modified eagle medium
DNA = deoxyribonucleic acid
ECM = extracellular matrix
FBS = fetal bovine serum
FGFs = fibroblast growth factors
FGFR = fibroblast growth factor receptor
FGF-2/bFGF = fibroblast growth factor 2
FGF-18 = fibroblast growth factor 18
GAG = glycosaminoglycan
HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IGF = insulin growth factors
MFC = meniscus fibrochondrocyte
MMP = matrix metalloproteinase
MMP-1 = matrix metalloproteinase - 1
MMP-3 = matrix metalloproteinase - 3
MSC = mesenchymal stem cell
OA = osteoarthritis
P0 = passage 0
P2 = passage 2
P4 = passage 4
PBS = phosphate buffered saline
PCL = posterior cruciate ligament
PDGF = platelet-derived growth factor
qRT-PCR = quantitative real time polymerase chain reaction
SD-MSC = synovium-derived MSC
SEM = standard error of the mean
T3 = TGFβ-3 control condition
T3F18 = TGFβ-3 + FGF-18 condition
TEM = transmission electron microscopy
TGFβ = transforming growth factor beta
TIMP = tissue inhibitor of metalloproteinases
TIMP-2 = tissue inhibitor of metalloproteinase 2
YWHAZ = Monooxygenase Activation Protein, Zeta Polypeptide
3D = three dimensional

Chapter 1: Introduction

1.1 Overview

Osteoarthritis (OA) is the most common degenerative joint condition, affecting >10% of Canadians aged 15 years and older[1]. The cost of treating and managing arthritis on the Canadian healthcare system and economy is estimated to be in excess $33 billion/annum when taking into consideration the prescribed medications, treatments, and loss of productivity due to the condition[2]. Although this statistic encompasses rheumatoid arthritis, osteoarthritis, and inflammatory arthritis, osteoarthritis is the most prevalent of all three and accounts for a significant portion of these costs[2]. The average age of diagnosis for osteoarthritis in Canada is 50.4 years (95% CI: 49.4-51.4) and is associated with chronic pain & inflammation, obesity, and decreased quality of life[1,3,4]. Due to the progressive nature of the disease and the correlation between age and severity of the condition, an aging population is expected to cause an increased burden on the healthcare system and Canadian economy[1,5,6]. Although the causal factor for OA remains elusive, a number of risk factors that have been identified including: acute trauma, obesity, sedentary lifestyles, increasing age, and chronic joint stress[3,7–9]. Current treatment of OA focuses on pain management and lifestyle changes following diagnosis. Thus, osteoarthritic research developing new curative and preventative treatments could make a substantial difference in the quality of life for affected patients[1,10]. Pertaining to the knee joint, current research supports the notion that osteoarthritis is a condition affecting all tissues comprising the joint, including the subchondral bone, articular cartilage, synovium, and meniscus[3,11]. Extensive research on articular cartilage and its implication in OA has been conducted, in which it is now common knowledge that the degradation of articular cartilage is directly linked with OA diagnosis and progression[12]. The meniscus, on the other hand, is understudied however its implication in OA is still highly relevant.

The menisci are semi-lunar cartilaginous wedges found within the tibiofemoral compartment of the knee joint that have major functions of transmitting stress across the joint during weight-bearing and joint stabilization[13–16]. Despite the previous misclassification that menisci are vestigial structures in the knee joint that serve no specific function, it is now clear they are critical for the health and integrity of the knee joint[13,17]. A compromised meniscus, whether due to physical trauma or idiopathic meniscal degeneration, is consistently correlated with OA diagnosis of the knee joint[18–21]. Likewise, early onset OA is frequently observed in patients following partial meniscectomies, a common treatment for meniscus trauma[22–25]. Because meniscal lesions are one of the most common issues in the orthopaedic practice and regeneration is notoriously limited to the vascular region of the meniscus, new repair techniques/methodologies are being developed due to the poor efficacy of current treatments[14,26]. The menisci's biomechanical properties are largely derived from the extracellular matrix (ECM), which is synthesized by the cells comprising the tissue. New treatment strategies aim to mimic the microarchitecture to produce a construct with similar biomechanical functionality. One potential approach is cell-based meniscus tissue engineering, in which the generation of a functional meniscus could be possible through the development of an *in vitro*-derived construct. Through the process of cell expansion, induced meniscus-like ECM formation, and growth factor supplementation, developing a tissue engineered meniscus construct with functional biomechanical properties may be possible. In this thesis I will cover a review of the meniscus, cell-based meniscus tissue engineering strategies, and the role of fibroblast growth factors and their designated receptors in the context of generating functional meniscus tissue. Lastly, we will investigate the redifferentiation capacity and ECM-forming phenotype of expanded meniscus fibrochondrocytes exogenously supplemented with Fibroblast Growth Factor 18 in a three dimensional cell aggregate model.

1.2 Anatomy of Human Meniscus

Gross Morphology and Composition

As can be seen in Figure 1.1, the superior aspect of either meniscus have a concave curvature that conforms to the convex femoral condyles and are relatively flat on the inferior surface due to conformation with the tibial plateaus[13]. The menisci are primarily composed of water (~72%) and organic matter (28%)[27]. In regards to organic matter of interest, extracellular matrix (ECM) proteins collagen and glycosaminoglycan's encompass 22% and ~0.8% respectively of the total wet-weight[2a7]. These ECM proteins have direct relation to the biomechanical properties of the meniscus which will be discussed in later sections. Despite the medial and lateral meniscus having similar functions, they have notable variations in shape and mobility. The medial meniscus appears C-shaped, whereas the lateral meniscus appears O-shaped (albeit not a complete circle)[28]. The subtle differences between the medial and lateral menisci are believed to be related to the differences in injury susceptibility between either meniscus.

Lateral Meniscus

The lateral meniscus is nearly circular, covering up to 80% of the articular surface between the lateral femoral condyle and the corresponding region of the tibial plateau[14]. The lateral meniscus, when compared to the medial, is also smaller and more mobile[29]. There are several attachment points holding the lateral meniscus in place, specifically to the intercondylar fossa anteriorly and to the PCL and medial femoral condyle via the Wrisberg and Humphrey ligaments posteriorly[14]. The lateral meniscus is typically injured comorbidly with ACL-related injuries[30]. Baker, Pupparo, and Sanborn (1985) found that 61 per 100,000 related meniscus injuries required some form of

meniscectomy. Of the surgeries performed, 81% were on the medial meniscus and the remaining 19% on the lateral meniscus[31].

Medial Meniscus

The medial meniscus covers less of the total articular surface than its lateral counterpart (60% versus 80%, medial meniscus: lateral meniscus)[18]. The anterior horn of the medial meniscus is attached to the tibia plateau by the intercondylar fossa, whereas the posterior horn is attached to the posterior intercondylar fossa of the tibia[14]. The medial meniscus has larger surface area than the lateral meniscus and is also thicker posteriorly. Due to these properties, there may be less synovial diffusion, making the medial meniscus more susceptible to degeneration because there is a decreased healing capacity coupled with increased nutritional demands[32]. The medial meniscus is more frequently injured than the lateral meniscus and typically affect posterior regions[30].

Cell Morphology and ECM Composition

The principal cell type comprising the meniscus are collectively referred to as meniscus fibrochondrocytes (MFCs), however their morphology and ECM composition changes as a function of region. Specifically, collagen comprises the majority of the dry weight (DW) of the meniscus (~75% DW), but the type and distribution is not constant[33]. In the vascularized periphery MFCs appear fibroblastic in morphology, with an elongated spindle-like cell body[27,34,35]. These MFCs produce ECM predominately comprised of type I collagen, and are surrounded by extensive connective tissue[27,36]. The outer 2/3 of the meniscus is approximately 80% collagen, with collagen I as the predominant form with only trace amounts of collagen II, III IV and VI[27]. These collagen fibres are oriented parallel to the peripheral regions of the meniscus, connecting the anterior horn

insertional ligament to the posterior horn insertional ligament[18]. These fibres transmit axial loading stress by converting it into hoop stresses within the meniscus tissue, contributing to the tensile strength of the tissue[13,19,37–39]. The regions of circumferentially-aligned collagen I fibres are interspersed with radial fibres which function to prevent splitting of the longitudinal array of the circumferentially oriented collagen I fibres[27,40]. The inner region of either meniscus is composed of cells similar to those of articular cartilage and are termed chondrocytes or fibrochondrocytes, which are morphologically round with increased collagen II production[12,27]. The ratio of type I: type II collagen in the inner 2/3 of the meniscus is approximately 2:3[35]. Another major component of the ECM in meniscus tissue is proteoglycans, specifically aggrecan[41]. Proteoglycans contribute to the viscoelastic properties of the meniscus and its ability to withstand compressive forces[42,43]. Through assays targeting chondroitin sulphate glycosaminoglycans, a carbohydrate-based substituent associated with the core protein in aggrecan, it is known that aggrecan content is highest in the inner portion of the meniscus. Aggrecan provides a hydrated gel structure with high viscoelasticity, thus aiding in the weight-bearing properties of the tissue and facilitates the tissues ability to resist compressive loading[42,44].

1.3 Physiology

Load Transmission

The primary function of the menisci is to transmit load across the tibiofemoral joint during weight bearing[13–16]. Due to the wedge-shaped conformation of the meniscus, it increases congruency of the knee joint and contact area, thus decreasing contact stress on the articular cartilage[45]. By transmitting force over a larger area, the resultant stress on the articular cartilage is reduced, acting as a protective factor for osteoarthritis. It has been documented that during knee extension, 40-

60% of the load is transmitted to the meniscus and up to 90% during flexion[46]. Loading on the meniscus can be intuitively understood by considering how force vectors are transmitted through the tissue. Upon axial loading on the knee, the wedge-shaped menisci experience a force with both vertical and horizontal components. The menisci transmit and distribute the vertical force component due to their congruency with the femur and tibia: the vertical, downward force on the upper meniscal surface from the femur is balanced by the vertical, upward force from the tibia. However, the horizontal force component must also be balanced to prevent lateral extrusion of the tissue from the joint. This is accomplished by the peripheral circumferential collagen I bundles and the rigid meniscal horn attachments to the midline region of the tibial plateau. As the meniscus is loaded, it initially displaces laterally, but the stiff collagen bundles experience tensile loading with a net force in the opposite direction, thereby balancing out this force as well. This phenomenon results in partial conversion of axial forces loaded on the knee joint into hoop stresses within the tissue[18]. The meniscus is critical in this function, for it is known that following meniscectomies significant degenerative changes occur in the knee joint[47]. More specifically, the subchondral bone flattens, joint space narrows, the formation of osteophytes occur, and articular cartilage degenerates[14,47]. Excision of meniscal tissue causes increased contact-point stress on the articular cartilage leading to their progressive degeneration and increases the risk for osteoarthritis development[48].

Secondary Functions

The meniscus also has several secondary functions related to the overall homeostatic balance within the knee joint. Shock absorption is commonly believed to be a function of the menisci, however conflicting theories currently persist[49,50]. On one hand, Voloshin and Wosk (1983) found that in patients who underwent meniscectomies there was approximately 20% decrease in overall shock-absorption ability based off accelerometry data measuring bone vibrations in the proximal tibia during gait. It is postulated that this is due to the viscoelastic properties of the proteoglycans present in the inner portion of the menisci. Proteoglycans absorb water and have excellent compressive capabilities, theoretically making them suitable for shock absorption[14]. Consequently, removal of meniscal tissue decreases the shock-absorbing properties of the remaining tissue. A review performed by Andrews et al (2011), however, suggests the contrary. Energy dissipation in viscoelastic tissue occurs in the form of molecular rearrangements or the movement of fluid that generates heat. Because elastic potential energy is not a form of shock-absorption (eg. the viscoelastic properties of the meniscus), it cannot be interpreted as such.

The menisci are also involved in joint stability, proprioception, and play a vital role in the overall health of the surrounding articular chondrocytes[13,14,16,19].

Regional Vascularity

The menisci receive their vascularization from branches of the popliteal artery, specifically the medial, lateral and middle geniculate arteries, forming a capillary network perfusing the peripheral region of the meniscus and joint capsule of the knee[26]. This blood supply, however, is limited to a small portion of the outer periphery which poses significant implications in the healing capacity of the menisci. Towards the peripheral region, only 10-30% and 10-25% of the medial and lateral meniscus, respectively, have direct blood supply from the blood vessels mentioned above[18,51]. The remaining 65%-75% is avascular and receives nourishment via diffusion through synovial fluid[13]. Based off the regional differences in vascularization and cell types comprising these regions, the meniscus is divided into three distinct zones. The vascularized peripheral regions are termed the red-red region and are conducive of a healing response following injury, whereas the avascular inner region is the called the white-white region and has an impaired healing response (Figure 1.2)[14,18]. The intermediate zone, called the red-white zone, has characteristics of both regions. This has major implications for overall joint physiology for meniscal injuries of the avascular region are ineffectively repaired, inevitably leading to progressive OA[23]. Just as the vascularity is related to different functionality of the meniscus in terms of healing capacity, we see a similar relationship in regard to cell morphology and ECM composition while moving from the outer periphery to the inner regions.

1.4 Meniscus Injury, Classification and Treatments

Overview

Meniscal tears are one of the most common injuries affecting the knee joint[19,52]. Because intact functional menisci are essential for proper knee joint integrity, repair of meniscal tears is imperative. Each year over 1 million surgical interventions are performed due to meniscal tears[53]. The two primary forms of meniscal injury result from trauma or gradual degeneration[54,55]. Meniscal injuries typically occur from combined compressive and rotational forces at the tibio-femoral joint as the knee changes from flexion to extension, particularly during pivoting-like movements[13]. Several risk factors for symptomatic meniscal tears have been recognized, including BMI > 25kg/m^2, male gender, and occupations or sports requiring chronic kneeling, squatting, and stair climbing[30]. Following meniscal tears, especially in the lateral meniscus, a 'pop' or 'clicking' sound is commonly reported when ACL tears occur comorbidly[16]. Depending on a variety of factors such as the type, location, and reducibility of the lesion (tear characteristics), and patient-specific traits such as age and weight, different treatment modalities are optimal[18].

Classification of Meniscal Tears

Meniscal tears are classified based off the orientation of the lesion. As can be seen in Figure 1.3, there are five types of tears known to occur in the meniscus, which are: vertical longitudinal, vertical radial, horizontal, oblique, and complex/degenerative[18]. Due to the specific arrangement of collagen fibres throughout the meniscus (as discussed previously), certain types of tears are more common in some regions than others and have a range of healing outcomes. In particular, tears affecting the outer region, such as vertical longitudinal tear, are associated with the best healing outcomes. These tears can be symptomatic because the biomechanical function of the

meniscus isn't drastically affected: the meniscus can still effectively convert tibio-femoral load into hoop-stresses within the tissue[13,18] Likewise, because these tears occur in the vascular region of the meniscus, repair is possible. This can be achieved through endogenous healing mechanisms or surgical approaches such as suturing depending on the severity of the lesion. A problematic version of this tear occurs when a longitudinal tear twists, forming a "buckethandle" tear[13,18]. These tears are generally symptomatic, unstable and are corrected via meniscectomies. Tears of the avascular region, such as radial tears, begin at the inner portion of the meniscus and propagate towards the peripheral regions[56]. When symptomatic, these types of tears are common targets of meniscectomies due to the avascularity of the inner region and inability to effectively heal. Complete vertical radial tears perturb the circumferential collagen fibrils in the periphery, impairing the ability of the meniscus to transmit load across the tibiofemoral joint[18]. Thus, even a minor radial tear can significantly disrupt meniscus function[57]. Horizontal tears split the meniscus into an upper and lower portion and are most often found in the posterior horn[18,58]. These types of tears typically occur in patients >40 years of age with no precipitating traumatic incident, and as such are sometimes categorized as a type of degenerative tear[58]. Patients affected with horizontal tears typically report symptoms of pain during periods of increased physical activity. These types of tears are normally stable but may give rise to flap tears[18]. Oblique tears are mechanically unstable and are targets of surgical resection in attempts to preserve biomechanical functioning in the remaining tissue[18]. Lastly, complex/degenerative tears occur when more than one tear is present in the same meniscus or a tear occurs in more than one plane[18,59]. These types of tears are typically symptomatic and result from repeated knee trauma[51].

Meniscus Tear Treatment

Historically, damaged menisci and the corresponding symptoms were believed to be alleviated via a complete meniscectomy. Progressive OA consistently followed the surgical procedure and was thought to be a result of insufficiently performed complete meniscectomies so surgeons advocated for the removal of more tissue. At the time, the meniscus was believed to be vestigial, having no apparent function in the knee and was found in the tibiofemoral compartment as an embryological remnant[17]. Current literature, however, posits that the meniscus is critical in the proper functioning of the knee joint, evident by the dysfunctional time-course following meniscectomies which inevitably lead to OA development[60]. Baratz et al (1986) found that following total medial meniscus meniscectomy there was an overall 75% decrease in intra-articular contact area and the peak contact pressure was elevated by approximately 235%[48]. Likewise, in a separate study Ahmed and Burke (1983) found that following a meniscectomy there was a 100-200% increase in contact pressure[61]. In light of these findings, current treatment methods focus on restoring meniscus function following injury and to perform meniscectomies only when absolutely necessary. Currently, the gold standard for meniscal lesions, particularly those affecting the inner regions, is the partial meniscectomy.

Partial meniscectomies are a common treatment for meniscal tears, particularly for symptomatic ruptures found within the white-white avascular region that is either incapable of healing or is irreparable[18,51]. When tears in this region occur surgeons enter the knee joint arthroscopically and resect the damaged portion of the affected meniscus, attempting to preserve as much of the intact, functional regions of the meniscus. Northmore-Ball, Dandy, and Jackson (1983) found that arthroscopically-assisted partial meniscectomies had increased overall patient satisfaction 4.3

years post-operation compared to open total meniscectomies[62]. Partial meniscectomies are most suited for meniscal tears affecting the avascular region of the meniscus, for it is unethical and illogical to remove the peripheral vascularized portion of the meniscus that is capable of self-healing (with exceptions). Partial meniscectomies are ideal for a variety of meniscal tears, in particular flap, bucket-handle, radial horizontal, and degenerative tears[13]. These tear types are most suited for debridement for a number of regions. Firstly, if the collagen fibre arrangement is disrupted (as is the case for flap tears, bucket-handle tears, and radial tears) the biomechanical function of the meniscus tissue is impaired. If the lesion is in the white-white region the healing potential is low through endogenous mechanisms or surgical intervention. Lastly, if the tear is likely to become degenerative it is more logical to perform the partial meniscectomy before any other adverse joint alterations take place[13,63]. Optimal prognosis for full recovery include age <40, one simple lesion, and minimal time differential between injury and data of surgical correction[64]. Partial meniscectomies are an effective treatment method for pain alleviation and reduce joint swelling, however there are long term repercussions[35]. One major drawback of partial and complete meniscectomies is an increased incidence of knee OA[47]. The progression and development of knee OA is more severe in cases of full meniscectomies, however there are physiological alterations to the knee joint in either case that predispose an individual for OA development. One explanation for this is due to the increase contact pressure on the articular chondrocytes. As can be seen in Figure 1.4, the state of the meniscus is closely related to the overall health of the joint. On the lateral femoral condyle there is clear evidence of subchondral bone degradation that coincides with the degenerated meniscus. On the medial condyle, which is fully intact, a healthy meniscus was dissected with no indication of subchondral bone breakdown. Despite this being a porcine knee joint, it is still relevant to the physiological state in humans.

