Models for Determining Estrogen Effects on Meniscal Fibrochondrocytes

By

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Abstract

Introduction: An estimated 242 million people in the world are living with symptomatic and activity-limiting osteoarthritis (OA). Women are 2-6X more likely to experience musculoskeletal injury and degeneration, highlighting a sexual dimorphism in diseases such as OA. One major cause of knee osteoarthritis is damage or injury to the fibrocartilaginous meniscal discs**.** The most common treatment for meniscal injury is partial or complete resection of the meniscus by meniscectomy. With resection of the meniscal tissue, the meniscus structural benefits to the joint are severely limited. Estrogens are steroid hormones that function to promote gene transcription and downstream activity and to play a role in musculoskeletal cell proliferation and new extracellular matrix production. The overall purpose of this thesis is to develop models for understanding the in-vitro response of meniscal cells to estrogen treatment to both determine a mechanism for the sex differences in OA and identify therapeutic strategies to promote new meniscal tissue growth by capitalizing on estrogen signaling.

Methods: Meniscus cells were harvested from the lateral, and medial meniscal discs of bovine (17 months old) and human samples (male and female, 18 years and 47 years old) and separated based on region (inner vs outer). The effect of hormone-free media composition, estrogen dosing concentration and kinetics, and 2D vs 3D on cell proliferation and extracellular matrix production was determined using assays to assess cell number and quantify RNA and protein level changes to extracellular matrix components. Specifically, the effect of hormone-free media components, namely charcoal/dextran treated FBS (Char FBS) and phenol red-free basal media (prfDMEM), on cell proliferation and ECM production was assessed to establish baseline readings prior to estrogen studies. Estrogen dosing at low and high doses and pulsed or continuous dosing kinetics was completed. Lastly, scaffold-free spheroid culture models were established using a hanging drop method to assess the role of estrogen in 3D.

Results: Both 2D and 3D in-vitro models were established in hormone-free culture media for estrogen dosing studies. Proliferation at day one in both 10 % and 15 % Char FBS was not

statistically different from the control group. No statistically significant changes to total GAG and collagen content were observed due to media composition or estrogen treatment. Phenol red-free DMEM (prfDMEM) significantly reduced cell proliferation compared to cells in charcoal FBS and normal FBS medium. Estrogen treatment showed no observable differences between conditions at 24 and 72 hours from inner and outer meniscus in cell proliferation. Gene expression of extracellular matrix components showed preliminary evidence that estradiol exhibited dose-dependent effects in both male and female cells. There are significant differences between sexes in cell proliferation but not significant differences in gene expression of matrix macromolecules.

Conclusions: 2D and 3D culture models were established to assess the role of estrogen on bovine and human MFCs. Using these models, it was determined that E2 promotes the upregulation of ACAN and Col2a1 gene expression while decreasing Col1a1 expression, at concentrations of 10^{-8}M. Further, in the spheroid culture model, 10^{-8}M E2 decreased significantly less in diameter compared to the 0M E2 group. Overall, these results highlight that MFCs, although partly responsive, are likely not the cell type responsible for responding to estrogen treatment at these dosages and culture conditions. The models developed in this thesis will continue to be explored to expand our working knowledge and develop better strategies to probe the effects of E2 on other cells in the knee joint to determine their role in knee health and joint tissue homeostasis.

Acknowledgements

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- Jesus G Gonzalez Flores

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1. Introduction

An estimated 242 million people in the world are living with symptomatic and activity-limiting osteoarthritis (OA). Within this population, an estimated 31 million people suffer from OA in the US. Women are 2-6X more likely to experience musculoskeletal injury and degeneration(Felson & Nevitt, 1998), highlighting a sexual dimorphism in diseases such as OA. One major cause of knee osteoarthritis is damage or injury to the fibrocartilaginous meniscal discs. Recently, two meta-analysis studies (>1,000 meniscus injuries analyzed) have indicated that sex plays a role in meniscal injury and repair. First, meniscal injury rates are higher for female high school athletes compared to males comparing data from gender-comparable sports(Mitchell et al., 2016). Second, data analysis has shown that females required repeat surgeries 2.2X more frequently than males after initial meniscal repair(Patel, Mundluru, Beck, & Ganley, 2019). These differences point towards meniscal repair rates dependence on sex hormones, namely estrogen. Understanding the role of estrogen on meniscus health is necessary to both determine a potential

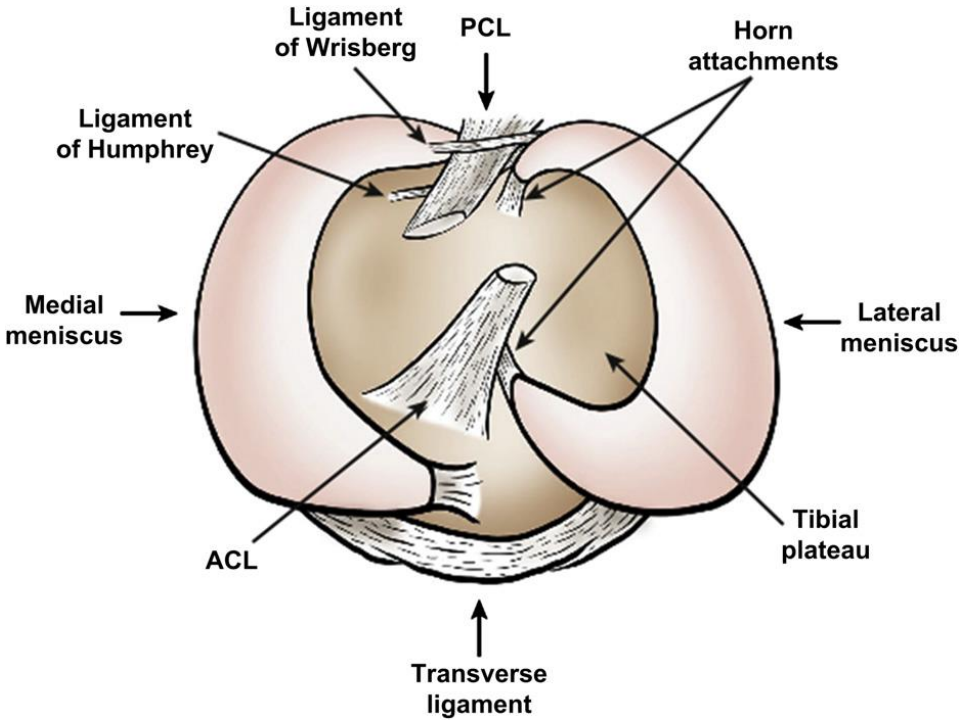

Figure 1 *Anatomy of the meniscus: superior view (Makris, Hadidi, Athanasiou, 2011)*

role in osteoarthritis and develop a regenerative strategy to provide tissue homeostasis and reduce the onset of OA.