Taken altogether, it is clear that better meniscus repair and replacement strategies are needed since meniscus debridement through partial meniscectomies results in detrimental changes to the knee joint, predisposing the patient for OA development. Other repair strategies that have varying efficacies include suturing, rehabilitation, conduit abrasion healing, and platelet rich plasma therapy[66–69].

Meniscus Repair

Meniscus repair is another approach to preserve knee functionality following meniscus injury. Suturing is a method of choice for symptomatic meniscal lesions affecting the vascularized periphery[70]. In the 1980s it was discovered that surgically-induced transverse medial meniscal lacerations in canines could be corrected with one Dexon suture, where a 94% healing rate 4 months post-operation was observed in a sample size of 20 canines[71]. Remarkably, healing was sufficient to protect underlying articular cartilage suggesting the preservation of meniscal tissue and facilitating the repair process is a better alternative than removing the damaged tissue (when possible)[71]. Longitudinal lacerations in the avascular inner portion, however, were incapable of healing. This further supports the notion that vascularity is directly related to the healing capacity of the meniscus and that suturing would only be effective for lesions affecting the red-red or red-white regions of the menisci.

Treatments Conclusion

Although a multitude of meniscal-injury related treatments are currently available the overall efficacy is low. Damaged menisci are a predisposing factor for progressive articular cartilage degeneration and other pathological changes to the knee joint which inevitably lead to OA development[23]. Because the success of meniscus repair strategies relies heavily on numerous factors (including age, type of tear, location of tear, physical activity level) and degenerative or non-repairable tears in the white-white region cannot be effectively corrected other than through partial meniscectomies, new techniques are being developed that focus on cell-based strategies to overcome these shortcomings. Meniscus tissue engineering is one promising avenue that blends numerous scientific disciplines with the intention of developing new meniscus replacement and repair strategies.

1.5 Meniscus Tissue Engineering

1.5.1 Overview

The aim of meniscus tissue engineering is to develop functionally equivalent meniscus replacements or efficacious repair strategies to restore normal joint function and reduce the risk of osteoarthritis development[35]. Due to the high degree of heterogeneity of the meniscus, in regards to regional ECM composition and biological function, some current techniques favor the use of multiple cell types in hopes of recapitulating the native meniscus properties[14,35]. It is known that the inner regions of the meniscus are primarily composed of aggrecan and type II collagen producing chondrocyte-like MFCs compared to the peripheral region which is rich in type I collagen producing fibroblast-like MFCs[27,42]. The compressive integrity of the meniscus is related to its proteoglycan content, whereas the tensile strength is due to collagen I circumferential fibres

in the periphery[27]. It is postulated that the regional differences in mechanical properties are the result of different cell phenotypes and niche. Cell-based meniscus tissue engineering strategies combine: 1) cell source; 2) an experimental model and; 3) growth factor supplementation. These variables are collectively used in attempts to synthesize functional meniscus tissue. Each will be considered in turn.

1.5.2 Cell Sources for Meniscus Tissue Engineering

A variety of cell types have been investigated for meniscus tissue engineering purposes, including: meniscus fibrochondrocytes and articular chondrocytes, as well as several types of mesenchymal stem cells (MSCs) such as bone marrow MSCs, adipocyte derived MSCs, and synovial-fluid/membrane derived MSCs[35]. These cell types have different characteristics including their anatomical site, morphology, and differentiating capacity, and have potential for meniscus tissue engineering.

Meniscus fibrochondrocytes (MFCs)

The principal cell type comprising the meniscus are collectively referred to as meniscus fibrochondrocytes (MFCs)[72,73]. These cells are ideal for meniscus tissue engineering since they are pre-programmed to have an ECM-forming phenotype[27,73]. MFCs naturally reside in the meniscus and produce the ECM that confer biomechanical functionality to the tissue. Specifically, the outer fibroblast-like MFCs deposit extensive type I collagen arranged in circumferential fibres in contrast inner chondrocyte-like MFCs that produce higher proportions of type II:type I collagen and increased proteoglycan synthesis which correspond with tensile and compressive properties, respectively[27,36]. Due to the availability of meniscus tissue excised during partial meniscectomies,

MFCs are an abundantly available cell type for meniscus tissue engineering purposes. Instead of discarding debrided meniscus tissue, research laboratories can isolate the MFCs for experimental use. MFCs display robust meniscus-like ECM-forming phenotypes and are responsive to growth factor supplementation and oxygen tension in culture[74]. When partial meniscectomized meniscus biopsies are received, 100% MFC isolation yields is unlikely due to cell death. In addition, only having a portion of the whole meniscus necessitates *in vitro* cell expansion. This process will be discussed in later sections but is characterized by a loss of ECM-forming capacity, a detrimental outcome when ECM is critical for proper biomechanical functioning[41]. MFCs also demonstrate trilineage potential, where MFCs isolated from meniscal tissue have been shown to differentiate towards chondrogenic, adipogenic and osteogenic lineages suggesting at least a portion of the MFCs exist in a plastic state[75]. Another useful characteristic of MFCs is that since they are an autologous cell source, this reduces the likelihood of immune rejection when newly synthesized constructs are implanted into the patient.

Articular Chondrocytes

Articular chondrocytes (ACs) are cells that comprise articular cartilage and are used in meniscus tissue engineering for a variety of reasons[76]. First, due to the low cell availability of MFCs, as mentioned previously, co-culturing MFCs with another cell type may be necessary[35]. ACs are known to produce an ECM with high type II collagen and proteoglycan content, resembling the ECM found within the inner meniscal regions[35,76]. Co-culturing ACs with MFCs may promote a superior ECM forming phenotype than either cell type alone by mitigating the need for excessive cell proliferation *in vitro* which is associated with a change in ECM-forming capacity[35]. In a study by Yang et al (2012), the researchers performed an indirect co-culture with bone marrow MSCs

and healthy articular chondrocyte and found that the ACs drove the differentiation of BM-MSCs to chondrocyte-like phenotypes through a proposed paracrine mechanism. Thus, it appears ACs secrete chondro-inductive biomolecules that may lead to a superior ECM phenotype when cultured with MSCs. All in all, ACs are promising cell source for meniscus tissue engineering and may have potential utility in a coculture model.

Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are a type of stem cell derived from connective tissues. This cell type has the capacity to differentiate into somatic cells including osteoblasts (bone cells), chondrocytes (cartilage cells), adipocytes (fat cells), and myocytes (muscle cells)[77]. In the context of meniscus tissue engineering, MSCs may be useful for their extensive proliferative ability, multilineage differentiation, and anti-inflammatory characteristics[78–80]. MSCs are also useful via their trophic effects. In particular, MSCs secrete growth factors and other cytokines that act in autocrine or paracrine fashion, stimulating anti-apoptosis, angiogenesis, differentiation, and proliferation[81]. These characteristics of MSCs may be exploited for restorative purposes of the meniscus. Zellner et al (2010) demonstrated the effectiveness of MSCs to heal meniscus defects[82]. 2mm meniscal "punches" were perforated through the avascular region of rabbit menisci and either left empty or filled with hyaluronan-collagen composite matrices. The holes or matrices were then left empty or filled with a number of different cell types. The punches that were filled with non-precultured mesenchymal stem cells integrated completely and showed the best prognosis compared to any other condition. The International Society for Cellular Therapy has encouraged the adoption of a stringent set of criteria to help identify MSCs used in experimental settings. MSCs must adhere to plastic in

culture, positively stain for CD105, CD73, and CD90, and have the potential to differentiate into osteoblasts, adipocytes, and chondrocytes[83]. MSCs can be collected from a variety of sources, including bone marrow, synovial membrane/fluid, and fat tissue[78]. In the following sections we will look at these in turn and their use in meniscus tissue engineering.

Bone Marrow Mesenchymal Stem Cells

Bone marrow mesenchymal stem cells are a type of multipotent stem cell isolated from bone marrow that have the capacity to differentiate into tissues of mesoderm origin[84]. In the context of meniscus tissue engineering, BM-MSCs appear to have a great deal of potential for facilitating repair. Numerous studies, both *in vivo* and *in vitro* design, suggest BM-MSCs promote effective repair in macro and microscopic meniscal injury[78,85–87]. It appears BM-MSCs differentiate towards chondrogenic lineages and produce a fibrocartilage-like ECM, however preservation of biomechanical functionality is not observed which necessitate further investigation. A major drawback with using BM-MSCs is cell hypertrophy, a cardinal stage of the osteogenic differentiation pathway that eventually leads to bone formation[88]. Additionally, the highly invasive procedure for bone marrow aspiration make BM-MSCs less accessible than other sources.

Synovium-derived Mesenchymal Stem Cells (SD-MSCs)

SD-MSCs are one of the cell types comprising the synovial membrane in articulating bones[89]. This stem cell population SD-MSCs has been implicated in meniscus tissue engineering due to their chondrogenic properties[35]. It was observed that these cells, when cultured *in vitro,* produce an ECM composition similar to that of chondrocytes. Likewise, SD-MSCs appear to respond to

fibroblast growth factors with increased collagen II expression and other chondrocyte-like

phenotype similarities. Not only do SD-MSCs display high chondrogenic potential, but direct

injection at the site of meniscal defect appears to promote healing. In a study by Mizuno et al

(2008) the researchers found that following injection of exogenous SD-MSCs into an induced

meniscal defect in a rat model, the SD-MSCs were not only recruited to the site of damage but

also differentiated into cartilage cells[90]. Additionally, SD-MSCs display multi-lineage potential

and have been implicated as a potential cell source for musculoskeletal regeneration of a variety

of tissues including meniscus, cartilage, tendon, and ligaments[91]. In addition to mesenchymal

stem cells present in the synovial membrane, there is also evidence for MSCs in the synovial

fluid. These cells, termed synovial fluid (SF) fibroblasts or SF-MSCs, display similarities to

MSC in terms of their chondrogenic potential[92]. Interestingly, the origin of these cells is

currently unknown. Literature posits that the MSCs present in synovial fluid may migrate from

the synovial membrane[92,93]

Adipose-Derived Mesenchymal Stem Cells:

Adipose-derived MSCs (AD-MSCs) are another popular cell source used in meniscus tissue

engineering based off high availability, minimally invasive isolation procedures, and

chondrogenic potential[94–97]. AD-MSCs have displayed self-renewing capacity and the ability to

undergo chondrogenic, osteogenic, adipogenic, and myogenic differentiation and as such appear

to have stem-cell like characteristics[96,97]. Isolation of adipose tissue typically occurs through

lipoaspiration or removal of fatty tissue during other surgical procedures. Adipose tissue in

humans are either classified as brown or white, depending on location and function. White-

adipose is used to store triglycerides for energy and the main target of lipoaspiration in contrast

to brown-adipose which is produced during development and virtually non-existent in mature adult humans[94]. As such, white adipose tissue is typically used for meniscus tissue engineering based off abundance and availability. One noteworthy point using AD-MSCs for meniscus tissue engineering is there is some degree of heterogeneity in experimental responses based off anatomical site of isolation.

1.5.3 Cell culture models

To assess the capacity of the cells mentioned above to undergo chondrogenesis and form new fibrocartilage resembling native meniscus ECM composition, the next step in meniscus tissue engineering is to select an optimal cell culture model. Two commonly used models include a 3-dimensional pellet model or larger scale scaffolds. Following selection of a cell type and expanding it *in vitro* to increase cell numbers, expanded cells may be centrifuged to form cell aggregates or seeded onto scaffolds and cultured *in vitro* or *in vivo* depending on the study.

3-Dimensional Pellet Model

The 3 dimensional (3D) pellet model is a useful in meniscus tissue engineering to promote the formation of quality, cartilage like tissues as well as test the effects of biological substituents such as growth factors[98]. This model mimics naturally occurring developmental processes that take place during embryogenesis. In particular, mesoderm-derived tissue formation, including cartilage and bone, are initiated during cell condensation[99–101]. By reducing intracellular space and allowing cells to come in close proximity to each other, the regional microenvironment promotes terminal differentiation towards cartilage and bone formation. In the 3D pellet model, this biological process is recapitulated and promotes chondrogenesis. During expansion de-

differentiating processes occur (will be covered in section 1.7), however these effects can be mitigated to some degree using a 3D pellet model. Specifically, this model has been shown to promote the re-differentiation of expanded cells into chondrocyte-like phenotypes and increase ECM synthesis by driving chondrogenesis[102,103]. In addition, by keeping all conditions constant in exception of a particular growth factor being supplemented, the effects can be measured with hopes of identifying anabolic growth factors that promote differentiated ECM-forming phenotypes. For the purposes of my study we will be using this model as a means of assessing redifferentiating capacity as measured by ECM-formation.

Scaffold

Scaffolds are another model used in meniscus tissue engineering, particularly as a means of recreating the microarchitecture within the meniscus. Created from a variety of materials, the main purpose of scaffolds is to provide a structural network that facilitates cell proliferation, ECM deposition, diffusion of nutrients, and other biomechanical considerations[104,105]. The long-term objective is to seed the scaffolds with cells and induce chondrogenesis such that the construct will have similar biomechanical properties as the native meniscus. Meniscus fibrochondrocytes, articular chondrocytes, and MSCs have all been used in scaffold studies due to their ability to promote the formation of chondro-specific ECM. The scaffold provides a structural framework in which the seeded cells may proliferate on, subsequently taking on the scaffold conformation. Thus, the scaffold serves as a template for the final shape of the newly derived engineered tissue. Most scaffolds are designed to slowly resorb over time so the final product is entirely cell-derived[8]. A variety of scaffolds are currently being used in meniscus tissue engineering, including hydrogels, decellularized ECM, collagen derived scaffolds, and

synthetic polymers such as polycaprolactone[106–110] . Currently there are two types of scaffolds

being used in clinical studies which are the CMI-menaflex and Actifit[111].

1.5.4 Bioactive Agents

Growth Factors Implicated in Meniscus Tissue Engineering:

A third component for consideration in meniscus tissue engineering is selection of bioactive

agents such as growth factors[112]. Growth factors are extracellular proteins or steroid hormones

that bind target cell receptors and induce expression of genes through second messenger systems

or signaling pathways that are involved in regulating cell growth, proliferation, and death[112–114].

In the context of meniscus tissue engineering, growth factors serve a variety of important

functions. They can be used to promote cell proliferation, ECM synthesis, prevent de-

differentiation during cell expansion, and increase cell survival[74,115,116]. With long-term goals of

creating a cell-derived meniscus replacement, the cellular response to growth factors is of

particular interest. By inducing anabolism and promoting ECM synthesis, creating functional

tissue that has ideal functional mechanical properties may be possible. Growth factors can be

supplemented at multiple levels during meniscus tissue engineering, such as expansion phase or

in chondrogenic culture model that was selected. Investigating the role of specific growth factors

in vitro used in meniscus tissue engineering will hopefully promote the development of

efficacious treatments for meniscus degeneration and trauma. A large number of growth factors

have been investigated, including insulin growth factors (IGF), bone morphogenic proteins

(BMPs), connective tissue growth factor (CTGF), platelet derived growth factor (PDGF) and a

number of others to varying degrees of success[115,117–119]. Two noteworthy growth factor families,

the transforming growth factor β superfamily and fibroblast growth factor family have displayed promising results and will be considered in closer detail.

Transforming Growth Factor β Superfamily

Transforming growth factor β's (TGF-β) are a superfamily of polypeptide cytokines that function to regulate development, growth, and inflammation[120,121]. As of 1994, 74 TGFβ-like sequences have been identified which are believed to represent approximately 23 genes[122]. These sequences were isolated from a range of species including mammals, insects, and amphibians which highlights the evolutionary conservation of these cytokines. Members of the TGFβ superfamily are essential for life, where knockout experiments demonstrate a lethal phenotype[123]. A group of researchers used transgenic mice with a null TGFβ-1 mutation such that no TGFβ -1 was produced, even at a transcription level and found that 50% of rats died in-utero. Furthermore, of the remaining 50% of the progeny that made it to birth, chronic and excessive states of inflammation were highly prevalent two weeks post-parturition and the rats eventually succumbed to the chronic inflammation. Members of the TGFβ family have been implicated in every stage of cartilage development, from embryogenesis through to maintaining and regulating adult cartilaginous tissues which suggests a homeostatic function[124]. As such, TGFβs are commonly investigated in the context of meniscus and cartilage tissue engineering. In cases of osteoarthritis, TGFβ signalling pathways appear to be disrupted and may play a role in the progression of the disease[125]. Two particular members, TGFβ-1 and 3, are regularly mentioned in meniscus tissue engineering research due to their anabolic and differentiating effects on MFCs, promoting stable ECM-forming phenotypes rich in type II collagen and proteoglycans[126]. Because proteoglycans and collagens are key components of the ECM that directly relate to the

biomechanical functioning of meniscus tissue, supplementation of TGFβ during chondrogenesis yields desirable phenotypes.

1.6 Fibroblast Growth Factors and Receptors

The fibroblast growth factors are a group of secreted polypeptides that are known agonists of tyrosine kinase receptors[127,128]. Twenty-three FGFs have been identified thus far with protein weight ranging from 17 kDa to 34kDa and are widely expressed in variety of adult cells[127–129]. FGFs have been implicated in a number of cellular functions including repair, cell proliferation, differentiation, neuronal protection, metabolism, osteogenesis modulation, and development[128,130–132]. Based off knockout experiments targeting FGF/FGF receptor signalling pathways, and the occurrence of a wide variety phenotypic irregularities, current literature supports the notion that FGF/FGFR signalling is involved in development and tissue homeostasis[127]. There are 4 known FGF receptors, FGFR1-4[127,128]. These receptors are comprised of 3 domains, specifically an extracellular binding domain, a single transmembrane domain, and an intracellular tyrosine kinase domain[40]. Following receptor activation by binding with an FGF ligand, the receptor dimerizes leading to autophosphorylation of the intracellular domain[127]. Activated FGFRs have been implicated in regulating a variety of pathways including the RAS-MAPK pathway, P13K-AKT pathway, and PLCγ[127,129,133]. Cellular response is variable depending on other dynamic conditions, however the RAS-MAPK pathway has been implicated in the mitogenic response of cells to FGFs, the PI3K-AKT pathway appears to regulate cell survival, and the PLCγ pathway is involved in cell motility[132–136]. An interesting characteristic of FGF receptors are the various splice variants/isoforms that exist. Following alternative splicing of the primary mRNA transcript, different IgG-like extracellular domains denoted as IgG IIIa,

IgG IIIb, and IgG IIIc can be the derived for FGF receptors 1-3, which alters ligand-receptor specificity and affinity[129,137]. Some proposed roles of FGFs that may be beneficial in meniscus tissue engineering include induction of anabolism, states of anti-catabolism, mitogenesis, and increasing the redifferentiation capacity of expanded MFCs[74,138,139]. Thus, FGF members are potential growth factor candidates for optimizing current meniscus tissue engineering strategies and may promote the development of new therapeutic applications altogether. In the following section we will consider FGF-2 and FGF-18 in detail and their role in meniscus tissue engineering.

1.6.1 Fibroblast growth Factor 2

Also referred to as basic fibroblast growth factor (FGF-2), this growth factor is primarily used in tissue engineering to increase cell proliferation rates[130,140–142]. The downstream effects of cell expansion using FGF-2 is dependent on the cell type, however. For example, one study showed culturing human articular chondrocytes isolated from knee cartilage in alginate beads supplemented with FGF-2 resulted in increased proliferation rates accompanied with a 50% decrease in proteoglycan synthesis[143]. These results suggest FGF-2 mediates a mitogenic and catabolic effect resulting in elevated proliferations rates and increased ECM degradation in terms of proteoglycan content. In a separate experiment by another group of researchers, expansion of meniscus fibrochondrocytes isolated from the avascular region of an osteoarthritic knee joint (primarily containing chondrocyte-like MFCs) in the presence of FGF-2 provided contrasting results[74]. In particular, FGF-2 expanded MFCs had increased proliferation rates during expansion and the production of key ECM components including type II collagen and aggrecan during chondrogenic induction were significantly upregulated. These results were aligned with previous

findings by Tumia and Johnstone (2004) where the researchers found that regardless of the region MFCs were isolated from within the meniscus (eg. any of the three zones), proliferation rates and glycosaminoglycan production were increased when MFCs were expanded in the presence of FGF-2[140]. Thus, the effects of FGF-2 supplementation may be dependent on cell type, where FGF-2 appears to induce a mitogenic and catabolic response in articular chondrocytes in contrast to MFCs where a mitogenic and increased redifferentiating capacity effect is observed. Based off immunofluorescent FGF receptor (FGFR) detection, Yan et al (2011) identified the primary ligand-receptor interaction of FGF-2 with its designated FGF receptor (FGFR). Although FGF-2 has the capacity to activate all four FGFRs, it has highest affinity for FGFR-1 and FGFR-3. To determine if the catabolic effects of FGF-2 in articular chondrocytes was mediated through FGFR1 or FGFR3, the researchers included an FGFR1 inhibitor while culturing the articular chondrocytes in the presence of FGF-2. The presence of the FGFR1 inhibitor resulted in proliferation rates and matrix production at comparable levels to the control condition, suggesting the catabolic and mitogenic effects of this growth factor are mediated through FGFR1 agonism. Additionally, culturing AC in the presence of FGF-2 also downregulated expression of FGFR3, suggesting an interaction with this receptor was taking place however did not appear to modulate a mitogenic or catabolic response seen with agonism of FGFR1.

1.6.2 Fibroblast Growth Factor 18

Fibroblast growth factor 18 (FGF-18) is a more recently discovered member of the FGF family

that has been implicated in a variety of cellular processes including the induction of

chondrogenesis and anabolism, differentiation, and regulation of cartilage homeostasis[131,138,144].

Because the ECM synthesized by meniscus fibrochondrocytes (MFCs) endows the tissue with its

biomechanical properties, identifying anabolic growth factors that facilitate ECM production

may be valuable for efficacious treatments to be developed in meniscus tissue engineering.

Although a multitude of research has been performed studying the effects of FGF-18 in various

cell types, the effects of FGF-18 supplementation on human MFCs is underrepresented.

Nevertheless, by considering the effects of FGF-18 in related cell types, including articular

cartilage and mesenchymal stem cells, this may provide insight on the potentially beneficial

effects of FGF-18 supplementation in MFCs. In particular, FGF-18 has been documented to

induce a chondrogenic response when supplemented in micromass cultures of pre-differentiated

embryonic mesenchymal stem cells. In a study by Davidson et al (2005), the researchers

developed knockout mice at the FGFR3 locus and isolated embryonic mesenchymal stem cells

from developing limb buds and cultured these cells in the presence or absence of FGF-18. They

found that in the presence of FGF-18 in the FGFR3 wildtype strain, there was an increase in type

II collagen immunofluorescence compared to the basal group (Figure 1.5). Additionally, the

knockout-derived micromass cultures had no trace of type II collagen whatsoever, regardless of

the presence or absence of FGF-18. This suggests that FGF-18 upregulates type II collagen

synthesis at the protein level and mediates its effect at least partially through interacting with

FGFR3. In another study, Yan et al (2011) demonstrated that FGF-18 supplementation in

articular chondrocytes promotes anabolism in terms of proteoglycan synthesis. The researchers

also showed that articular chondrocytes respond to FGF-2 supplementation by increasing rates of cell division, mediated through FGFR1. Because it is known that FGF-2 can induce effects in MFCs, this suggests not only that MFCs express the necessary FGF receptor but also that FGF-18 may be able to confer some sort of benefit in terms of anabolism and ECM synthesis as well. Another group of researchers indicated that FGF-18 induces anti-catabolism in articular chondrocytes through the upregulation of Tissue inhibitor of metalloproteinases (TIMPs). TIMPs function to inhibit Matrix metalloproteinases (MMPs), which are known for remodelling ECM in a catabolic fashion. By upregulating TIMPs and inhibiting MMPs, a state of anti-catabolism was observed, promoting the formation and deposition of quality ECM while minimizing its degradation. FGF-18 has been shown to have affinity for FGFR1-3, however most research documenting the anabolic effects of FGF-18 to be mediated through agonism of FGFR3[129]. As of late, three isoforms have been characterized for this receptor, specifically the FGFR3 IIIa, IIIb, and IIIc isoforms[137]. The IIIa isoform is a secreted extracellular protein in contrast to the IIIb and IIIc which are membrane bound[145]. Following activation of the IIIa variant, no known signalling pathways appear to be activated. The IIIb isoform of FGFR1-3 is preferentially expressed in epithelial tissue in contrast to the IIIc isoform which is expressed in mesenchyme derived tissues[145]. Due to the various FGF isoforms, and their differential expression pattern, FGFs role and modulatory effects depend on stage of development.

In the context of therapeutic application in humans, a clinical trial using recombinant FGF-18 (trade name Sprifermin) was approved in 2010 but was terminated early. Here we briefly highlight the trial below:

MERCK Clinical Trial

In February 2010, a phase 2 clinical trial was approved that aimed to provide a treatment to alleviate the symptoms of progressive knee joint osteoarthritis through intra-articular (IA) injection of Sprifermin (recombinant FGF-18) into the knee joint of affected patients. This trial was funded by MERK and used humans at various stages of osteoarthritis development with hopes to identify an optimal concentration of Sprifermin that would lead to the best prognostic outcomes. The clinical trial was terminated early due to low recruitment, however. Looking at the experimental results provided on the clinical trials website, this may be due to the questionable efficacy of the treatment as opposed to low recruitment numbers that the authors claimed. In the primary outcomes measured, testing 0, 10, 30, and 100µg Sprifermin IA injections, the 12 month change in cartilage defect volume relative to baseline was relatively equal between the experimental and control conditions, where the decrease in cartilage thickness was comparable between all groups. Growth factor availability and clearance could potentially be a confounder in this study which highlights why it is important to develop effective delivery mechanisms for *in vivo* studies.

Overall, FGF-18 supplementation appears to promote cartilage-like ECM-forming phenotypes that may be beneficial in meniscus tissue engineering. In light of this, we decided to investigate the role of FGF-18 in expanded meniscus fibrochondrocytes. No research has been published using human MFCs *in vitro* and we are excited to be the first.

1.7 Cell Expansion and De-differentiation

Now that a cell type has been selected (meniscus fibrochondrocytes), a model (3D cell pellet),

and the bioactive agent to be tested (FGF-18), the final phase in *in vitro* meniscus tissue

engineering is experimental execution. This is achieved by initially inducing cell division *in vitro*

followed by a chondrogenic induction period (eg. 3D pellet model). Because partial

meniscectomy biopsies are only a fraction of the whole tissue, deriving an entire meniscus

replacement with equivalent cellularity would be impossible without initially inducing cell

division. As such, meniscus fibrochondrocytes (MFCs) are frequently passaged in monolayer to

allow the acquisition of sufficient number of cells for experimental use and replacement

construct production. As MFCs are expanded, however, a phenotypic change occurs at multiple

levels. This process, termed de-differentiation, is associated with the loss of a differentiated

ECM-forming phenotype at the transcriptional and translational levels[41,73]. As passage (and

population doublings) increases, there is a respective decrease and increase in type II and type I

collagen mRNA expression (Figure 1.6). This is also observed at the protein level, where Liang

et al (2017) showed that type II collagen and proteoglycan synthesis is reduced as a function of

passage and population doublings during chondrogenic culture (Figure 1.7)[73]. These de-

differentiating effects are detrimental for meniscus tissue engineering because the biomechanical

integrity of the tissue is derived from its ECM.

1.8 Chondrogenesis

Chondrogenesis is the process in which cartilage is formed through mesenchyme-derived progenitor cell condensation and differentiation[146]. In meniscus tissue engineering, the step that frequently follows cell expansion is chondrogenic induction– eg. chondrogenesis. This is achieved through cell condensation in the form of a 3-dimensional cell aggregate/pellet model or through scaffold seeding and cellular infiltration (as discussed previously). The de-differentiating effects from monolayer expansion can be mitigated to some degree using a chondro-inductive experimental model. Adesida et al (2006) demonstrated this, where de-differentiated/expanded MFCs isolated from the meniscus of an osteoarthritic knee joint had evidence of de-differentiation at the gene expression level, specifically the downregulation of type II collagen and increased type I collagen. By expanding the MFCs in the presence of bFGF, during the chondro-inductive period using the 3D pellet model it was found that the de-differentiated MFCs had an increased redifferentiation capacity and could still form inner meniscus-like ECM despite going through a significant number of population doublings. This suggests that members of the FGF family may help retain the ECM forming phenotype of expanded MFCs even though the cells have undergone de-differentiation. Additionally, this provides further support that MFCs express the necessary receptors for FGF-FGFR signalling pathways. Taken altogether, we decided to investigate the potential effects of FGF-18 supplementation in expanded MFCs for the prospective use of preserving functional ECM-forming phenotypes to further counteract de-differentiation processes from monolayer expansion.

The objective of this study was to investigate the effects of FGF- supplementation in expanded MFCs. FGF-18 has been shown to promote ECM synthesis, specifically aggrean and type II collagen[138, 143]. If similar effects occur in MFCs, then supplementation following monolayer expansion may mitigate the effects of dedifferentiation and preserve functional ECM formation. We hypothesize that FGF-18 would agonize FGFR3, leading to an anabolic response and preserve the MFC's ECM forming capacity which could potentially translate into increased biomechanical functionality.

Taken altogether, we decided to investigate the potential use of FGF-18 supplementation in expanded MFCs for the prospective use of preserving functional ECM-forming phenotypes to counteract de-differentiation processes from monolayer expansion.

1.9 Summary

The menisci are complex fibrocartilaginous wedges found within the tibiofemoral compartment in either knee joint. Due to the high occurrence of meniscal damage from traumatic injury, the degenerative changes that occur as a result of aging, and the lack of effective repair techniques, new treatment methodologies are being developed. Meniscus tissue engineering is one avenue displaying true potential in regard to the development of an effective treatment. By utilizing chondro-inductive cell sources in experimental models, and testing the effects of supplemented bioactive agents, identifying new therapeutic applications in meniscus tissue engineering may be possible. The transforming growth factor β family and fibroblast growth factor family have been highly implicated as potential candidates to promote ECM-forming phenotypes conducive of biomechanical functionality. Fibroblast growth factor 18, despite its frequent mentioning in

cartilage regeneration and potent anabolic effects, has not been studied in isolation in human meniscus fibrochondrocytes. Thus, by isolating meniscus fibrochondroytes and inducing de-differentiation through monolayer expansion, FGF-18 supplementation during chondrogenic culture is being investigated to assess the potential utility of restoring a meniscus-like ECM-forming phenotype.

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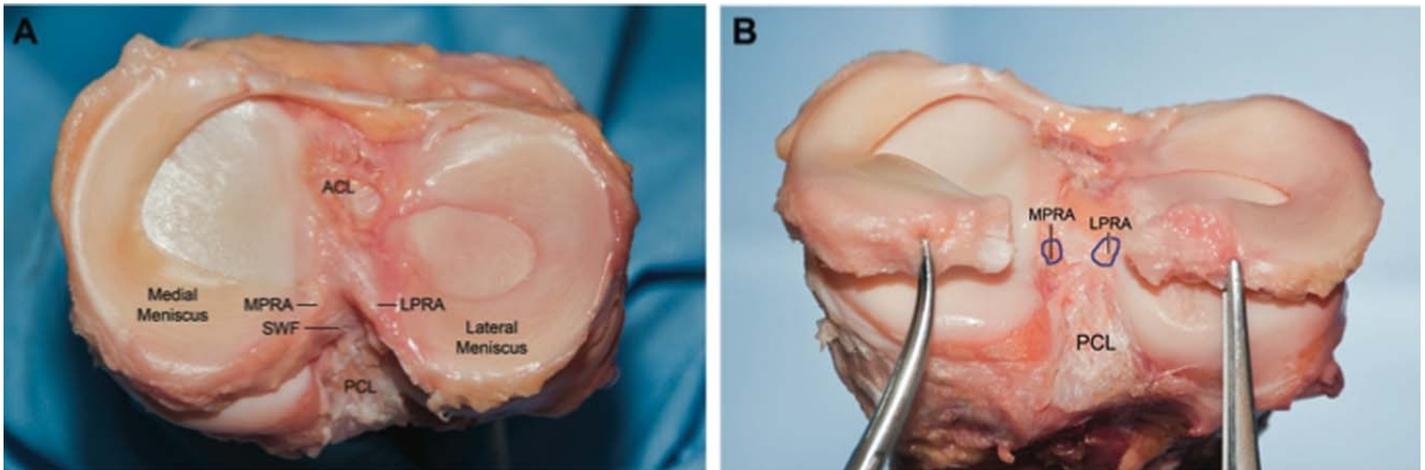
1.10 Figures

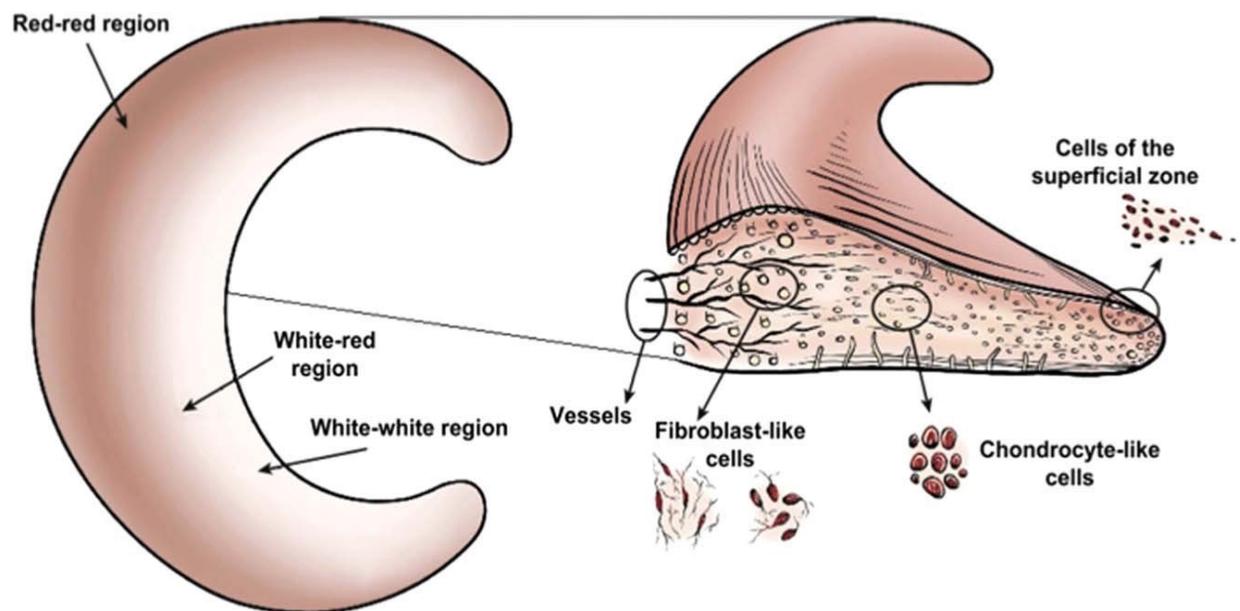Figure 1.1: (A) superior and; (B) supraposterior view of human medial and lateral menisci (Fox et al 2015).