1.1. The meniscus

The meniscus of the knee has two semi-lunar fibrocartilaginous discs as seen in **Figure 1**. The lateral and medial meniscus are wedged between knee joint and, in contrast to other cartilaginous disks, only cover part of the joint surface. The knee meniscus contributes significantly to the biomechanical function of the joint and functions to stabilize, distribute forces in the joint, and act as a shock absorber(Makris, Hadidi, & Athanasiou, 2011). The meniscal discs are composed of a tridimensional complex of primarily collagen, water, and proteoglycans(Maffulli, Longo, Campi, & Denaro, 2010).The meniscus has two distinct regions, the avascular (inner 1/3rd) and the vascular region (outer 2/3rd)(Maffulli et al., 2010; Makris et al., 2011) The vascularized zone of the meniscus is divided into the red-red, and the red-white zones as seen in **Figure 2**. The meniscal zones have distinct cell phenotypes. The outer vascularized zone contains cells with spindle-

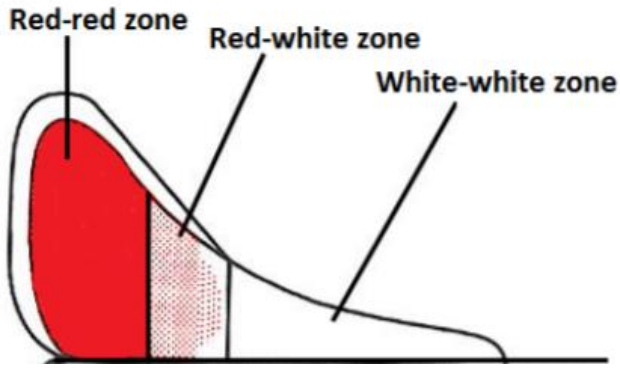

Figure 2 *Meniscus graphical representation of vascularity. Outer meniscus (red-red) and middle region (red-white) of the meniscus are vascularized while the inner (white-white) is non-vascularized.*

shaped, fibroblast morphology while the inner, avascular zone has cells with round, chondrocyte morphologies. **Figure 3** illustrates the meniscus cellular and extracellular matrix composition. The primary regional differences present in the tissue include collagen composition, collagen fiber alignment and their organization, and proteoglycans content. At the surface, the fibers are aligned

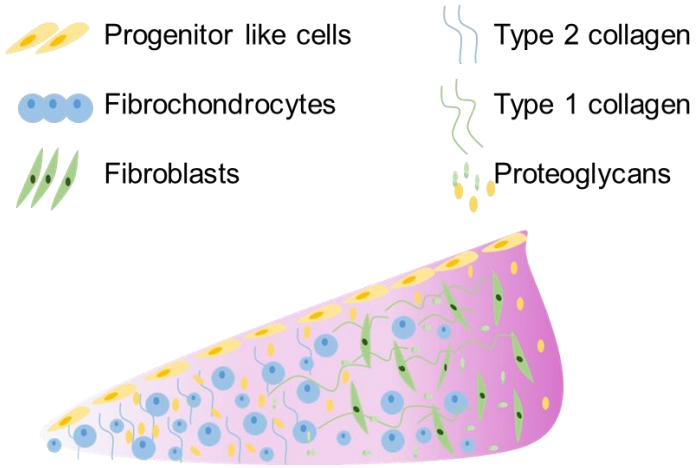

Figure 3 *Illustration of the cellular composition of the meniscus. The meniscus has a heterogeneous cell composition but there are regional differences as seen in the illustration. Recently it has been postulated that a small number of progenitor like cells are present in the surface of the meniscus. Inner meniscus is primarily composed of chondrocyte like fibrochondrocytes while the outer region is primarily composed of fibroblast like fibrochondrocytes.*

randomly, while the bulk of the tissue has circumferential and radial aligned fibers(Aspden, Yarker, & Hukins, 1985). Each region is made of fibers that are separated into multiple layers and

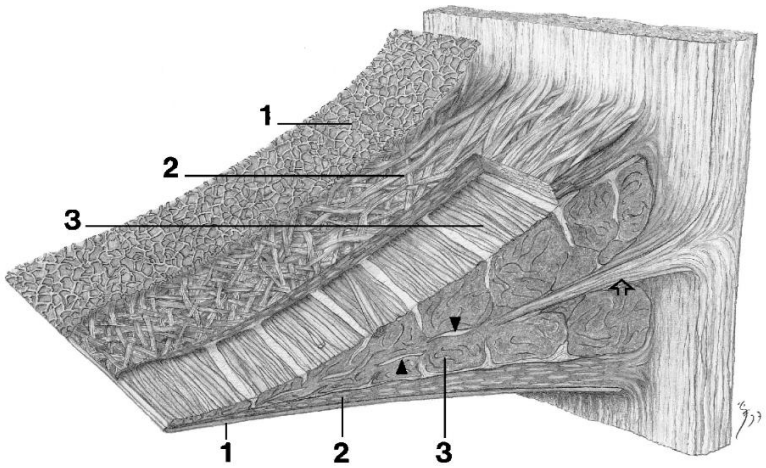

Figure 4 *Synoptic drawing. Scanning electron microscopy reveals three distinct layers in the meniscus cross section: (1) The superficial network: the tibial and femoral sides of the meniscus surface are covered by a meshwork of thin fibrils. (2) Lamellar layer: beneath the superficial network there is a layer of lamellae of collagen fibrils on the tibial and femoral surface. In the area of the external circumference of the anterior and posterior segments the bundles of collagen fibrils are arranged in a radial direction. In all other parts the collagen fibril bundles intersect at various angles. (3) Central main layer: the main portion of the meniscus collagen fibrils is located in the central region between the femoral and the tibial surface layers. Everywhere in the central main layer of the meniscus the bundles of collagen fibrils are orientated in a circular manner. In the region of the internal circumference a few radial collagen fibrils are interwoven with the circular fibril bundles (arrowheads). In the external circumference loose connective tissue from the joint capsule penetrates radially between the circular fibril bundles (arrow)(reproduced from Petersen & Tillmann, 1998)*

multiple orientations, which gives the tissue its impressive mechanical properties. **Figure 4** (Petersen & Tillmann, 1998)shows the orientation of these fibers. The circumferential orientation allows the dissipation of compressive forces through hoop stresses. Without the circumferential fibers, the meniscus would be extruded out of the joint. The radial fibers provide structural support by forming tie-ins to the circumferential fibers. They also dissipate the forces that could lead to longitudinal splitting in the meniscus. The tensile modulus of the meniscus in the circumferential fiber region is in the range of 100-300 MPa, while at the radial fibers is ten times lower(Makris et al., 2011) The shear modulus of the tissue is in the range of 120kPa(Makris et al., 2011).