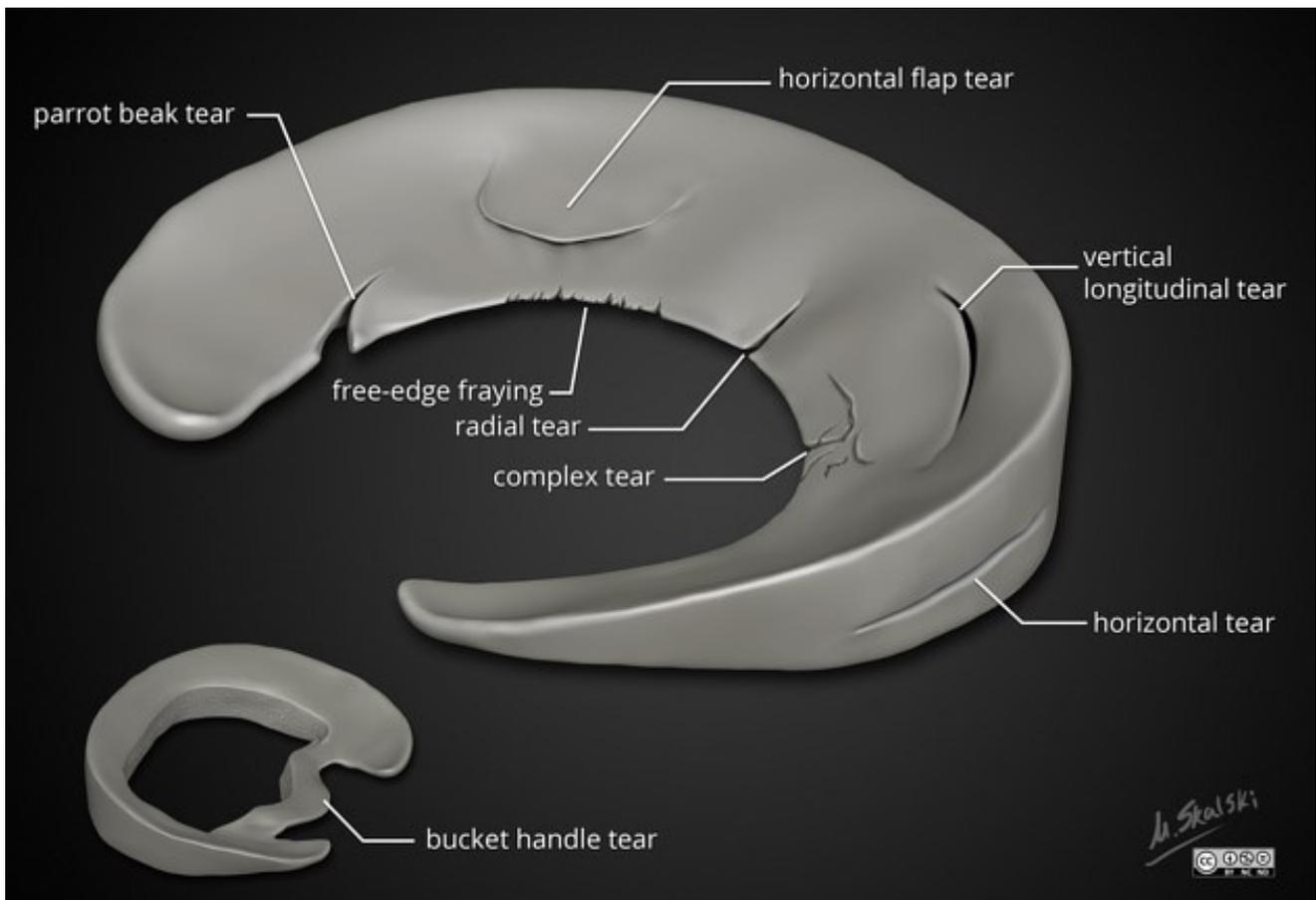Figure 1.2: Regional differences in vascularization and cell morphology of the meniscus (Makris et a 2011).

Figure 1.3: Classifying the different types of meniscal tears (Retrieved from https://radiopaedia.org/articles/meniscal-tear)
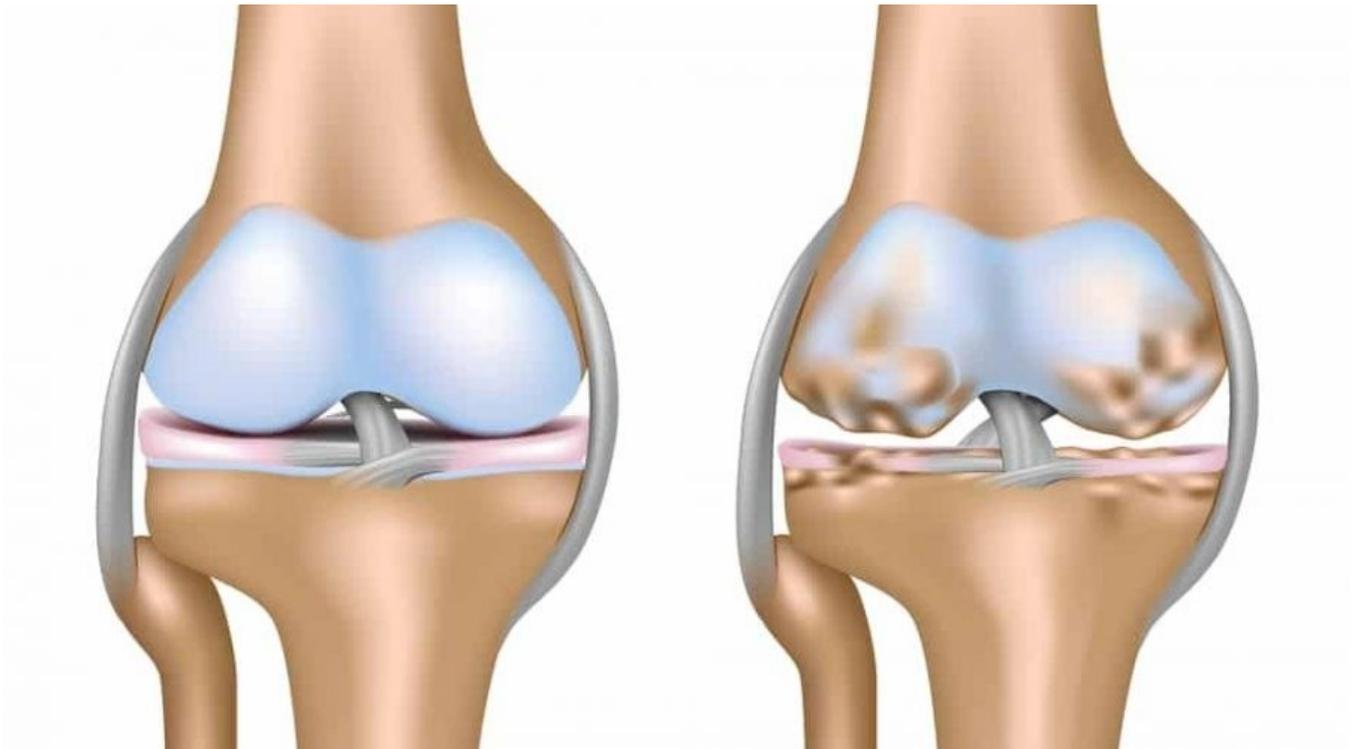
Figure 1.4: Cartoon rendition displaying the pathophysiological effects of degenerating menisci on all tissues comprising the knee joint. (Retrived from https://www.howardluksmd.com/sports-medicine/degenerative-joint-disease-treatments)

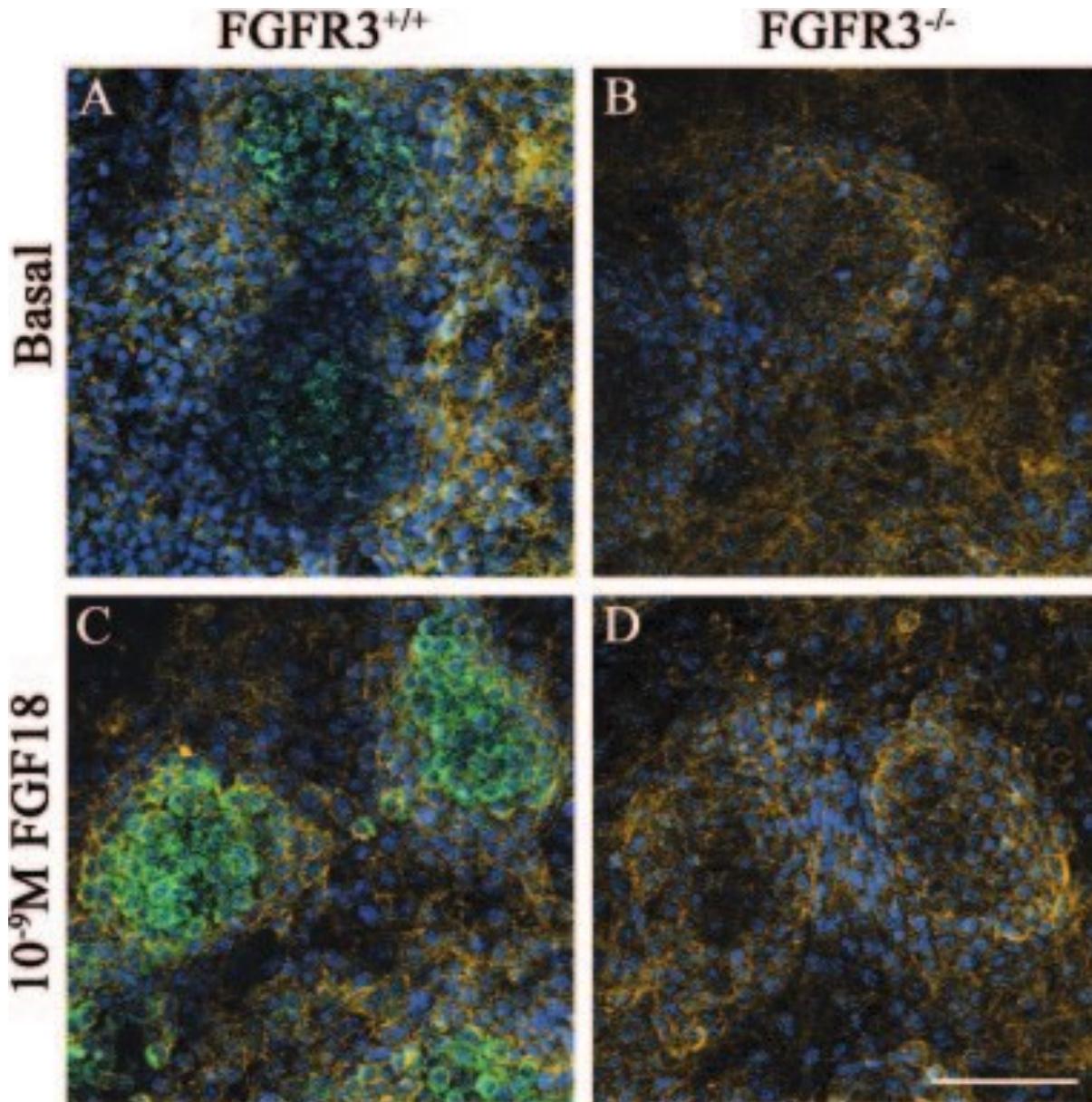Figure 1.5: Immunofluorescence of FGFR3 wild type (+/+) and knockout (-/-) embryonic mesenchymal stem cells cultured in high densityin the presence or absence of FGF-18 (10^{-9}M). Hoechst 33258 (blue), TRITC-phallodin (orange), type II collagen (green) (Davidson et al 2005).
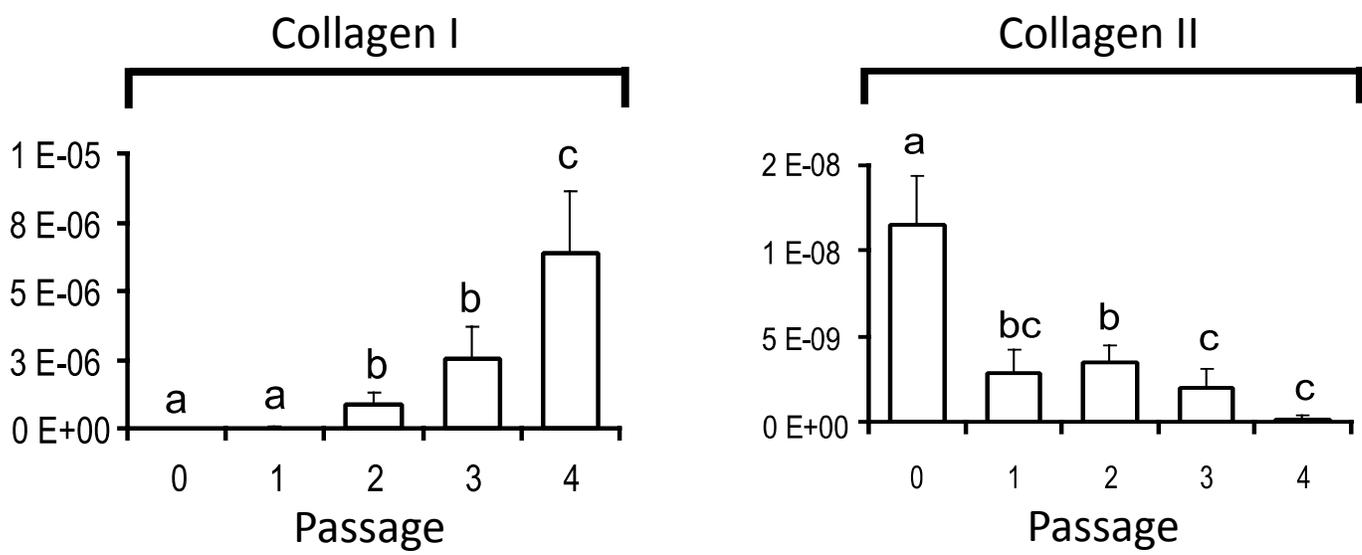
Figure 1.6: Type I and II collagen gene expression for bovine meniscus fibrochondrocytes expanded until passage 4 (Gunja et al 2009).
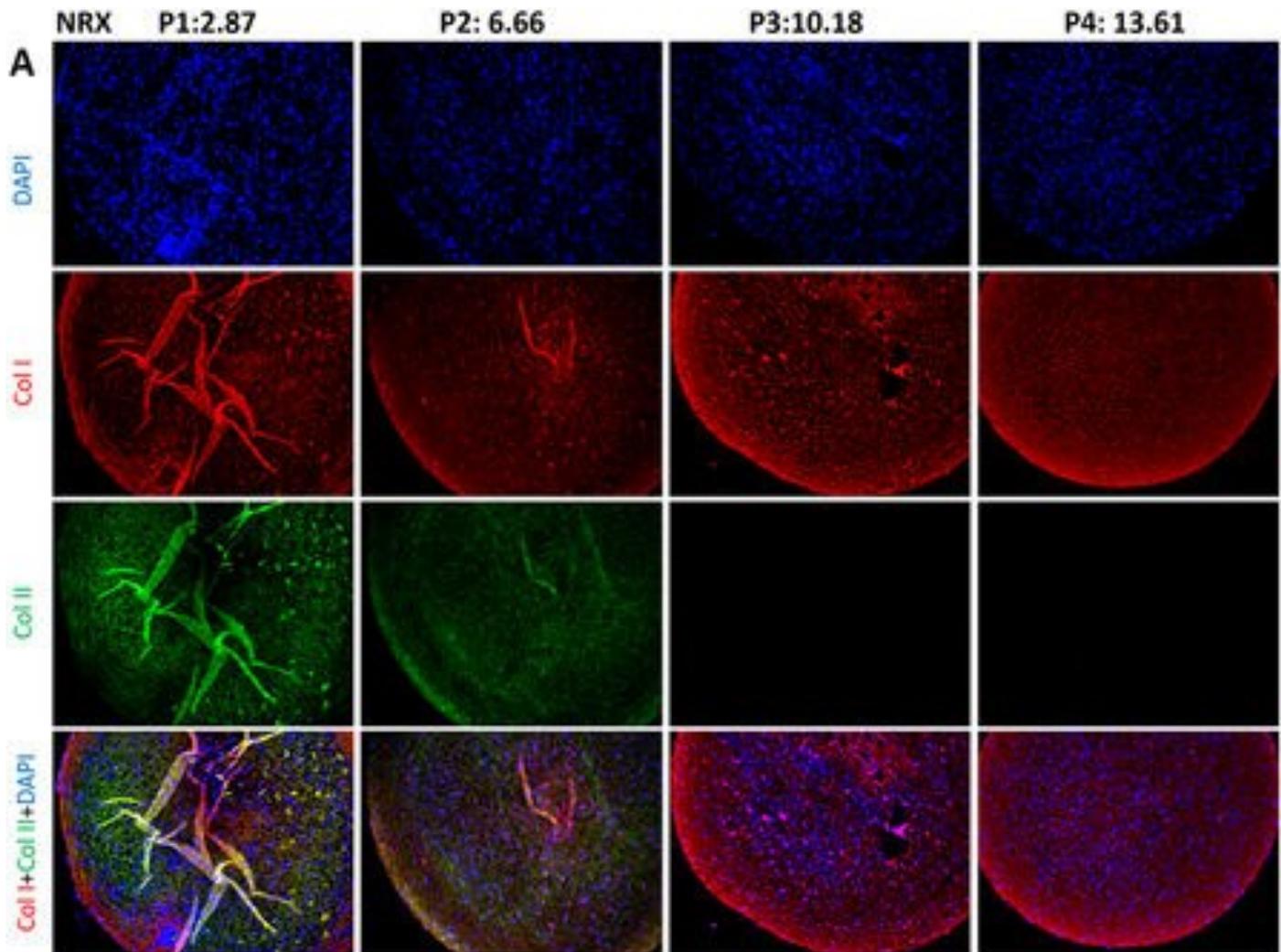
Figure 1.7: Immuofluorescence results of T1F2 expanded meniscus fibrochondrocytes chondrogenically-induced in a 3D pellet model at Passage 1-4. Red = Type I collagen, Green = Type II collagen, Blue = DAPI. (Liang et al 2017).

Chapter 2: Investigating the Role of Fibroblast Growth Factor 18 for Engineering Functional Meniscus Tissue

2.1 Introduction

The menisci are fibrocartilaginous wedge-shaped tissues that serve important functions including load distribution across the knee joint and protection of articular cartilage[1–3]. Due to the avascular nature of inner meniscal regions the healing response is limited following trauma[4,5]. A common treatment for inner meniscus injuries is arthroscopic partial meniscectomy, however, this compromises joint biomechanics and leads to degradation of articular cartilage lining the femoral condyle and tibial plateau, a predisposing factor for osteoarthritis (OA) development[6–10]. Due to an increasing prevalence of OA and costs for treating and managing the disease, orthopaedic surgeons and scientists are interested in developing more efficacious treatments for meniscus injuries[11–13].

Tissue engineering is one field being thoroughly investigated to promote meniscus healing or generate a replacement construct altogether. The cells populating the meniscus, known collectively as meniscus fibrochondrocytes (MFCs), may be an ideal source for meniscus tissue engineering since they have the capacity to synthesize the mechanically functional components of the tissue – its extracellular matrix (ECM)[14,15]. MFCs of the inner meniscus reside in an ECM rich in proteoglycans and types I and II collagen and appear round in morphology, phenotypically similar to articular chondrocytes[16]. These ECM components confer the functional compressive characteristics of the inner meniscal regions[1]. In contrast, cells of the outer region produce an ECM containing primarily type I collagen that is arranged in circumferential fibres. This type of arrangement endows the tissue with functional tensile properties and facilitates the conversion of axial loading forces into hoop stresses within the tissue[17–20]. Low cell isolation yields from tissues

obtained through partial meniscectomies, however, necessitates *in vitro* cell expansion. Cell expansion is associated with detrimental cellular processes collectively referred to as de-differentiation, and are characterized by the loss of a differentiated ECM-forming phenotype. These changes are detectable at the transcriptional and translational level, where a respective increase and decrease in type I and type II collagen is consistently observed[15,21].

Due to the similarity between cell phenotypes of the inner meniscus to that of articular cartilage, in terms of cell morphology and expression of aggrecan and type II collagen, a similar response to growth factor supplementation may also occur in MFCs[4]. In particular, fibroblast growth factor 18 (FGF-18) has been shown to stimulate anabolism in articular chondrocytes in regards to increased aggrecan synthesis, one of the principal ECM proteins of inner meniscal regions[22]. As such, we wanted to determine if similar anabolic effects could be induced in MFCs following supplementation with FGF-18. If a favorable effect was observed, then FGF-18 supplementation in expanded MFCs may promote the redifferentiation of expanded MFCs and retain their ECM-forming capacity despite dedifferentiation from monolayer expansion. These findings could potentially be applied to the development of novel meniscus tissue engineering strategies.

In **Part I** of this study we compared the ECM-forming phenotype of primary, differentiated MFCs (Primary, P0) to passage 4 (P4) expanded MFCs that had undergone de-differentiation from *in vitro* cell expansion using a 3 dimensional pellet model supplemented with FGF-18 to assess if this growth factor could promote a redifferentiated ECM-forming phenotype. In **Part II** of this study, P0 and P2 MFC pellets were cultured under the same experimental conditions in exception

of the [FGF-18] which ranged from 0-250ng/uL in attempts to identify i. an optimal concentration for FGF-18 to mediate its effects in MFCs and; ii. determine if a dose-response relationship exists.

2.2 Materials and Methods
Isolation and expansion of human meniscus fibrochondrocytes (MFCs)

Fresh meniscus specimens were obtained from 12 partial meniscectomy patients (ages 17-45, $\bar{X}_{age}\pm$STD = 29±8years) suffering from acute injuries after approval and waiver of informed consent of the local ethics committee at the University of Alberta. As described previously, wet meniscus tissues were weighed, aseptically cut into small pieces, and treated with trypsin-EDTA for 1h at 37°C[23]. This was followed by type II collagenase digest (0.15% w/v; 300 U/mg tissue) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% v/v fetal bovine serum (FBS) for 22h at 37°C to release primary meniscus fibrochondrocytes (MFCs). The tissue suspension was filtered using a 100 μm nylon-mesh filter to separate MFCs from undigested tissues. MFCs were then plated at 10^4 cells/cm^2 in polystyrene flasks (Sarstedt, DEU) in a standard expansion medium (high glucose DMEM (4.5 mg/mL D-glucose), 10% FBS, 100 U/mL penicillin, 100 μg/mg streptomycin, 2mM L-glutamine, and 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)) and were cultured in a humidified incubator at 37°C with 5% CO_2. After 48 hours (end of passage 0), cells were rinsed in phosphate-buffered saline (PBS) which was then aspirated to remove non-adherent cells. Adherent primary MFCs were detached using trypsin-EDTA (0.05% w/v) (Invitrogen, USA) and were quantified using a haemocytometer with trypan blue exclusion. Cells were then replated at 10^4 cells/cm^2 in fresh culture medium described above. Upon reaching 80-85% confluence, first-passage (P1) cells were detached with trypsin-EDTA and re-plated in polystyrene culture flasks equivalent to 2x the surface area as the previous passage, denoted as P2. This process was repeated until P4 for part I of the study and was

stopped at P2 for part II of the study. MFCs were quantified at the end of each passage to determine population doublings calculated as $\log_2(N/N_0)$, where N_0 is the number of cells plated at the beginning of a passage, and N is the number of cells counted at the end of a passage[24]. Cumulative population doublings were then calculated as the sum of population doublings at each passage. Population doublings per day were calculated by dividing the population doublings of each passage by the number of days in culture.

Induced Chondrogenesis in 3D Pellet Model

Chondrogenic differentiation was induced in a three-dimensional pellet model. **Part I:** Upon reaching 80-85% confluence at the end of P0 and P4, 5×10^5 MFCs were centrifuged at 1500 rpm for 6 minutes in sterile 1.5mL conical microtubes to form pellets. The pellets were cultured in 0.5mL of serum-free chondro-inductive media consisting of: high glucose DMEM containing 100 units/mL penicillin, 100µg/mL streptomycin, 2mM L-glutamine, 10mM HEPES, ITS+1 premix, 100nM dexamethasone, 365µg/mL ascorbate, 40µg/mL L-proline, 125 µg/mL human serum albumin and 10ng/mL transforming growth factor β3 (TGFβ-3). Experimental condition also contained 10ng/mL fibroblast growth factor 18 (FGF-18). Pellets were cultured at 21% O_2 and 37°C in a humidified incubator for 21 days with media changes occurring 2-3 times weekly. **Part II:** 2.5×10^5 P0 and P2 MFCs were centrifuged to form 3D cell pellets and cultured in the same chondro-inductive media mentioned above in exception of [FGF18]. In this experiment, pellets were cultured with 250µL of chondro-inductive media which was additionally supplemented with 0, 10, 50, 100, or 250ng/mL of FGF-18 under the same experimental conditions mentioned previously. Following completion of the 21 day culture period, pellet wet weights were recorded then assessed: biochemically to quantify glycosaminoglycan (GAG) and DNA content,

histologically and immunohistochemically to detect meniscus-specific extracellular matrix proteins, and quantitatively via qRT-PCR to compare gene expression profiles.

Histological Analysis

Day 21 pellets were transferred to a new sterile 1.7mL microtube and fixed in 10% v/v formalin overnight at 4°C. Following the fixation period pellets were rinsed in phosphate buffered saline (PBS), processed via ethanol dehydration and xylene treatment, and embedded in paraffin wax. 5µm sections were cut and dried overnight in a 37°C incubator. Dried histological sections were then used for safranin-O staining to visualize proteoglycan deposition and immunofluorescence to detect type I/II collagen, fibroblast growth factor receptor 3, and cell localization.

Safranin-O Staining

Dried sections were deparaffinised, rehydrated, stained with 0.1% w/v Safranin-O and counterstained with 1% w/v Fast-Green to visualize proteoglycan extracellular matrix proteins as described previously[25].

Immunofluorescence

Sections used for immunofluorescence were initially deparaffinised in Ultraclear xylene-substitute followed by submersion in ethanol solutions of decreasing concentration. Slides were then rehydrated in water and used for immunofluorescent detection described below.

Type I/II Collagen

As discussed previously, 5µm pellet sections were treated with Protease XXV (Thermo Scientific, CAN) and hyaluronidase (Sigma-Aldrich, USA) for 30 minutes each to expose the antigen binding sites for type I and II collagen[26]. Sections were incubated for 30 minutes at room temperature with a blocking solution containing 5% v/v bovine serum albumin (BSA) in PBS to prevent non-specific antigen binding. Sections were then incubated with rabbit anti-collagen I (CL50111AP-1, 1:200 dilution; Cedarlane) and mouse anti-collagen II (II-II6B3, 1:200 dilution; Developmental Studies Hybridoma Bank) primary antibodies overnight at 4°C. The next day sections were rinsed in PBS followed by treatment with secondary antibodies goat anti-rabbit IgG H&L Alexa Fluor 594 (ab150080, 1:200 dilution; Abcam) and goat anti-mouse IgG H&L Alexa Fluor 488 (ab150117, 1:200 dilution; Abcam) for 40 minutes at room temperature. After a wash in PBS, sections were counterstained with 4',6-diamidino-2- phenylindole (DAPI) (Cedarlane) and mounted with 1:1 glycerol-PBS and sealed with clear nail polish.

FGFR3

Antigen retrieval was performed using Tris-EDTA buffered to pH 9.0 for 40 minutes at 95°C using a steamer. Sections were subsequently rinsed in PBS with 0.01% v/v Tween to maintain cell permeabilization. Sections were then blocked using 5% BSA followed by incubation with rabbit anti-FGFR3 primary antibody (ab137084, 1:250 dilution; Abcam) overnight at 4°C. The following day slides were rinsed in PBS followed by treatment with the secondary antibody goat anti-rabbit IgG H&L Alexa Fluor 594 (ab150080, 1:200 dilution; Abcam) for 40 minutes at room temperature. Sections were then rinsed three times in PBS and counterstained with DAPI for cell visualization.

Lastly, sections were rinsed in PBS before mounting with 1:1 glycerol-PBS and sealed with clear nail polish.

All images were captured using an Eclipse Ti-S microscope (Nikon Canada, CAN) fitted with NIS Elements (version 4.20; Nikon Canada) and assembled using ImageJ.

Transmission Electron Microscopy (TEM)

TEM was performed on one representative donor for passage 4 expanded pellets to observe phenotypic differences between growth factor conditions. Upon reaching 21 days of culture, pellets were harvested and fixed overnight in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (CB) as described previously[27,28]. The next day, the partially fixed pellets were rinsed in 0.1M CB to remove any residual glutaraldehyde. Pellets were then transferred to a shell vial and fixed in 1% osmium tetroxide (OsO_4) for 1 hour. After this fixation step, OsO_4 was aspirated followed by 3 serial washes in 0.1M CB then 2 rinses in 0.1M sodium acetate buffer (5.2 pH). Enbloc staining was then performed in 1% uranyl acetate (UA) in 0.1M sodium acetate trihydrate solution overnight at 4°C. The following day the UA solution was aspirated and rinsed in 0.1M sodium acetate trihydrate followed by a rinse in Milli Q water. The fixed sample were then dehydrated in a serial rinses of increasing concentration of ice-cold ethanol (EtOH). Sample were then infiltrated with Spurr's resin diluted in 100% EtOH overnight. The following morning, Spurr's:EtOH solution was aspirated and replaced with absolute Spurr's resin for 8 hours. The next day samples were embedded in fresh absolute Spurr's and heat polymerized at 60°C for 48 hours. 800nm semi-thin sections were then cut and stained with 1% toluidine blue. 70nm ultrathin sections were cut and stained with 2% uranyl acetate and Reinold's lead citrate for

contrast enhancement. Sections were imaged using a Hitachi H-7650 transmission electron microscope at 60 kV with a 16 mega pixel EMCCD camera (XR111; Advanced Microscopy Techniques, USA) and imaging software (AMT version 600; Advanced Microscopy Techniques).

Biochemical Analysis

Following completion of the 21-day culture period pellets were rinsed in PBS to remove residual media. Pellets were digested with 250μL proteinase K (1 mg/mL in 50 mM Tris with 1 mM EDTA, 1 mM iodoacetamide, and 10 mg/mL pepstatin A; all from Sigma-Aldrich, USA) for 16 h at 56°C. The GAG content was quantified spectrophotometrically via 1,9-dimethylmethylene blue assay using chondroitin sulphate as the standard as previously described[29]. DNA was quantified using a kit from Invitrogen (CyQuant Cell Proliferation Assay) using λ bacteriophage DNA as the standard.

Quantitative Real Time PCR

Day 21 pellets were transferred to Trizol in sterile, RNase free microtubes and immediately frozen at -80°C. Once all pellets from one single donor were collected (eg. completion of P0/P4 in Part I or P0/P2 in Part II), cell lysate was formed by mechanically perturbing the cell membranes via crushing with molecular grinding resin. RNA was isolated by chloroform phase extraction and quantified using nanodrop. 100ng of clean RNA ($A_{260}/A_{280} \sim 2.0$) was used as template for cDNA synthesis, using GoScript reverse transcriptase (Fisher Scientific, CAN), and 1μg of oligo(Dt) primers in a 25μL reaction. Quantitative real-time polymerase chain reaction was performed in a CFX Connect Real Time PCR machine (Biorad, USA) using hot start Taq and SYBR Green

detection (Eurogenetec Northa America Inc., USA). Primer sequences were obtained from previously published work and purchased from Invitrogen, Canada. Table 2.1 summarizes candidate genes and primer sequences that were used. mRNA expression levels for each primer set were normalized to the expression level of β-actin, YWHAZ and B2M for Part I and to β-actin and B2M for Part II using the $2^{-\wedge ct}$ method[30].

2.3 Results
Cell yield and expansion

Meniscus tissue was obtained from 12 donors (ages 17-45) who underwent partial meniscectomy arthroscopy due to acute traumatic injury. Non-identifying donor information is summarized in Table 2.2. Mean wet weights (± STD) of meniscus tissue pre-digestion were 2.1 ± 1.1g. Following collagenase digest and 48 h recovery period, viable MFCs isolated were 4.79 ± 2.49 million cells per gram of wet weight meniscus tissue.

Viable MFCs were expanded in standard culture media (see methods) in a humidified incubator set to 37°C, 21% O_2 tension, and 5% CO_2. Primary cells (P0) appeared predominately round in morphology compared to P2 and P4 expanded MFCs which were more spindle-like and elongated, resembling fibroblasts. Mean population doublings per day (±STD) at P1, P2, P3, and P4, respectively, were: 0.26±0.08, 0.21±0.05, 0.13±0.06, and 0.13±0.06 (Figure 2.1B). Mean cumulative population doublings (±STD) from P1-P4 were 1.66±0.44, 3.02±0.43, 3.81±0.40, and 4.70±0.53 (Figure 2.1A).

Results – Part I

Wet Weights

After attaining 80-85% confluence, 5×10^5 P0 and P4 cells were independently centrifuged at 1500 rpm for 6 minutes to form pellet aggregates and cultured in chondrogenic media supplemented with TGFβ-3 (T3) or FGF-18+TGFGβ-3 (T3F18) at [10ng/mL] for 21 days with media changes occurring 2-3 times per week. Following completion of the 21 day culture period, pellet wet-weights were recorded then immediately processed for analysis. Mean P0 pellet wet weight (±STD) were 1.2 ± 0.5mg for both the T3and T3F18 condition (Figure 2.2A). The mean P4 pellet wet weight was 3.7±1.8mg and 3.6±1.6mg for the T3 and T3F18 condition, respectively. No significant differences were detected between conditions at either passage, however a significant increase from P0 to P4 was observed (p = .006).

Biochemical

To evaluate the potential anabolic effect of FGF-18 in expanded MFCs, biochemical analysis was performed to quantify the amount of glycosaminoglycan (GAG) and DNA present in the pellets following the 21 day culture period. GAG content did not vary substantially between T3 and T3F18 condition at either passage (Figure 2.2B). A 3.3 fold increase in GAG content was detected comparing P0 to P4, however, this was most likely due to TGFβ3 supplementation as there are no measurable differences between the control condition and the FGF-18 supplemented experimental group *(p = 0.003)*. DNA content was quantified spectrophotometrically to assess for evidence of cell proliferation. As can be seen in Figure 2.2C, DNA was relatively uniform across all conditions and passages, however a slight increase was observed when comparing P0 to P4. This was present in both P4 conditions suggesting this effect may have been induced by TGFβ-3. Standardizing

GAG values to cellular DNA content (GAG/DNA), a similar trend was observed that closely resembled the relationship seen in GAG and WW (Figure 2.2D). Within each passage no significant differences were observed, however there was a significant increase in the GAG/DNA ratio from P0 to P4 ($p = 0.002$).

Histology

Safranin O

In passage 0 pellets from both conditions (T3 and T3F18), trace amounts of pink staining were detected along the peripheral region which closely coincided with the relatively low GAG content for this passage (Figure 2.3A). A portion of cells comprising the inner region of each P0 pellet for both conditions appeared fibroblastic, with an elongated cell morphology in contrast to the outer region which were predominately round and chondrocyte-like. Passage 4 pellets, regardless of condition, had robust pink staining distributed throughout the entirety of the stained section, with the most intense staining concentrated in the inner regions, decreasing gradually towards the peripheral border (Figure 2.3A). Cells of the inner regions appeared round with lacunae-like pericellular matrices. In the P4 MFC group, T3F18 condition appeared to have an increased prevalence of round chondrocyte like cellular morphologies in contrast to the T3-alone group which was heterogeneously mixed with round cells and elongated fibroblast-like cells.

Type I and II Collagen

Fluorescence microscopy was performed to visualize cell localization with DAPI and ECM proteins using primary antibodies to type I and II collagen. Cells appeared to be uniformly distributed throughout the entirety of pellet, regardless of condition or passage (Figure 2.3D).

Passage 0 pellets appeared to be at a higher density compared to the P4 pellets, regardless of condition, which was most likely attributed to less overall ECM synthesized in these pellets. Type I collagen was positively detected in all conditions and was distributed uniformly across all pellets (Figure 2.3B). In passage 4 pellets, the lacunae-like structure was evident, with round pericellular matrices being detected surrounded by type I collagen and DAPI located centrally. Trace amounts of type II collagen was only detected in the P0 pellets, for both the T3 and T3F18 conditions, along the peripheral region which can be seen with the red arrows included in the diagram (Figure 2.3C). No positive type II collagen fluorescence was observed in any P4 pellets.

FGFR3

Immunofluorescence was performed to assess the effects of expansion at the protein level for FGF receptor 3 (FGFR3). FGFR3 immunofluorescent detection was highly prevalent at passage 0 (P0) for both conditions (T3 and T3F18), with an even distribution across the entirety of the section (Figure 2.3F). DAPI localized to sites of FGFR3 detection, with no FGFR3 being observed in the absence of a DAPI overlay suggesting the receptor is localized to the membranes of these cells. Passage 4 (P4) pellets had significant downregulation of the FGFR3, as can be seen in Figure 2.3F panels 3 and 4, in comparison to either condition at P0.

Transmission Electron Microscopy (TEM)

TEM was performed based off observations in P4 derived pellets from previous donors (unpublished data). In particular, P0-derived pellets appeared phenotypically similar regardless of growth factor condition. At P4, however, subtle variations in cellular morphology were apparent. This was also the case for the representative donor presented in Figure 2.4. As can be

seen in the Safranin-O stained sections, P4 pellets cultured in T3 alone appeared to have a higher proportion of fibroblast-shaped MFCs, which was most evident along the peripheral edge of the pellet (Figure 2.4A). In contrast, the P4-T3F18 condition were more chondrocyte-like and round in morphology. This was corroborated with TEM imaging, where again we saw the P4-T3 group had a higher proportion of elongated MFCs in contrast to P4-T3F18 group, which were primarily rounded (Figure 2.4B). It appears that the actin fibre arrangement in the T3 condition is highly organized and directional, which may contribute to the elongated cell morphology. This contrasted with the P4-T3F18 condition, where this arrangement was less prevalent and appeared somewhat disorganized. Based off these findings it may be possible that FGF-18 is involved, to some degree, in cytoskeletal reorganization. In eukaryotic cells, actin is one of the most copious intracellular proteins and plays an integral role in cell shape and morphology.[31]

Gene Expression Analysis

Assessment of extracellular matrix (ECM) and catabolic-related gene expression was performed via quantitative real time polymerase chain reaction in primary (P0) and passage 4 (P4) expanded meniscus fibrochondrocytes using the delta CT method. Each sample was normalized to three house keeping genes, β-actin, B2M, and YWHAZ. The gene expression results for the chondrogenic-related genes aggrecan (*ACAN*)*,* type II collagen (*COL2A1*)*,* type I collagen (*COL1A2*)*,* and the chondrogenic transcription factor (*SOX9*) as well as catabolic related genes matrix metalloproteinase 1 and 3 (*MMP1, MMP3)* and tissue inhibitor of metalloproteinase 2 (*TIMP2*) are presented in Figure 2.5.