1.2. The extracellular matrix

The extracellular matrix (ECM) is composed of a complex, interlocking fibrous proteins and glycosaminoglycans supporting cells. Of these proteins, collagen is the number one constituent of the ECM, making up roughly 75%(Herwig, Egner, & Buddecke, 1984; Proctor, Schmidt, Whipple, Kelly, & Mow, 1989). The meniscus ECM is composed primarily of collagen type 1(Abraham & Donahue, 2013; Aspden et al., 1985; Makris et al., 2011) but an assortment of other types of collagen variants are present in the tissue. Other important components of meniscus ECM are proteoglycans, which make up about 17% of the total organic matter of the meniscus(Herwig et al., 1984; Proctor et al., 1989); aggrecan is the major large proteoglycan in this matrix(Scott, Nakano, & Dodd, 1997). Proteins such as fibronectin and collagen VI serve as a link between ECM and meniscus cells.

When considering the environment for cells one should consider the hierarchical composition of tissues. The ECM is composed of multiple layers that range from fibers in the micro scale to whole tissues at the macro scale. The ECM anchors cells within its fibrillar proteins and polysaccharides to provide a specific environment providing structural support and cell adhesion. The ECM and cells feed each other signals, where the forces generated by the ECM are equivalent to the level of tension provided by the cytoskeleton in the cells(Bao, Xie, & Huck, 2018). The ECM sequesters

proteins and peptides via charge as well and thus provides mechanical and chemical cues to the cells. The extracellular matrix can be composed of different materials depending on the tissue or organ in question. Due to the feedback between cells and substrate, one should consider not only

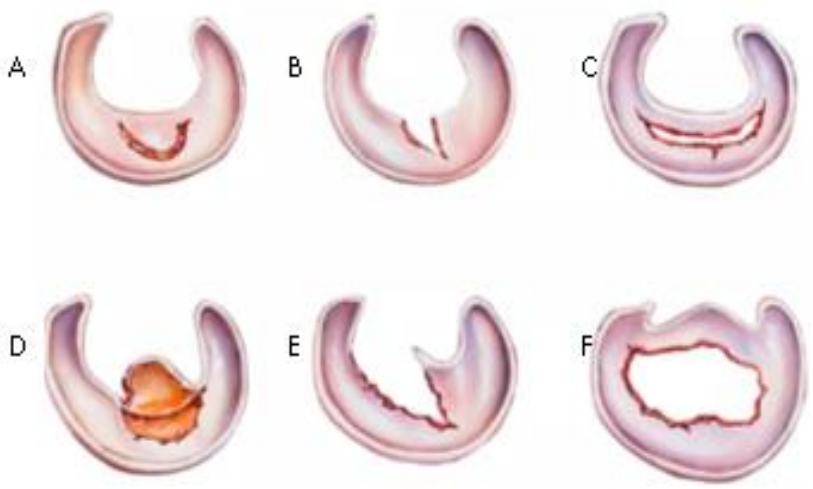

Figure 5 *Common meniscal tear types. (A) horizontal, (B) radial, (C) vertical, (D) flap, (E) parrot beak, and (F) bucket handle.*

materials, but also overall properties that have an effect on the cells when designing regenerative strategies for the meniscus.

1.3. Clinical need of meniscus regeneration

Meniscal injuries occur at the rate of 1 million cases every year in the US alone. It has been reported that the incidence of meniscal injury resulting in meniscectomy is 61 per 100,000, of which medial versus lateral meniscus injury was 81 versus 19%, respectively(B. E. Baker, Peckham, Pupparo, & Sanborn, 1985). The vast majority of these surgeries include the resection of the meniscal tissue, taking away the structural benefits of the tissue and its function from the area. This used to be the gold standard in the treatment of meniscal tears(Abraham & Donahue, 2013; Butler, Juncosa, & Dressler, 2004; Maffulli et al., 2010; Makris et al., 2011) up until recently(Gillquist, Hamberg, & Lysholm, 1982). This procedure leads to the degradation of the cartilaginous tissue and potential development of osteoarthritis. With the recent understanding of

meniscus properties and role in joint health, partial meniscectomy is now the standard of care for meniscus tears that cannot be sutured or repaired. Meniscal injury patterns are shown in **Figure 5**. The types of tears that can occur are **5A** horizontal, **5B** radial, **5C** vertical, **5D** flap, **5E** parrot beak, and **5F** bucket handle. These injuries can be combinatorial or just one depending on the severity of the injury. Overall, the type of tear and subsequent lack of meniscus repair is associated with OA development(Jarraya et al., 2017). The reconstruction of these injuries is primarily done by replacing the injured site with an autogenous or allogeneous soft tissue. The problem that arises from this approach is that these do not remodel as the previous attachment site(Abraham & Donahue, 2013; Apostolakos et al., 2014; Deymier et al., 2017), as well as there being limited resources due to the lack of cadaveric allografts that make up the majority of the repairs. Meniscal repair in the avascular zone with most surgical procedures give mixed results as to the efficacy of treatment(Dutton, Choong, Goh, Lee, & Hui, 2010). Even in the vascularized zone of the meniscus, re-rupture of the tissue occurs at a rate of 30%(Gillquist et al., 1982).

1.4. Male vs female dimorphism in other tissues

Estrogens are steroid hormones that function to promote gene transcription and downstream activity. Estrogens bind with a high affinity to estrogen receptors alpha (ERα) and beta (ERβ). These receptors have been shown to be present within several cell types including meniscal fibrochondrocytes (W. Wang, Hayami, & Kapila, 2009) . Estrogen has been shown to regulate differentiation of mesenchymal stem cells into osteoblast and osteoclast(Almeida et al., 2016) . Similarly, growth plate chondrocytes exhibit sex specific response to treatment(Elbaradie, Wang, Boyan, & Schwartz, 2013).The differences in response to estrogen treatment could be due to the receptors associated with the stimulus as intracellular receptors and membrane associated receptors have been shown to produce a different response in chondrocytes (Dienstknecht, 2010).