Chondrogenesis

Aggrecan expression was relatively equal across all passages and conditions, with a slight decrease seen for the P0 T3F18 condition (Figure 2.5A). Type I collagen was relatively equal between growth factor conditions at either passage, however an insignificant increase was detected comparing P0 to P4 however there was a large degree of variation which is reflected in the p-value (Figure 2.5B, *p* = 0.42). This result suggests the MFCs at P4 may have been in a more de-differentiated state, as type I collagen upregulation is a cardinal characteristic of this process. This was further stipulated when looking at type II collagen expression (Figure 2.5C), where P0 pellets had a non-significant decrease in expression compared to P4 (*p* = 0.14). The presence of FGF-18 appeared to slightly reduce type II collagen expression relative to the control group at both passages (Figure 2.5C).

Catabolic-Related Gene Expression

Assessment of pro-catabolic genes *MMP-1* and *MMP-3* displayed similar expression profiles, with relatively equal expression regardless of growth factor supplementation within each passage (Figure 2.5D, 2.5E). When comparing P0 to P4, there was a statistically significant decrease in *MMP1* and *MMP3* expression (*p* = 0.037, 0.035, respectively). Gene expression for the anti-catabolic gene, *TIMP2*, which is a known regulator of MMPs, was relatively equal across all conditions and passages, with no statistically significant differences observed for any comparison (Figure 2.5F).

Overall, no significant difference in gene expression for any of the ECM- or catabolic-related genes was observed between the growth factor conditions at either passage.

Results: Part II

Wet weight

After attaining 80-85% confluence, 2.5×10^5 P0 and P2 cells were independently centrifuged at 1500 rpm for 6 minutes to form pellet aggregates and cultured in chondrogenic media supplemented with TGFβ3-alone [10ng/mL] (T3F18-0) or TGFβ3 [10ng/mL] and FGF-18 at 4 different concentrations: 10ng/mL (T3F18-10), 50ng/mL (T3F18-50), 100ng/mL (T3F18-100), and 250ng/mL (T3F18-250). Following completion of the 21 day culture period, pellet wet-weights were recorded then immediately processed for analysis. Mean pellet wet weights are presented in Figure 2.6A. Overall, no statistically significant differences in wet weights were observed within each passage, regardless of the [FGF-18]. A significant increase was detected for comparing P0 to P4 ($p = 0.01$).

Biochemical (see methods)

Glycosaminoglycan (GAG) content followed a similar trend seen with wet weights, in which no significant differences were detected within either passage (Figure 2.6B). There was an insignificant increase in overall GAG content comparing P0 to P4 ($p = 0.19$). The presence of FGF-18, regardless of concentration, did not increase overall GAG content. DNA content was relatively equal within each passage, however an increase was detected between P0 and P2 that approached significance (Figure 2.6C, $p = 0.05$). Normalizing GAG content to total DNA (Figure 2.6D), a decreasing ratio was observed, where the higher the [FGF-18], the lower the overall ratio. This trend was observed both at P0 and P2. No significant differences in GAG/DNA ratio was observed between growth factor conditions or passage, however the ratio increased overall comparing P0 to P2 regardless of condition.

Safranin-O

For all 5 growth factor conditions, positive pink staining was detected in primary (P0) and passage 2 (P2) derived MFC pellets (Figure 2.7A). P0 pellets appeared approximately equal in diameter across conditions, with pink staining most pronounce in the inner core region of each pellet. No major macroscopic differences were observable between conditions at passage 0, however, the amount of positive pink staining increased comparing P0 relative to P2. Staining in P2 appeared to be most intense in the inner region, decreasing incrementally moving towards the periphery (as was seen in Part I of this study). P2 pellets also appeared to display a lacunae-like structure, in which round areas where cells are localized (blue staining) are devoid of pink staining. This round morphology was nearly absent in the T3-alone group and was most prevalent in the P2-T3F18-50 condition. With the higher [FGF-18] groups, ie. 100 and 250ng/mL, the rounded morphology began to decrease and were more fibroblastic in appearance. Overall, no major differences were evident in proteoglycan staining and deposition, suggesting FGF-18 does not play a major anabolic role in this ECM protein's synthesis in primary or expanded MFCs undergoing chondrogenic-induction in a 3D pellet model.

Type I/II Collagen

Type I collagen was distributed throughout the entirety of each pellet at both passages (Figure 2.7B). All P0 pellets appeared to have the most type I collagen along the peripheral border, as determined by an increased brightness in type I collagen immunofluorescence. This result was found in all P0 pellets regardless of the growth factor condition. P2 pellets had a uniform distribution of type I collagen throughout the entire section in contrast to the P0 pellets which had a pronounced peripheral ring. Overall, no major differences in type I collagen immunofluorescence

was observed within P0 or P2. Positive detection of type II collagen was only present in primary (P0) derived 3D pellets (Figure 2.7C). Detection was most pronounce along the inner regions which corresponded with the same area as proteoglycan deposition as was demonstrated by the Safranin-O staining (Figure 2.7A). P2 pellets were entirely devoid of positive type II collagen detection. Overall, it does not appear that FGF-18 supplementation enhances the synthesis and deposition of type I or II collagen in this 3D pellet model.

FGFR3

Immunofluorescence targeting FGFR3 was performed as described earlier. Part I of this study showed FGFR3 downregulation by passage 4 (P4) suggesting the dedifferentiating effects of *in vitro* expansion also effects FGFR3 production at the protein level. As such, MFCs were used in their primary state (P0) and passage 2 (P2) expanded for Part II in hopes expanded MFCs retain their FGFR3 expression and translation. Positive FGFR3 immunofluorescence was detected for all growth factor conditions (T3F18-0, T3F18-10, T3F18-50, T3F18-100, T3F18-250) in primary (P0) derived pellets to varying degrees (Figure 2.7F). In particular, the control group devoid of FGF-18 (T3F18-0) had the highest intensity, which was most pronounce towards the peripheral edges of the section. As the concentration of FGF-18 increased, the overall FGFR3 detection decreased, suggesting an inverse relationship exists. Overlaying with DAPI (Figure 2.7G), we can see that FGFR3 detection was only occurring in sites with DAPI overlap suggesting once again that the location of these receptors was localized to the membranes of these cells. (Blue+red = pink). In contrast to P0, passage 2 (P2) pellets were almost entirely devoid of FGFR3 immunofluorescence. The T3F18-10 and T3F18-250 conditions had some positive detection along the right portion of the image, but was less intense than at P0. Additionally, it appears the section

may have a small fold, which would double the number of receptors normally occupying that region. Overall, it appears that increased [FGF-18] downregulates FGFR3 synthesis at the protein level in P0 pellets, and that by P2 expansion, MFC synthesis of FGFR3 is significantly downregulated even in a 3D pellet model.

Semi-Quantitative Immunofluorescence was applied to the FGFR3 immunofluorescent results in Figure 2.6F. Positive detection of FGFR3 was normalized to DAPI content then plotted as a function of [FGF-18] (Figure 2.9). Interestingly, FGFR3/DAPI ratio appeared to decrease at an exponential rate with higher concentrations of FGF-18 ($R^2 = 0.93$).

qRT-PCR

Gene expression analysis for the same 6 genes in Part I of this study were assessed. In contrast to Part I, aggrecan expression increased from P0 to P2 (Figure 2.8A). Within P0, no significant differences in expression was observed. At P2, an insignificant downward trend was observed, where higher [FGF-18] was associated with a decrease in *ACAN* expression. Type I collagen expression was approximately equal across all conditions and passages with no trends or statistically significant differences being observed for any comparison (Figure 2.8B). This suggests the P2 MFCs were less de-differentiated than the P4 MFCs that were used in Part I. Type II collagen expression was variable at P0. At P2, however, higher [FGF-18] was associated with decreased type II collagen expression, closely mirroring the trend seen with *ACAN* (Figure 2.8C). *MMP1* and *MMP3* gene expression contrasted with the trend seen in Part I of this study, where *MMP1* decreased as seen before, however, *MMP3* increased from P0 to P2 (Figure 2.8D/E). No significant differences were observed from growth factor conditions at either passage, however a

statistically significant decrease in *MMP1* expression was seen comparing P0 to P2 (Figure 2.8D, p = 0.03). *MMP3* displayed a downward trend at P2, with higher [FGF-18] associated with less *MMP3* expression. *TIMP2* expression was approximately equal for all growth factor conditions at P0 (Figure 2.8F). Looking at the effects of passage, again we saw a downward trend where higher [FGF-18] groups consistently expressed less *TIMP2*. Overall, it appears that FGF-18 supplemented groups are responding to this growth factor, however it does not appear to upregulate any of the ECM-related proteins needed for the biomechanical functionality of meniscal tissue.

2.4 Discussion
The purpose of this study was to 1) determine if FGF-18 supplementation in expanded MFCs using a 3D pellet model can promote the redifferentiation of MFCs to maintain or upregulate ECM synthesis and; 2) identify the optimal concentration of FGF-18 to mediate an effects in expanded MFCs. Our findings from Part I of the study using primary (P0) and passage 4 (P4) expanded MFCs cultured in a chondrogenic media supplemented TGFβ3 with or without FGF-18 (10ng/mL for both growth factors) showed FGF-18 had no effect at a concentration of 10ng/mL in terms of the ECM matrix proteins of interest. Comparing growth factor conditions at P0 and P4, no discernable trends were suggestive of an anabolic effect from FGF-18 supplementation. Passaging effects were evident, however, where an increase in type I collagen and decrease in type II collagen at the translational and expressional level were observed comparing P0 to P4 (Figure 2.3 and 2.5). These findings suggest the P4 MFCs had undergone dedifferentiation as they no longer had the capacity to synthesize type II collagen. Quantified GAG content showed no statistical difference between growth factor conditions at both passages while a significant increase was detected comparing P0 to P4 (Figure 2.2B, p = 0.003). Gene expression for aggrecan, the principle proteoglycan synthesized by MFCs of inner meniscal regions, was relatively uniform across

passages despite a 3.3 fold increase in GAG content comparing P0 to P4. This may be due to increased proteoglycan catabolism, specifically aggrecan, at P0 relative to P4. There was a significant increase in wet weight, glycosaminoglycan (GAG) content, and GAG/DNA ratio comparing P0 to P4, but this trend was present in both growth factor conditions suggesting this effect was most likely mediated through other signalling pathways, possibly TGFβ.

Safranin O staining uncovered something quite interesting. At passage 4, the cellular morphology appeared to be variable between growth factor conditions. In the T3 supplemented group, MFCs were more fibroblastic, especially towards the edge of each stained section (Figure 2.3A/2.4A). Transmission electron microscopy results supported these findings, where we saw predominately fibroblastic cellular phenotype in the P4-T3 condition compared to round or circular morphologies in P4-T3F18 (Figure 2.4B-C). Thus, it may be possible that FGF-18 has a direct or indirect role in actin polymerization and cytoskeletal reorganization.

It is proposed in literature that FGF-18 mediates its anabolic effects on embryonic mesenchymal stem cells and articular chondrocytes through agonism of FGFR3[32,33]. To further elucidate the role of FGF-18 in expanded MFCs and determine why no effect was observed, FGFR3 was visualized by immunofluorescence. Immunofluorescent results showed downregulation of FGFR3 at the protein level, where P0 derived pellets from both growth factor conditions produced the receptor in abundance in contrast to P4 where minimal positive detection was observed (Figure 2.3F/G). Overall, the results from Part I of this study suggest: 1) 10ng/mL FGF-18 is insufficient to induce an anabolic effect in P4 expanded MFCs; 2) P4 expanded MFCs have undergone dedifferentiation, as determined by decreased type II collagen and increased type I collagen synthesis at the protein

and transcriptional level; 3) FGFR3 production at the protein level is downregulated as a function of passaging/monolayer cell expansion and; 4) FGF-18 may play a role in cytoskeletal reorganization.

The results of Part I of this study inspired the follow-up concentration study (Part II), wherein 2.5×10^5 primary (P0) and passage 2 (P2) expanded MFCs were independently centrifuged to form a 3D cell aggregates and cultured in chondrogenic media supplemented with 10ng/ml TGFβ3 + 0, 10, 50, 100, or 250ng/mL FGF-18 (T3F18-0, T3F18-10, T3F18-50, T3F18-100, T3F18-250, respectively). The initial cell quantity in pellets was reduced by 50% due to low primary MFCs availability accompanied with an increase number of experimental groups. The results from Part II suggest P2 MFCs had undergone less de-differentiation compared to the P4 MFCs used in Part I. Specifically, type I collagen expression was relatively uniform across all conditions from P0 and P2 however in Part I we saw an increase in type I collagen expression from P0 to P4 (Figure 2.8B). Furthermore, type II collagen detection was only seen in P0 pellets (as was the case in Part I), suggesting some degree of de-differentiation did occur. This was corroborated at protein level, where positive type II collagen detection was only seen at P0 for all growth factor conditions (Figure 2.7C). Higher [FGF-18] appeared to downregulate *ACAN*, *COL2A1*, *MMP3*, and *TIMP2* expression only at P2 (Figure 2.8). Of all the quantitative metrics that were assessed (GAG, DNA, GAG/DNA), no significant differences were seen between growth factor conditions at either passage. Safranin O staining was relatively equal in all conditions at P0, however the cell morphologies appeared to be influenced by [FGF-18] at passage 2 (Figure 2.7A). As can be seen, the P2-T3 condition appeared to have mainly fibroblastic morphologies in contrast to the P2-T3F18-50 group which were mainly rounded. The highest [FGF-18] at 250ng/mL, however, were

more similar to the T3 group. As such, the effect of FGF-18 on cytoskeletal reorganization may have an optimal concentration to induce this effect, after which the cellular response may be dampened.

FGFR3 immunofluorescence was nearly absent in all P2 pellets, an effect most likely due to passaging as this was also seen in the control group with no FGF-18 present (Figure 2.7F). P0 pellets, however, showed an interesting trend. The higher the concentration of [FGF-18] the less positive FGFR3 detection was observed. Semi-quantitative immunofluorescence was performed, normalizing FGFR3 to DAPI content to control for section depth inequalities, and plotted against [FGF-18]. An exponential trend was observed with an R^2 of 0.929 (Figure 2.9). Thus, it appears FGF-18 is inducing an effect, at least at the receptor level, where higher concentrations resulted in FGFR3 downregulation. From a cell biology perspective, this is a logical response as it the cell's attempt to re-establish a homeostatic balance. Literature posits the FGFR3 exists in three isoforms: the FGFR3 IIIa, IIIb and IIIc variants. The IIIa variant is a secreted extracellular peptide, the IIIb variant is expressed in epithelial cells, and the IIIc is expressed in mesenchymal-derived tissues[34,35]. Taking into consideration the lack of an anabolic response, it quite possible there are additional FGFR3 splice variants in addition to the IIIa, IIIb, and IIIc that have yet to be characterized.

Overall, the results of study II suggest that FGF-18 mediates an effect on MFCs, however not in terms of the ECM or catabolic-related genes and proteins that were analyzed. The most significant evidence of this is derived from the P0 FGFR3 semi-quantitative immunofluorescent results, where we see a negative exponential relationship with FGFR3/DAPI ratio as a function of [FGF-18]

(Figure 2.9). This, in addition to changes in gene expression for the panel of genes that were assessed, as well as the subtle variations in cellular morphology, suggest FGF-18 has an effect in primary and expanded MFCs, however for the purposes of meniscus tissue engineering it may not be worthwhile investigating the specific pathways it regulates.

The effects of passaging and induced de-differentiation, in terms of the loss of type II collagen production at the translational level and reduction at the gene expression level for P2 and P4 expanded MFCs suggest expansion could be used to promote a fibroblast-like phenotype. This may be useful in the context of zonal recapitulation, which aims to produce regionally-specific ECM deposition such that it recapitulates the microarchitecture normally found within the meniscus[36]. Theoretically, using a meniscus-shaped scaffold and seeding expanded MFCs on the peripheral regions and primary, differentiated MFCs on the inner region, deriving ECM with high proportions of type I collagen on the outer region with type II collagen and proteoglycan rich inner regions may be possible. This may confer similar biomechanical properties as the native tissue, but generated in an *in vitro* setting. This would have to be verified experimentally, however.

2.5 Conclusion

This study aimed to characterize the effects of supplemented human FGF-18 on primary and expanded meniscus fibrochondrocytes. Due to the poor healing capacity of inner meniscal regions and ineffective treatments following meniscal trauma, meniscus tissue engineering strategies are under development to minimize pathological changes to the knee joint that inevitably lead to osteoarthritis progression. By characterizing the effects of growth factor supplementation on MFCs *in vitro,* this may help develop new cell-based repair strategies that create meniscal tissue with functional ECM formation. Here we demonstrated that FGF-18 supplementation could not mitigate the dedifferentiating effects of monolayer expansion and retain a differentiated ECM-forming phenotype in a 3D pellet model. This can potentially be due to an undiscovered FGFR3 splice variant that may be preferentially expressed in adult meniscal tissue. Future studies may aim to characterize the specific FGFR3 isoforms produced in MFCs and determine the validity of this claim.