1.5. Dosing kinetics

Estrogen affects transcriptional status by activating its corresponding nuclear receptor, it is also understood that it can elicit cellular reactions within minutes that do not conform to the transcription mediated effects of estrogen(Azuma & Inoue, 2012). These reactions are considered "non-genomic". The classical pathway of transcription results in estrogen binding to the ERα or ERβ which then elicits a series of changes including conformational change, dimerization, and translocation to the nucleus(Beato, Herrlich, & Schütz, 1995). The receptor complex will then bind to the estrogen response element and act as an enhancer attracting co-factors and to promote gene transcription(Carroll et al., 2006; Klinge, 2001). The biological effects of estrogen are the result of a complex interplay of transcriptional and non- transcriptional signaling

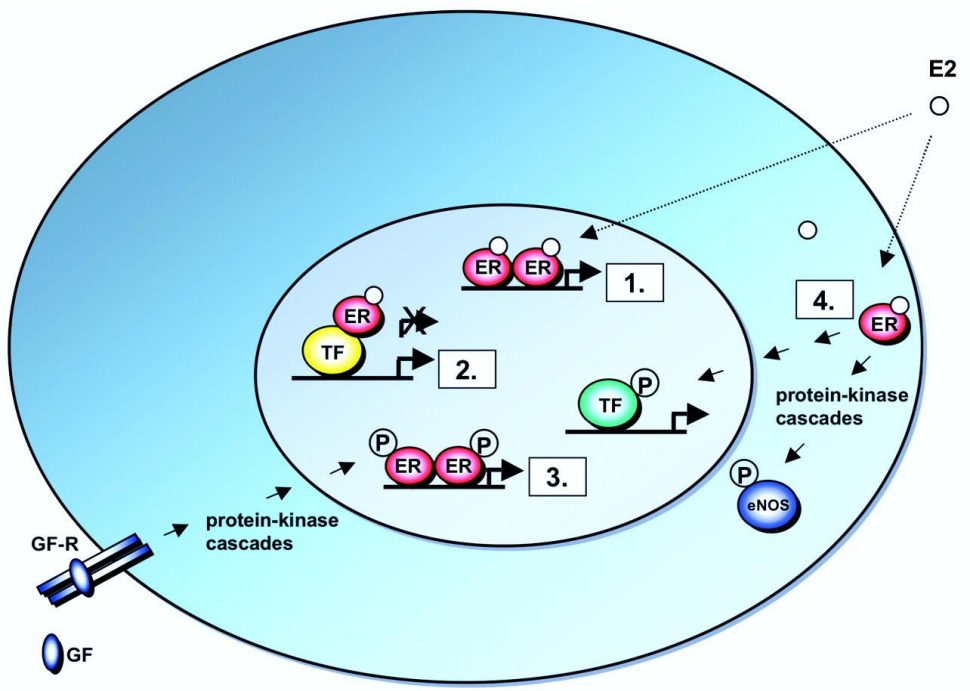

Figure 6 *Schematic Illustration of ER Signaling Mechanisms 1. Classical mechanism of ER action. Nuclear E2-ERs bind directly to EREs in target gene promoters. 2. ERE-independent genomic actions. Nuclear E2-ER complexes are tethered through protein-protein interactions to a transcription factor complex (TF) that contacts the target gene promoter. 3. Ligand-independent genomic actions. Growth factors (GF) activate protein-kinase cascades, leading to phosphorylation (P) and activation of nuclear ERs at EREs. 4. Non-genomic actions. Membrane E2-ER complexes activate protein-kinase cascades, leading to altered functions of proteins in the cytoplasm, e.g. activation of eNOS, or to regulation of gene expression through phosphorylation (P) and activation of a TF(reproduced from Björnström & Sjöberg, 2005).*

mechanisms. A report by Simoncini et al. showed that the effect of dosing kinetics produces

different responses from human umbilical vein endothelial cells(Simoncini et al., 2005). The effect seen was that the estrogen administered in a pulsed treatment elicited a non-genomic response in which the response elicited was quick after contact with the estrogen and the receptor. This study expanded on previous research that demonstrated that the dosing kinetics produce a similar effect on a mouse animal model(Devissaguet, Brion, Lhote, & Deloffre, 1999) as well as normal and cancer cell lines in proliferation(Cavaillès et al., 2002). In another study it was shown that the dosage administration of estrogen caused gene expression differences between pulsed and continuous treatments and had a significant increase in apoptosis and proliferation related cell cycle pathways(Li et al., 2008). Estrogen and its release kinetics can then be hypothesized to produce a response in regards to administration and dosage. The current understanding of the events caused by estrogen are summarized in **Figure 6**(Björnström & Sjöberg, 2005). In a recent study using an ovariectomized rat model, estradiol was administered at a pulse or continuous treatment; in this study, they analyzed the effects of this treatment in the bone, uterus, liver and mammary glands of the rats. In its results the researchers demonstrated that the pulsed release had a significantly reduced incidence of stimulatory effects of estrogen(Otto et al., 2012). The effects of E2 treatment in a pulsed treatment was seen to successfully prevent ovariectomized induced bone loss(Otto et al., 2012). This method of administration can be exploited when treating different conditions where local delivery at a control rate is desired to reduce the risk of estradiol's negative effects as a systemic treatment.

The overall purpose of this thesis was to develop models for understanding the *in vitro* response of meniscal cells to estrogen treatment to both determine a mechanism for the sex differences in OA and identify therapeutic strategies with estrogen. The overall hypothesis was that estrogen treatment would promote proliferation of cells and ECM protein upregulation at 17β-estradiol (E2) treatment conditions. In the context of the overall hypothesis, three specific hypotheses were tested. First, it was hypothesized that a hormone-free media composition would be determined to

produce the same or higher proliferation of cells. In the following study, it was hypothesized that estrogen treatment would increase proliferation and gene expression of extracellular matrix (ECM) proteins. Lastly, it was hypothesized that dosage kinetics would not produce an increase in proliferation but that estrogen dosage would. Also understanding the appropriate dosage to elicit an appropriate response to provide the local population of cells the adequate cues for homeostasis is vital. The studies presented in this thesis aimed to determine the role of estrogen on bovine and human primary meniscal fibrochondrocyte proliferation and extracellular matrix production as a function of age, sex, and region. Techniques were used to determine changes in cell phenotype at the RNA and protein level to get an overall picture of the cells' response to estrogen treatment.