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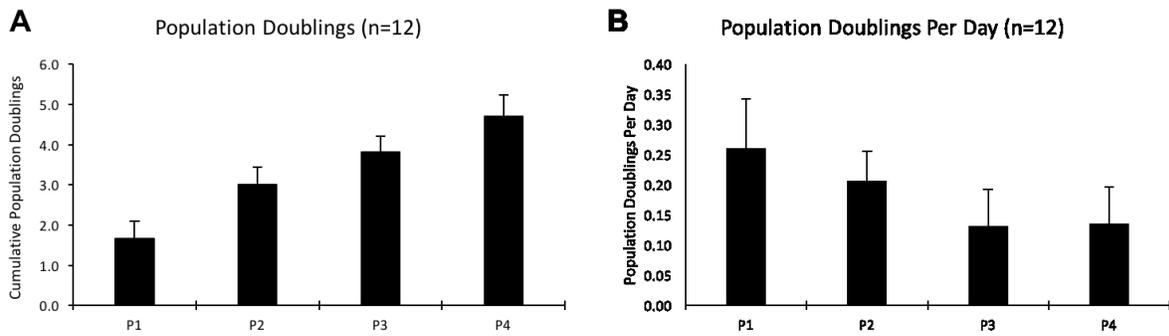
2.7 Figures

Figure 2.1: Assessment of meniscus fibrochondrocyte proliferation during monolayer expansion. (A) Cumulative population doublings (± STD) and; (B) population doublings per day (± STD) for passage 1-4 expanded meniscus fibrochondrocytes (MFCs) cultured in a standard expansion media under normoxia (21% O_2).
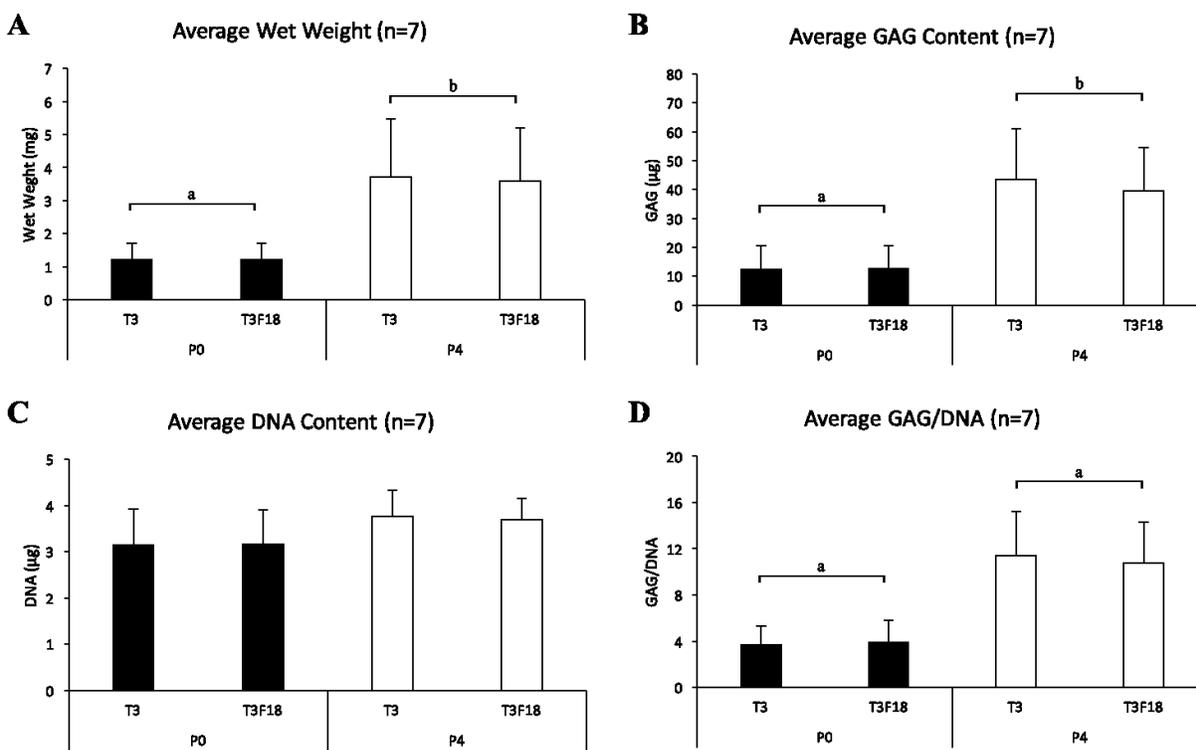
Figure 2.2: Pellet wet weight, glycosaminoglycan (GAG), and deoxyribonucleic acid (DNA) content in primary (P0) and passage 4 (P4) expanded MFCs. P0 and P4 expanded MFCs were redifferentiated in a three dimensional (3D) pellet model and cultured in a defined media supplemented with TGFB3 with or without FGF-18 for 21 days of chondrogenesis. Afterwards, DNA and GAG were measured. (A) Average pellet wet weight, (B) GAG content, (C) DNA content, (D) GAG/DNA ratio. Statistical analysis was performed using 2-way ANOVA with repeated measures. Groups with different letters are significantly different (p<0.05). All values represented as mean±STD.
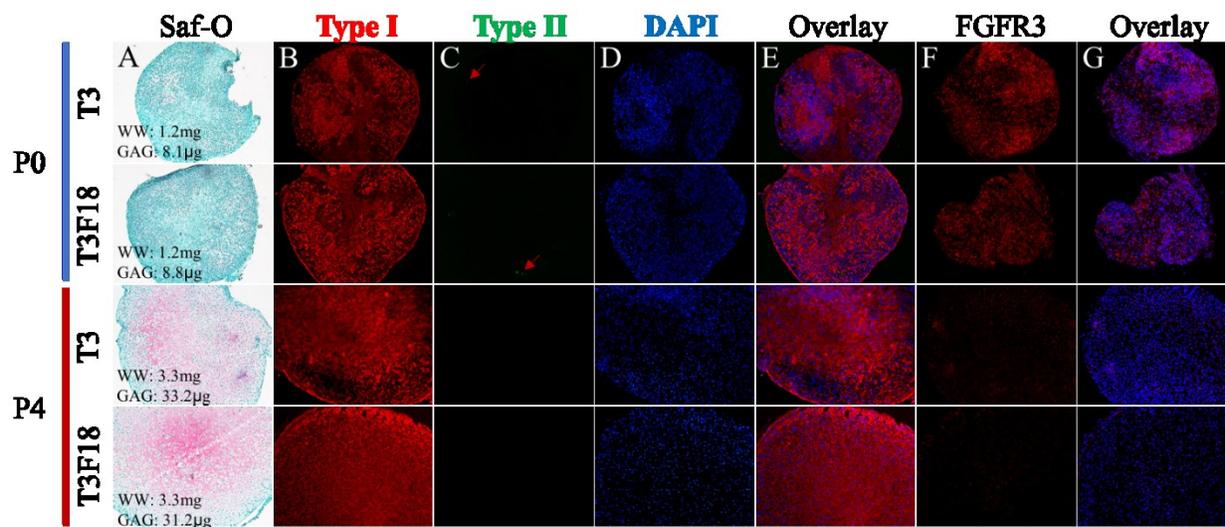
Figure 2.3: Histological assessment for meniscus-like ECM formation and visualization of FGF receptor 3 protein expression (FGFR3). 3D cell pellets were formed using primary (P0) and passage 4 (P4) expanded meniscus fibrochondroytes (MFCs) and cultured in a defined media (see methods) containing TGFB3-alone (T3) or additionally supplemented with FGF18 (T3F18) for 21 days. One representative donor is presented (male, 25 years old). (Column A) Safrannin O staining with glycosaminoglycan (GAG) value and wet weight (WW) presented in the bottom left corner, (Column B) Type I collagen immunofluorescence using Texas Red secondary antibody (red), (Column C) Type II collagen immunofluorescence using FITC (green), (Column D) DAPI (blue), (Column E) Type I, II collagen, DAPI overlay, (Column F) FGFR3 immunofluorescence using Texas Red secondary antibody (red) (G) FGFR3 immunofluorescence (red) overlayed with DAPI (blue). Images were generated using ImageJ. Red arrow indicates positive detection of type II collagen. Scale bar:100µm.
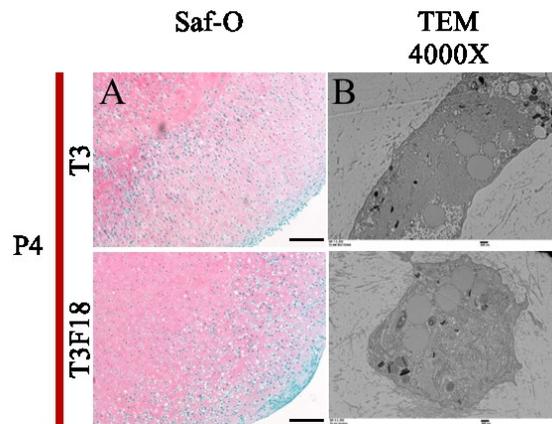
Figure 2.4: Safranin O staining and transmission electron microscopy results for passage 4 expanded MFCs cultured in a standard chondrogenic media supplemented TGFβ-3 or TGFβ-3 and FGF-18 from one representative donor. (A) Safranin O staining (scale bar: 200μm), (B-C) Transmission electron microscopy images viewed at 4000x magnification (scale bar:500nm).
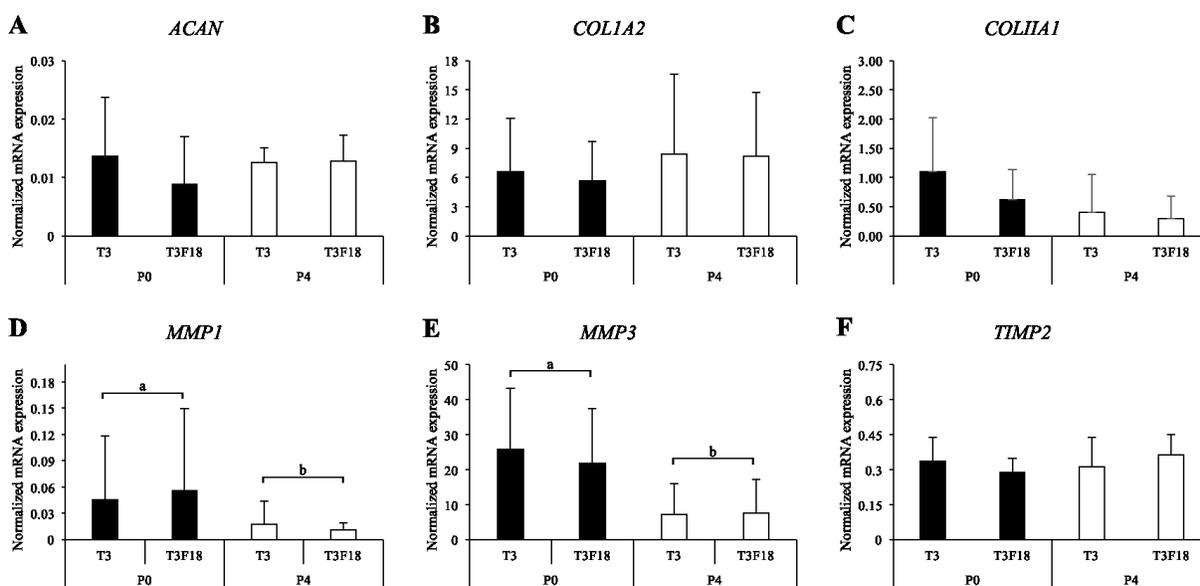
Figure 2.5: Quantitative real time PCR results for primary (P0) and passage 4 (P4) expanded MFCs that were chondrogenically-induced in 3D cell pellet model supplemented with TGFβ-3 (T3)-alone or TGFβ-3 +FGF18 (T3F18) for 21 days. Assessment of extracellular matrix-related genes are presented in A-C: Aggrecan (A), type I collagen (B), Type II collagen (C). Catabolic-related genes are presented in D-F: matrix metalloproteinase 1 (D), matrix metalloproteinase 3 (E), Tissue inhibitor of matrix metalloproteinase 2 (F). CT values were normalized to three house keeping genes, B-actin, B2M, and YWHAZ. Statistical analysis was performed using 2-way ANOVA with repeated measures. Groups with different letters are significantly different (p<0.05). All values represented as mean±STD.

A Average Wet Weight (n=4)

B Average GAG Content (n=4)

C Average DNA Content (n=4)
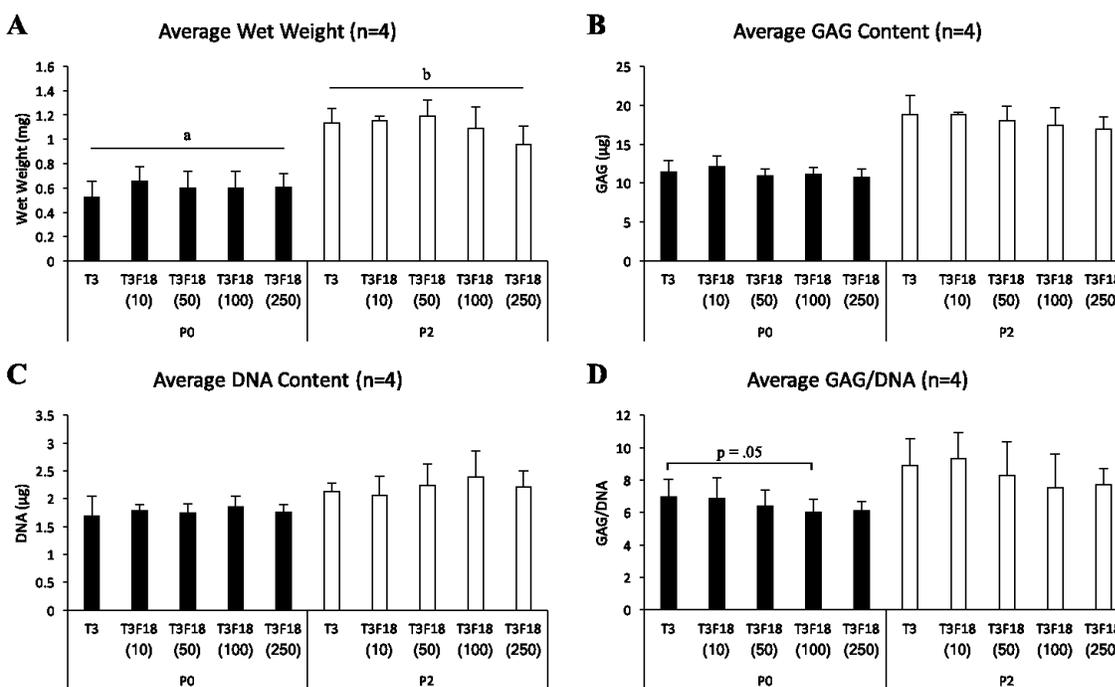
D Average GAG/DNA (n=4)

Figure 2.6: Pellet wet weight, glycosaminoglycan (GAG), and deoxyribonucleic acid (DNA) content in primary (P0) and passage 2 (P2) expanded MFCs. P0 and P2 expanded MFCs were redifferentiated in a three dimensional (3D) pellet model and cultured in a defined media supplemented with TGFβ-3 alone or additionally supplemented with FGF-18 (10, 50, 100, or 250 ng/mL) for 21 days of chondrogenesis. DNA and GAG were then measured. (A) Average pellet wet weight, (B) GAG content, (C) DNA content, (D) GAG/DNA ratio. Statistical analysis was performed using 2-way ANOVA with repeated measures. Groups with different letters are significantly different (p<0.05) unless stated otherwise. All values represented as mean±STD.
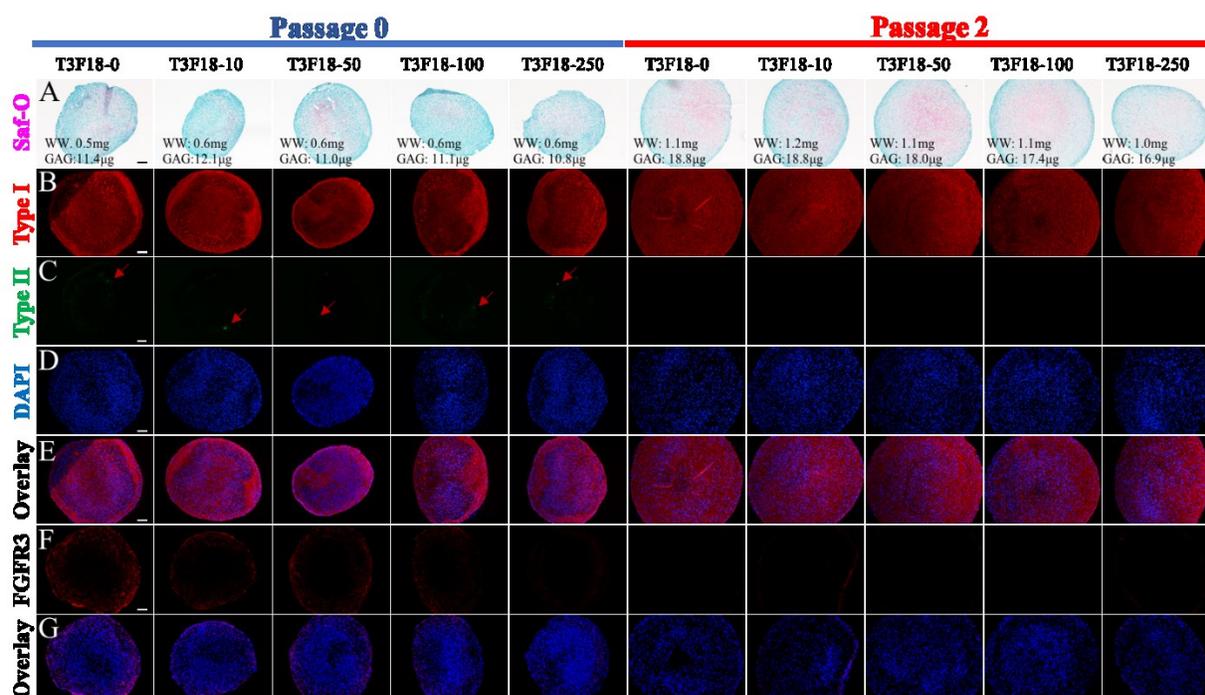
Figure 2.7: Histological assessment for meniscus-like ECM formation and visualization of FGF receptor 3 protein localization (FGFR3). 3D cell pellets were formed using primary (P0) and passage 2 (P2) expanded meniscus fibrochondroytes (MFCs) and cultured in a defined media (see methods) containing TGFβ3 alone [10n/mL] (T3) or additionally supplemented with FGF18 ([FGF-18] - 10, 50, 100, or 250 ng/mL) for 21 days of chondrogenesis. One representative donor is presented (Female, 21 years old). (Row A) Safranin O staining with glycosaminoglycan (GAG) content and wet weight (WW) presented in the bottom left corner, (Row B) Type I collagen immunofluorescence using goat anti-rabbit secondary antibody (red), (Row C) Type II collagen immunofluorescence using goat anti-mouse secondary antibody (green), (Row D) DAPI (blue), (Row E) Type I and II collagen with DAPI overlay, (Row F) FGFR3 immunofluorescence using goat anti-rabbit secondary antibody (red) (Row G) FGFR3 (red) with DAPI (blue) overlay. Images were generated using ImageJ. Red arrows indicate positive detection of type II collagen. Scale bar: 200μm.
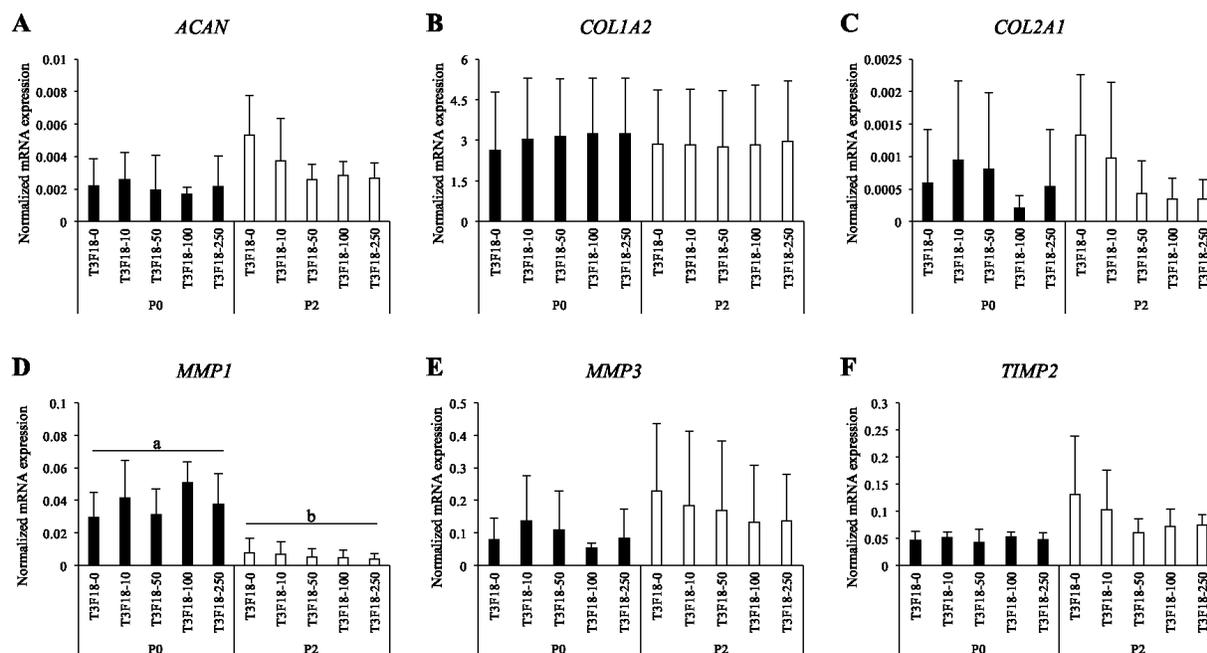
Figure 2.8: Quantitative real time PCR results for primary (P0) and passage 2 (P2) expanded MFCs that were chondrogenically-induced in 3D cell pellet model. P0 and P2 expanded MFCs were redifferentiated in a three dimensional (3D) pellet model and cultured in a defined media supplemented with TGFβ-3 alone or additionally supplemented with FGF-18 (10, 50, 100, or 250 ng/mL) for 21 days of chondrogenesis. Assessment of extracellular matrix-related genes are presented in A-D: Aggrecan (A), type I collagen (B), Type II collagen (C). Catabolic-related genes are presented in D-F: matrix metalloproteinase 1 (D), matrix metalloproteinase 3 (E), Tissue inhibitor of matrix metalloproteinase 2 (F). CT values were normalized to two house keeping genes B-actin and B2M. Statistical analysis was performed using a 2-way ANOVA with repeated measures. Groups with different letters are significantly different (p<0.05). All values represented as mean±STD.
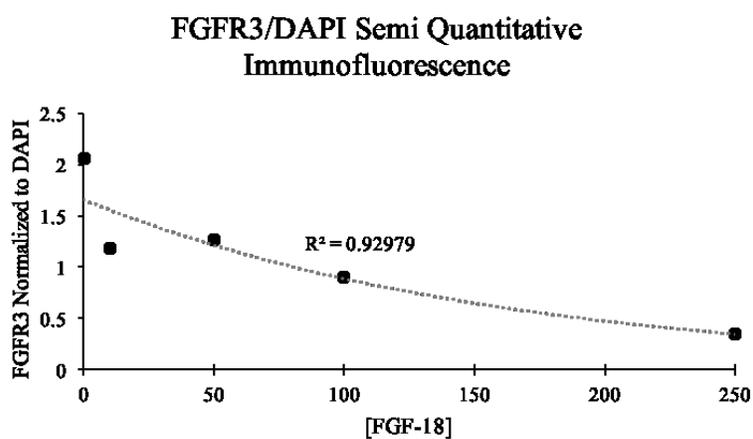
FGFR3/DAPI Semi Quantitative
Immunofluorescence