2. Materials

Media and solutions for cell culture

Table 1 *Media composition for cell culture experiments*

Solution	Composition
Live dead solution	2uM Calcein 4uM ethidium homodimer-1 PBS
Freezing Cell Media	10% (v/v) DMSO 90% (v/v) FBS
10% FBS Cell culture Media	88% DMEM 10% FBS 1% Pen-strep 1% L-Glutamine
10% Char FBS Culture Media	88% DMEM Phenol Red (-) 10% Char FBS 1% Pen-strep 1% L-Glutamine
15% Char FBS Culture Media	83% DMEM Phenol Red (-) 15% Char FBS 1% Pen-strep 1% L-Glutamine
10^{-6}M E2 Culture Media	83% DMEM Phenol Red (-) 15% Char FBS 1% Pen-strep 1% L-Glutamine 10^{-6}M E2
10^{-8}M E2 Culture Media	83% DMEM Phenol Red (-) 15% Char FBS *(continued)*

Solution	Composition
	1% Pen-strep
	1% L-Glutamine
	10^{-8}M E2
10^{-9}M E2 Culture Media	83% DMEM Phenol Red (-)
	15% Char FBS
	1% Pen-strep
	1% L-Glutamine
	10^{-9}M E2
$24*10^{-8}$M E2 Culture Media	83% DMEM Phenol Red (-)
	15% Char FBS
	1% Pen-strep
	1% L-Glutamine
	$24*10^{-8}$M E2
$24*10^{-9}$M E2 Culture Media	83% DMEM Phenol Red (-)
	15% Char FBS
	1% Pen-strep
	1% L-Glutamine
	$24*10^{-9}$M E2
10ng/ml TGFB3	83% DMEM Phenol Red (-)
	15% Char FBS
	1% Pen-strep
	1% L-Glutamine
	10ng/ml TGFB3

Table 2 *Primers for gene expression*

Gene	Species	Gene ID	Lot#	Provider
Gapdh	Bovine	Bt03210913_g1	P180522-016 D06	Life Technologies
Col1a1	Bovine	Bt1463861_g1	1700337	Life Technologies
Col2a1	Bovine	Bt03251861_m1	P180522-016 D08	Life Technologies
Col1a2	Bovine	Bt03214860_m1	P180522-016 D07	Life Technologies
ACAN	Bovine	Bt03212186_m1	P180522-016 D09	Life Technologies
Gapdh	Human	Hs99999905_m1	1688723	Life Technologies
Col1a1	Human	Hs00164004_m1	1717939 A7	Life Technologies
Col2a1	Human	Hs0500163_m1	P180927-011 B03	Life Technologies
Col1a2	Human	Hs0016099_m1	1690104 A5	Life Technologies
GPER	Human	Hs00173506_m1	1602319 A7	Life Technologies
ESR1	Human	Hs01046812_m1	1504917 F9	Life Technologies
ESR2	Human	Hs01100353_m1	1729637 E5	Life Technologies
ACAN	Human	Hs00153936_m1	1717962 A9	Life Technologies

Table 3 *List of biological material*

Species	Sex	Tissue	Age	Use
Bovine	Male	Meniscus	17 months	Media Composition Study
Bovine	Male	Meniscus	17 months	Estrogen Study
Bovine	Female	Meniscus	Unknown	Estrogen Study
Human	Male	Meniscus	47 years	Dosage Kinetics Study
Human	Female	Meniscus	47 years	Dosage Kinetics Study
Human	Male	Meniscus	18 years	Regional Meniscus separation

3. Methods

Table 4 *Experimental groups for media composition study*

Subject	Treatment	Number of repeats	Time Points (Days)	Regional separation
Male Bovine	10% FBS	5	1, 3, 7	Inner Meniscus
Male Bovine	10% Char FBS	5	1, 3, 7	Inner Meniscus
Male Bovine	15% Char FBS	5	1, 3, 7	Inner Meniscus
Male Bovine	10% FBS	5	1, 3, 7	Outer Meniscus
Male Bovine	10% Char FBS	5	1, 3, 7	Outer Meniscus
Male Bovine	15% Char FBS	5	1, 3, 7	Outer Meniscus

Table 5 *Experimental groups for pulsed versus continuous study*

Group	Treatment	Meniscus	Time points (days)
Control	0 E2	Lateral Meniscus	1, 3, 7
High Pulsed	$24e10^{-8}$ E2	Lateral Meniscus	1, 3, 7
High Continuous	10^{-8} E2	Lateral Meniscus	1, 3, 7
Low Pulsed	24e 10^{-9} E2	Lateral Meniscus	1, 3, 7
Low Continuous	10^{-9} E2	Lateral Meniscus	1, 3, 7
Control	0 E2	Medial Meniscus	1, 3, 7
High Pulsed	$24e10^{-8}$ E2	Medial Meniscus	1, 3, 7
High Continuous	10^{-8} E2	Medial Meniscus	1, 3, 7
Low Pulsed	24e 10^{-9} E2	Medial Meniscus	1, 3, 7
Low Continuous	10^{-9} E2	Medial Meniscus	1, 3, 7

Table 6 *Experimental groups for regional separation study*

Group	Treatment	Meniscus Region	Time points (days)
Control	0 E2	Inner	1, 7
Pulsed E2	$24e10^{-8}$ E2	Inner	1, 7
Continuous E2	10^{-8} E2	Inner	1, 7
Control	0 E2	Middle	1, 7
Pulsed E2	$24e10^{-8}$ E2	Middle	1, 7
Continuous E2	10^{-8} E2	Middle	1, 7
Control	0 E2	Outer	1, 7
Pulsed E2	$24e10^{-8}$ E2	Outer	1, 7
Continuous E2	10^{-8} E2	Outer	1, 7

3.1. Tissue harvest

Stifle joints from a steer were procured from a local butcher shop (Bichelmeyer Meats, Kansas City). The animals were estimated to be 17 months old, the joints procured for this study were

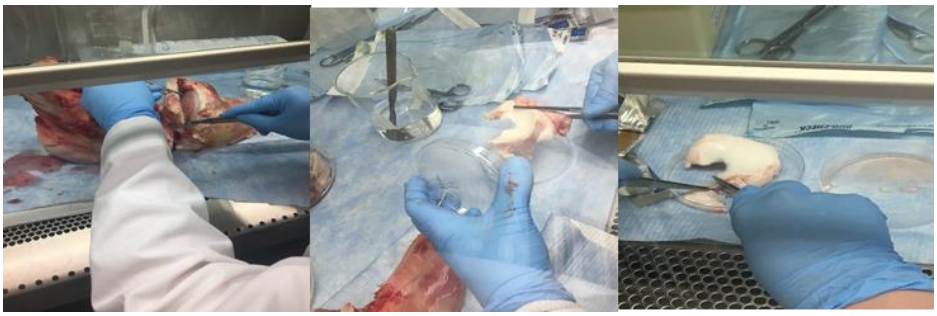

Figure 7 *Bovine meniscus harvest and preparation for tissue digestion*

both male. The steer was sacrificed days prior to collecting the joints, the joints were kept under refrigerated condition but not frozen. Once retrieved the knees were sterilized by submerging in a 1% weight/volume(wt/v) solution of Alconox detergent (Fisher Scientific, Lot#A4G2) for 40 minutes, and subsequently submerged for 20 minutes in a 70% Ethanol (Fisher Scientific Lot#175343) solution for 20 minutes. Tissue extraction was performed in a sterile biosafety cabinet. The biosafety cabinet was first scrubbed with a Clorox solution followed with a 70% ethanol solution, and then it was lined with absorbent padding to prevent fluids to be exposed to the surface. The padding was sterilized by turning on the UV light for 10 minutes on each side of the padding.

The bovine joint was introduced into the biosafety cabinet for dissection and opening of the capsule as seen in **Figure 7**. Removal of soft tissues and other ligament attachments for ease of access to the joint was performed. Discarded tissues were stored in biosafety bags and frozen for disposal through incineration as per the University of Kansas Animal Care Unit instructions. Once the menisci were excised from the joint, they were stored in sterile PBS to maintain them hydrated on 150 mm* 25 mm poly(styrene) petri dishes (Corning). A fine dissection was performed to remove excess soft and connective tissues based on previous methods

(Subramony, Su, Yeager, & Lu, 2014). The meniscus was separated into outer and inner regions with the mid white-red region being stored for other studies. The meniscus once separated into outer and inner regions were then chopped into 1-2 mm pieces. The meniscus tissue was then placed in pre-measured 50 ml conical tubes in 10 ml of PBS to maintain the tissues hydrated. Once the mincing process was completed the PBS was aspirated and the mass of the tissue was calculated to determine the appropriate concentration of collagenase media to produce. Collagenase media was prepared at a 1.5mg/ml concentration approximately following previous protocols. The minced tissues were stored in an incubator with the collagenase solution at 37 degrees Celsius (°C) and 95% humidity in an orbital shaker (Corning LSE) set at 2400 RPM for 16 hours. Digested tissue supernatant was removed and placed in fresh 50ml conical tubes; the tubes were then spun at 400G for 10 minutes to pellet the cells. The pellet was suspended in 20 ml of media and cells were plated separating medial and lateral meniscus. The cells were cultured to 90% confluency and passaged. Cell imaging was performed using an inverted light microscope (Zeiss inverted microscope) the images were collected at different passages using a 10X objective under bright field microscopy.

3.2. Live Dead Assay

Live/Dead assay (Invitrogen, L3224, Life Technologies) was performed as recommended by the manufacturer. To ensure the difference in media composition did not affect the cells viability (n=5). Briefly samples were cultured with the media compositions in the experiment for 24 hours and then the samples were rinsed with phosphate buffered saline (PBS; Sigma Aldrich), stained, and then imaged using a plate reader (Cytation 3, BioTek Instruments) using a 4X objective.

3.3. Growth Kinetics

In order to characterize the normal growth rate and behavior of the cells two ten day growth studies were performed. In the first growth study ten time points every 24 hours for ten days were taken of five replicates of healthy outer MFC's at passage three derived from an 18-year-old

deidentified male. In the second growth study, eight replicates of three different tissue culture media containing varying concentrations of phenol red were tested to determine what the effect of phenol red concentration is on mid MFCs at passage two and increase confidence in future results. Outer MFCs were thawed and were seeded at an initial density of 10^4 cells/mL in T75 flasks at their second passage. Once the cells reach confluency, cells were then seeded in 6-well plates at a density of 10^4 cells/cm^2 in premium DMEM (10% FBS) at passage three. They will then were allowed to culture for 24 hours after which the DMEM was replaced with phenol red-free DMEM. The cells were cultured in the phenol red-free medium for 24 hours prior to treatment. Phenol red free DMEM was used because it has been found that phenol red mimics nonsteroidal estrogen and will bind to estrogen receptors of estrogen sensitive cells. Phenol red free DMEM was used to incubate the cells 24 hours prior to study to make the cells naïve to any possible sources of estrogen and estrogen mimetic compounds.

Ten time points at passage three were seeded at a density of 10,000 cells/cm^2 in 6-well plates. The cells were allowed to grow until their collection time, which occurred every 24 hours from day one to day ten. Triton-X 100 at 0.1% v/v was added to each sample at the time of collection in order to lyse open the cell membrane to allow access to the nucleic acid for further data acquisition. After collection, the lysate samples were frozen at -20°Cuntil they were analyzed. Ten time points at passage two were seeded at a density of 10,000 cells/ mL in 48-well plates. The cells were separated into groups and were exposed to one of the three different types of media listed under **Figure 10**, which would represent exposure to different concentrations of various different estrogen mimetic compounds present in tissue culture media. The cells were collected every 24 hours from day one to day ten.

3.4. Cell proliferation

Initial seeding density for the study was 5000 cells/cm^2. Cell proliferation was determined by measuring total DNA content per sample using the Quant it ds DNA PicoGreen assay (p7589,

Life Technologies) this according to the manufacturer's suggested protocol. The samples in culture were rinsed using phosphate buffered saline and the cells were then lysed with 300uL of 0.1% Triton X-100 solution (Sigma, T8787). The cell lysates were stored at -20°C until the assay was performed. At the time of running the assay, an aliquot of 20ul was then added to the working solution of the PicoGreen assay. The fluorescence was measured with a microplate reader (Cytation5, BioTek Instruments) with wavelengths of 485nm and 535nm for excitation and emission respectively. The total cell number was determined by converting the total amount of DNA per sample to cell number using the conversion factor of 7.7 pg DNA/cell (Kim, Sah, Doong, & Grodzinsky, 1988) following previously established protocols(Jiang, Leong, Mung, Hidaka, & Lu, 2008).