Figure 2.9: Semi-quantitative immunofluorescent results comparing FGFR detection normalized to DAPI content and plotted against [FGF-18] in Passage 0 MFC pellets from Part II of this study.

2.8 Tables

Table 2.1: Summary of non-identifying donor

Donor	Age	Sex	Previous Medical History	Meniscus	Mass (g)	Total P0 Cells (x10^6)	Cell Yield (M cells/g of tissue)
NMEN 245	28	M	ACL deficiency	R-Medial	4.30	21.4	4.98
NMEN 251	31	M	right knee meniscal tear	R-Medial	1.82	4.25	2.34
NMEN 252	23	M	no medical hx	L-Medial	3.55	7.55	2.13
NMEN 266	39	M	no medical hx	L-Medial	1.78	9.92	5.57
NMEN 267	25	M	no medical hx	L-Medial	3.63	15.8	4.35
NMEN 291	27	M	no medical hx	R-Medial	2.17	5.61	2.59
NMEN 293	45	M	right knee meniscus repair	L-Medial	0.78	1.97	2.53
NMEN 294	30	F	no medical hx	L-Medial	1.90	9.95	5.24
NMEN 296	38	F	no medical hx	L-Lateral	1.4369	5.8	4.04
NMEN 297	21	F	previous ACL repair	L-Medial	1.7748	9.225	5.20
NMEN 303	17	F	no previous medical hx	R-Medial	0.898	7.175	7.99
NMEN 304	25	M	no previous medical hx	L-Medial	1.0343	10.89	10.53
Mean ± SEM	**29.1 ± 2.3**				**2.1 ± 0.3**	**9.1 ± 1.5**	**4.8 ± 0.7**

Table 2.2: Name, primer sequences, and NCBI reference numbers for the candidate and housekeeping genes assessed via qRT-PCR.

Gene	Primer sequences		NCBI Reference
β-actin (*ACTB*)	5'AAGCCACCCCACTTCTCTCTAA3'	Forward	NM_0011101
	5'AATGCTATCACCTCCCCTGTGT3'	Reverse	
β-2 microglobulin (*B2M*)	5'TGCTGTCTCCATGTTTGATGTATCT3'	Forward	NM_004048.2
	5'TCTCTGCTCCCCACCTCTAAGT3'	Reverse	
YWHAZ	5'TCTGTCTTGTCACCAACCATTCTT3'	Forward	NM_003406
	5'TCATGCGGCCTTTTTCCA3'	Reverse	
Aggrecan (*ACAN*)	5'AGGGCGAGTGGAATGATGTT 3'	Forward	NM55172
	5'GGTGGCTGTGCCCTTTTTAC3'	Reverse	
Collagen I (*COL1A2*)	5'TTGCCCAAAGTTGTCCTCTTCT3'	Forward	NM_000089
	5'AGCTTCTGTGGAACCATGGAA3'	Reverse	
Collagen II (*COL2A1*)	5'CTGCAAAATAAAATCTCGGTGTTCT 3'	Forward	NM_033150
	5'GGGCATTTGACTCACACCAGT3'	Reverse	
Matrix metalloproteinase 1 *(MMP1)*	ATGAGTCTTTGCCGGAGGAA	Forward	NM_002421
	GTGACACCAGTGACTGCACATG	Reverse	
Matrix metalloproteinase 3 *(MMP3)*	AGGCATCCACACCCTAGGTTT	Forward	NM_002422
	ATCAGAAATGGCTGCATCGAT	Reverse	
Tissue inhibitor of metalloproteinases 2 (*TIMP2*)	TGGGCGTGGTCTTGCAA	Forward	NM_003255
	GCCGCTGAATAGAACAGGCTAA	Reverse	

Chapter 3: Conclusion

3.1 Concluding Remarks, Implications, and Future. Directions

Osteoarthritis is a progression degenerative joint condition that significantly reduces a patient's quality of life due to chronic pain, inflammation, and reduced joint mobility[1–5]. A cardinal characteristic of the disease is degenerating articular cartilage on joint surfaces, leading to alteration of all tissues comprising the joint including subchondral bone breakdown[4,6–8]. Due to the progressive nature of the disease accompanied with the poor healing capacity of articular cartilage, identifying new therapies that maintain joint integrity is of extreme importance[1,9–11]. In the context of the knee joint the menisci play an integral role in proper joint physiology despite their previous misclassification as being vestigial structures[12–14]. Following meniscal trauma, particularly those affecting the inner avascular regions, the most common treatment is in the form of surgical debridement through partial meniscectomies[13,15–17]. This is a predisposing factor for osteoarthritis development, however, due to increased stress on articular cartilage[15,18–20].

The menisci are fibrocartilaginous wedge shaped tissues within the knee joint that have important functions including load transmission across the joint and protection of articular cartilage[13,21–23]. By dissipating axial loading forces across the joint into hoop stresses within the tissue, the resultant force experienced by articular chondrocytes is diminished, thus serving as a protective factor for articular cartilage health[17,24,25]. This biomechanical functionality of this tissue is believed to derived from the menisci's extracellular matrix composition, as seen in many other mesenchyme-derived tissues[24,26]. As such, new therapeutic options are being investigated that promote meniscus repair or replacement.

Cell-based meniscus tissue engineering is one area that aims to develop repair and replacement treatment options following meniscal injury. By combining chondro-inductive cell sources, experimental models, and bioactive agents, promoting a differentiated meniscus-like ECM forming phenotype may be possible in laboratory derived tissues[27–29]. A variety of cell types and growth factors have been analyzed to varying degrees of clinical applicability. Meniscus fibrochondrocytes (MFCs) may be an optimal cell source for meniscus tissue engineering since they are the principal cell type comprising meniscal tissue, are pre-programmed for an ECM-forming phenotype naturally, and reduce the likelihood of immunorejection when used as an autologous cell source[28,30]. Because only a fraction of the meniscus is targeted during partial meniscectomies, and generation of an entire replacement construct is the long term goal, inducing cell division to increase cell numbers is required. This process, termed monolayer expansion, is associated with detrimental processes including a diminished ECM-forming capacity known as de-differentiation[30,31]. As such, identifying novel growth factors that promote ECM-synthesis and the redifferentiation of expanded MFCs is of great interest. Fibroblast growth factor (FGF) – 18 has displayed promising results in a variety of related cell types including articular chondrocytes and embryonic mesenchymal stem cells by upregulating ECM proteins including type II collagen and proteoglycans, two major ECM proteins found within the inner meniscal regions[32,33]. These effects appear to be mediated through agonism of FGFR3.

In this study we investigated if FGF-18 supplementation could induce a differentiated ECM phenotype in expanded MFCs. Our results from Part I of this study suggest FGF-18 at 10ng/mL was insufficient to induce an anabolic effect, however it was clear FGFR3 was downregulated as a function of passage at the protein level when comparing primary MFC pellets to passage 4

expanded MFC pellets (Figure 3.1F). Additionally, when P4 expanded MFCs were supplemented with TGFβ-3 and FGF-18, cellular morphologies were primarily round and chondrocyte like in contrast to the TGFβ-3 alone group which were fibroblastic. This was observed in the Safranin-O stained sections and was further supported by transmission electron microscopy imaging.

Results from Part I inspired the follow up concentration study, using P0 and P2 expanded MFCs with a range of FGF-18 from 0-250ng/mL. In this study we saw no discernable anabolic effect of FGF-18 at any concentration using the ECM proteins of interest as indicators. Semi-quantitative immunofluorescent results displayed a negative exponential relationship with FGFR3:[FGF-18] at passage 0, suggesting FGF-18 was interacting with this receptor and induced a homeostatic shift to accommodate excess receptor agonist (FGF-18) present in the media (Figure 3.2 - $R^2 =$ 0.930). In passage 2 pellets, FGFR3 detection was nearly absent altogether in comparison to P0 (aligned with Part I), which suggested FGFR3 may be used as a marker for the state of de-differentiation in expanded MFCs. Overall, our results suggest that FGF-18 confers no benefit in terms of enhanced ECM-formation indicative of a differentiated phenotype. This may potentially be due to an uncharacterized FGFR3 splice variant preferentially expressed in adult meniscal tissue.

The passaging effects suggest there may be some downstream application of these findings through a process known as zonal recapitulation. This theory posits that seeding different cell types as a function of region may promote regional-specific ECM formation, similar to the ECM microarchitecture in the native tissue[28]. The downregulation of type II collagen at the transcriptional and translational level in the expanded MFCs used in this study suggest the

dedifferentiating effects from cell expansion were not reversible during chondrogenic induction in a three dimensional cell pellet model. Theoretically, seeding expanded MFCs that had undergone dedifferentiation (fibroblastic phenotype with primarily type I collagen ECM) on the outer regions and primary chondrocyte-like MFCs (fibrochondrocyte-like phenotype with type II collagen and proteoglycans) on the inner regions of a meniscus-shaped scaffold may recapitulate this ECM microarchitecture and lead to the derivation of a biomechanically functionally equivalent replacement construct.

The limitations of this study include only testing the effects of FGF-18 supplementation in one cell type (meniscus fibrochondrocytes). Future studies may evaluate the effects using a coculture model with articular chondrocytes and meniscus fibrochondrocytes in various ratios as well as in the presence or absence of FGF-18 to see if the anabolic effects could be induced as has been described in literature [32,34,35].

Another limitation of this study was the use of one solitary expansion protocol (described in methods, section 2.2). Expansion of meniscus fibrochondrocytes in the presence of TGFβ-1 and FGF-2 has been shown to increase proliferation rates while simultaneously preserving the redifferentiation capacity of expanded MFCs when cultured under hypoxia[30,36]. Using this expansion method followed by chondrogenic induction in a 3D pellet model with exogenously supplemented FGF-18 may induce an anabolic effect.

Overall, this study suggests that exogenously supplementing FGF-18 during 3D chondrogenic

culture of expanded MFCs does not promote a redifferentiated ECM-forming phenotype and

may not be useful for the purposes of meniscus tissue engineering.

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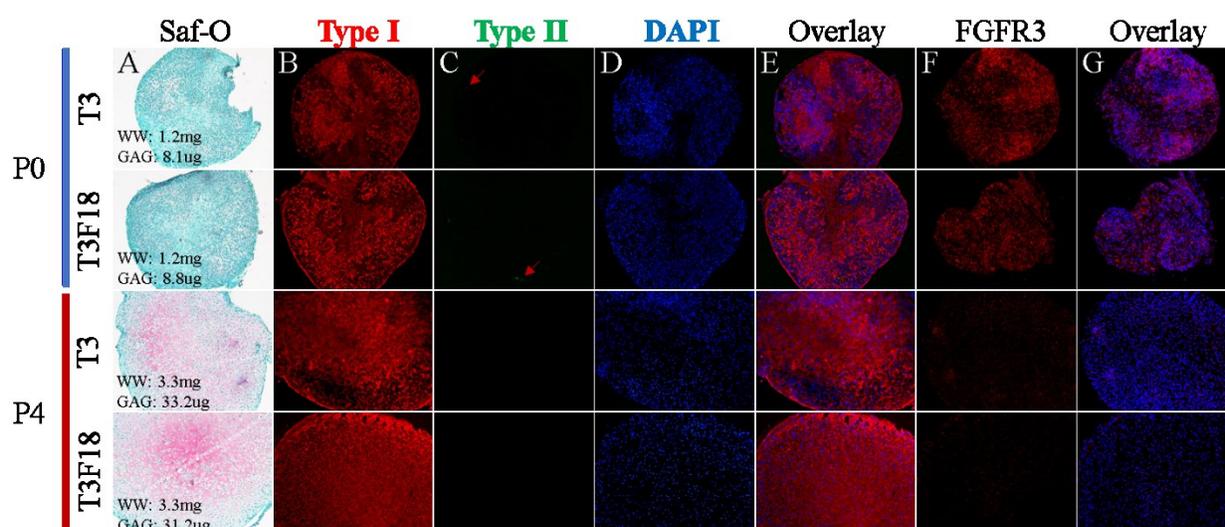
3.3 Figures

Figure 3.1: Histological assessment for meniscus-like ECM formation and visualization of FGF receptor 3 protein expression (FGFR3). 3D cell pellets were formed using primary (P0) and passage 4 (P4) expanded meniscus fibrochondroytes (MFCs) and cultured in a defined media (see methods) containing TGFB3-alone (T3) or additionally supplemented with FGF18 (T3F18) for 21 days. One representative donor is presented (male, 25 years old). **(Column A)** Safrannin O staining with glycosaminoglycan (GAG) value and wet weight (WW) presented in the bottom left corner, **(Column B)** Type I collagen immunofluorescence using Texas Red secondary antibody (red), **(Column C)** Type II collagen immunofluorescence using FITC (green), **(Column D)** DAPI (blue), **(Column E)** Type I, II collagen, DAPI overlay, **(Column F)** FGFR3 immunofluorescence using Texas Red secondary antibody (red) **(G)** FGFR3 immunofluorescence (red) overlayed with DAPI (blue). Images were generated using ImageJ and enhanced equally between conditions to minimize bias. Red arrow indicates positive detection of type II collagen.

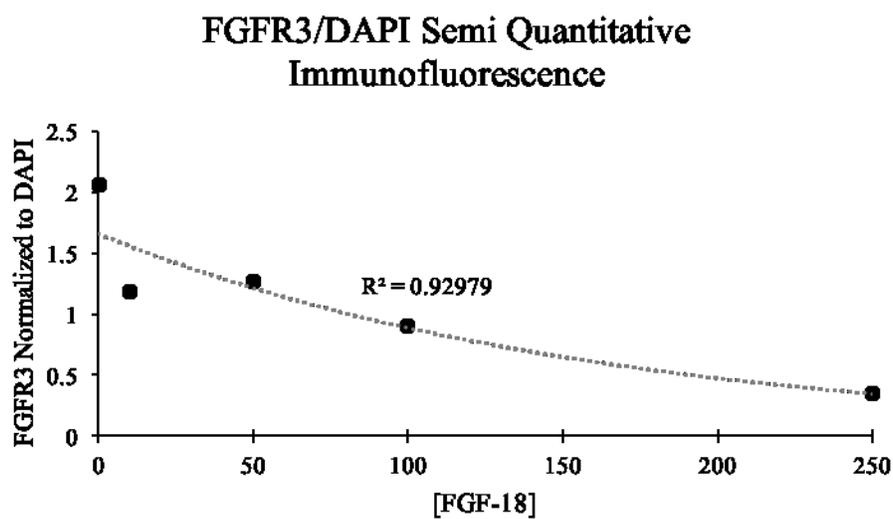## FGFR3/DAPI Semi Quantitative Immunofluorescence

Figure 3.2: Semi-quantitative immunofluorescent results comparing FGFR detection normalized to DAPI content and plotted against [FGF-18] in Passage 0 MFC pellets from Part II of this study.

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