3.5. Biochemical Analysis

Total collagen and GAG content per sample was measured by executing Hydroxyproline assay and DMMB assay respectively. These assays were performed after 16 hours of papain digestion (Sigma-Aldrich) to solubilize the cellular material in suspension. Acetate citrate buffer was prepared by diluting the following reagents into 250 ml of distilled water 60g of sodium acetate trihydrate (Sigma-Aldrich, S-7670), 23g of citric acid (Sigma-Aldrich, C-1909), 6mL acetic acid (Fisher, BP2401), 17g of NaOH (Sigma-Aldrich, S-5881). The solution was set for 30 minutes in a magnetic stirrer and monitored until complete dissolution of the reagents was observed. Once this was completed, the solution was brought to PH of 6.5 using either 1M NaOH. Once adjusted the solution was brought up to a final volume of 500mL. 0.056M Chloramine T solution was prepared the day the assay was performed. First in 20mL 50% reagent grade isopropanol 1.27g Chloramine T (Sigma-Aldrich, 857319) was dissolved, this was then brought to 100mL with Acetate-Citrate buffer. Ehrlich's Reagent was prepared 1n 100mL (2:1 v/v) isopropanol (reagent grade): perchloric acid (Sigma-Aldrich, 244252) (66.7mL isopropanol, 33.3mL perchloric acid) to which it was added 15g of p-dimethylaminobenzaldehyde (Sigma-Aldrich, 156477). Samples

were concentrated in a vacuum oven from the digestion lysate overnight. The sample was then hydrolyzed using 25ul of 2M NaOH and autoclaving the samples for 25 minutes. Then the samples were prepared in 2ml Eppendorf tubes by adding 225ul of the chloramine T into 25ul of the hydrolysate. The oxidation reaction was allowed to run for 25 minutes at room temperature. 250ul Ehrlich reagent was added to each sample and allowed to incubate at 65 C for 20 minutes. Once the reaction was complete the samples were thoroughly mixed using a vortex mixer and 200ul of the sample was plated into a 96 well plate. Absorbance was measured using Cytation 5 microplate reader at 555nm wavelength. A standard curve was prepared to correlate the content of collagen based on absorbance(DasGupta, 1970; Reddy & Enwemeka, 1996; Subramony et al., 2014) . The concentrations of the standard curve are summarized in **Table 7**.

Table 7 *Standard Curve values for Hydroxyproline Assay*

	1	2	3	4	5	6	7
Collagen (mg/ml)	0.001	0.005	0.01	0.05	0.1	0.5	1

Total GAG content per sample was measured by performing DMMB assay. Reagents were prepared ahead of time DMMB dye was prepared by dissolving 0.4 grams of sodium formate into 160 ml of distilled water. In a separate 15 ml conical tube, 4.2mg of 1-9-dimethylmethylene blue was dissolved into 1 ml ethanol. Formic acid was used to bring the pH to 1.5. Once adjusted the volume was brought to 200ml and stored in the fridge covering the container with foil to prevent light interaction with the Dye. A standard curve was prepared to correlate the GAG content based on the absorbance of the assay the concentrations of said standard are summarized in **Table 8**. Using a working solution of 50µg/ml following the volumes in the table below. Once the samples were digested, they were allowed to return to room temperature before preparing the plate. 50µl of the sample was plated after the tubes were thoroughly mixed by using a vortex mixer. 250µl of dye was added quickly to the sample as the GAG complexes will precipitate and become unreadable in a very short period of time. Preparation of the assay was done in dim light to

mitigate light exposure from affecting our absorbance readings. Absorbance was measured using Citation 5 microplate reader at a dual wavelength of 540nm and 595nm.

Table 8 *Standard Concentrations and Dilution Values for DMMB dye Assay*

Working Standard (µl)	**dH_2O (µl)**	**Standard Amount (µg)**
0	50	0
5	45	0.25
10	40	0.5
20	30	1
30	20	1.5
40	10	2
50	0	2.5

3.6. Human meniscus harvest and cell isolation

Knee menisci of human male and females was prepared by the National Disease Research Interchange (NDRI) these were sent to us on average two days post death of the individuals. The samples were sent on ice in DMEM with antibiotics for preservation of the integrity of the tissue.

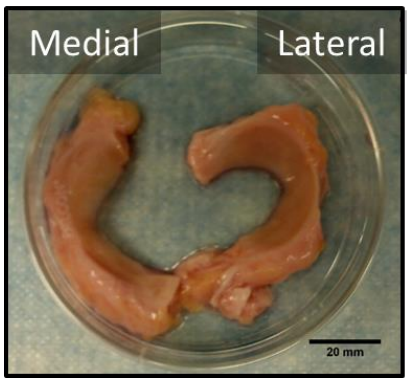

Figure 8 *Human Meniscus after fine dissection and removal of excess connective tissue*

A representative image from one of the samples can be seen in **Figure 8**. The samples were whole menisci as seen in **Figure 8**, which were dissected to remove excess soft and connective tissue. All this as previously mentioned in a sterile environment prepared ahead of time. The menisci for the 47-year-old male and female subjects were separated by lateral and medial

menisci. These were then processed for cell isolation following the same protocol performed for bovine tissue in section 3.2. With some adjustments as stated in the following section. The tissue was treated with 5ml of solution per gram of tissue. The solution was prepared by adding the collagenase type 1 (Gibco, Life technologies, 17100-017) into a 50 ml conical tube. This was measured as per the required mass to our required volume. The solution was prepared at 10% of our final volume to ease the filtration of the solution prior to diluting with Dulbecco's modified eagle medium (DMEM) (Gibco, 11995) with 2% Penicillin-Streptomyosin as an antibiotic. These were then placed on an orbital shaker at 2400 RPM (Corning LSE) for 16 hours inside an incubator (PHCBi, Panasonic) at 37C with 5% CO_2 and 95% humidity. Post digestion with collagenase 2 the tissue and cell supernatant was then taken and filtered using 60ml syringes with an assembled filter with 33um mesh (Spectrum Labs, Cat# 08670203) into a 15 or 50 ml conical tube depending on the volume of the solution. The supernatant was centrifuged at 500G for 10 minutes to collect the cells at the bottom of the conical tube, the remaining supernatant was removed with an aspirating pipette connected a vacuum and waste collector. The cells were suspended in two milliliters of media and counted using a hemocytometer (Hausser Scientific) according to previously stablished protocols. A 20 µl aliquot of the cell suspension is placed on a piece of parafilm and mixed with 20 µl of trypan blue dye (Corning, Lot# 03717004), 10 µl of this solution is the loaded on one of the ends of the hemocytometer for cell counting. Once cells are counted they are then platted at 2000 cells/cm^2.

3.7. Gene Expression qPCR

After in-vitro culture and treatment, mRNA was extracted to analyze the effects of estradiol on meniscal fibrochondrocytes on extracellular matrix protein markers and estrogen receptor markers. The mRNA was extracted with TRIzol reagent (Ambion, Life Technologies, 15596018) following the manufacturers protocol. After extraction, mRNA was treated with DNase treatment and removal kit (Ambion Life technologies) to eliminate any residual DNA contaminants. Reverse

transcription was performed on the mRNA samples to convert to cDNA utilizing the High capacity cDNA reverse transcription kit (Applied Biosystems) in RNAse free conditions.

Human primers used for the experiments included collagen type 1 (Col1a1, Col1a2), collagen type 2 (Col2a1), , aggrecan (ACAN), as well as estrogen receptor alpha (ESR1), estrogen receptor beta (ESR2), G-coupled estrogen receptor (GPER) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an endogenous control. All of the quantitative PCR analysis was done using TAQMAN chemistry assays (Life technologies) using a quantstudio 7 (Thermo Fisher Scientific). For the PCR reaction master mix was prepared using 2X Taqman buffer (Life technologies), this was then diluted with RNA, DNA free H2O. The total volume was then aliquot depending on the repeats of the experiment. Each aliquot would then be added their respective primer. Then the solution was mixed by pipetting gently up and down. 20µl of this was then plated in a MicroAmp Fast 96 reaction well plate (Themofisher). The plate would then be spun down briefly using a centrifuge at 500G for 1 minute to ensure the reaction volume is down and that no bubbles were present. A cycle threshold (CT) experiment was then performed for 45 cycles and the results were plotted and compared to the GAPDH housekeeping gene as an endogenous control with an untreated sample serving as the reference. A list of Primers used during the studies is on **Table 2.**

3.8. ddPCR Gene Expression

Similarly, to qPCR once in-vitro culture and treatment was performed, mRNA was extracted to analyze the effects of estradiol. The mRNA was extracted as previously discussed in qPCR gene expression section. The difference with this approach was the use of BioRad reagents to perform the studies. Primers used for these experiments are summarized in **Table 2**. The PCR plate follows the same preparation as in any other PCR reaction, the additional steps for this experiment consisted of the following: Briefly, 20µl of the PCR reaction are plated in DG8 cartridges (cat# 1864008, BioRad) with 70µl of Droplet generation oil for EvaGreen (Cat# 1864006, BioRad).

These are then covered with a Droplet generator gasket (Cat# 1863009, BioRad) and placed in the QX200 for droplet generation (QX200, BioRad). Once the droplets were formed, the resulting emulsion of 40µl was pulled and plated into a fresh 96well plate. The last steps were repeated until all samples were prepared. Then the plate was sealed with a pierceable foil and placed in the Thermal cycler for 40 cycles at a modified temperature of 105 °C to allow heat transfer with the addition of the oil. Once PCR reaction takes place the plate was transferred to a QX200 droplet reader for quantification of the gene expression.

To optimize the concentration of cDNA required to improve on the previous runs of qPCR a preliminary study was performed measuring the absolute quantification of the genes in measured concentrations of cDNA for the endogenous control and a gene of interest. This was performed by first measuring the cDNA content of each sample from lateral meniscus from the pulsed versus continuous study. These were measured using 1µl of cDNA loaded onto a Nanodrop 2000 spectrophotometer. The machine was set to zero by first running two blank samples (DNA, RNA free H2O). The values were collected on a spreadsheet and dilutions were performed afterwards. These dilutions were in the range of 200, 100, 50, 20, 10, 5, 2, 1, 0 ng/µl. As recommended by BioRad the cDNA content per sample run in a ddPCR experiment should not exceed 60ng/ul so the experiment tested to 50 ng/µl.

3.9. Spheroid formation

Spheroid formation was done by growing meniscal cells to 90% confluence. Cells were counted and suspended to a concentration of 2.5 x 10^6 cells/ml. Spheroids were prepared on a 150mm tissue culture petri dish. First, the lid was removed from a 150 mm tissue culture dish and 20 ml of PBS was placed in the bottom of the dish. This acted as a hydration chamber. The lid was then inverted, and a 20µl multichannel pipetter was used to deposit 20µl droplet onto the lid. Making sure that drops were placed apart to not come into contact with each other. Once the droplets were placed the lid was flipped onto the PBS-filled bottom chamber and incubated at 37°C/5%

CO_2/95% humidity. The droplet formation was monitored daily. The cell spheroids took on average three days to form. The spheroids were collected using a modified 1ml pipetter tip by aspirating the 20ul volume and placed onto a 96 non-adherent well plate. The spheroids then were monitored and imaged at day 1 and 7 of treatment. Spheroids were treated with 100 µl of treatment solution daily. The treatment conditions included a negative control (noE2) two experimental groups low E2 (10^{-8} M) High E2 (10^{-6}) and a 10ng/ml of TGFB3 as a positive control.

3.10. Spheroid Imaging

Spheroids were imaged and monitored at day one and seven. The images were taken with an inverted microscope at a 4X magnification. These images were then measured using ImageJ to determine their initial size prior to treatment and their size after treatment for 7 days of daily

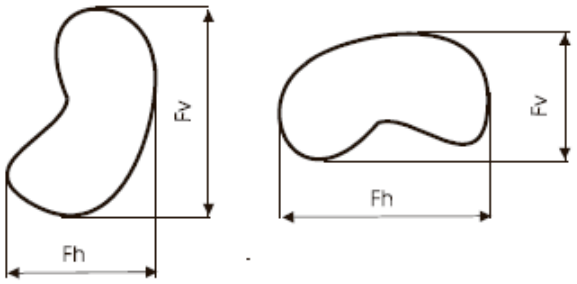

Figure 9 *Feret diameter measurement schematic. Measurements are taken in one plane across two parallel tangential lines.*

dosages. Due to the amorphous nature of the spheroids at the beginning, Feret diameter was used as the measurement for analysis. The diameter was measured based on the distance between two parallel tangential lines. As seen in the illustration in **Figure 9**.

3.11. Statistical Analysis

Statistical analysis was performed using SPSS and SAS, with these software ANOVA testing was performed, while analyzing the effects of media composition and the presence of prfDMEM a 2-way ANOVA was performed. Similarly when analyzing the data in the effects of estrogen and time a 2-way ANOVA was performed. To determine the effects associated with treatment, region and

time a 3-way ANOVA was carried out. A Tukey Post-Hoc Analysis was performed following the ANOVA tests.

4. Results

4.1. Effects of Hormone-free FBS composition on meniscal fibrochondrocytes

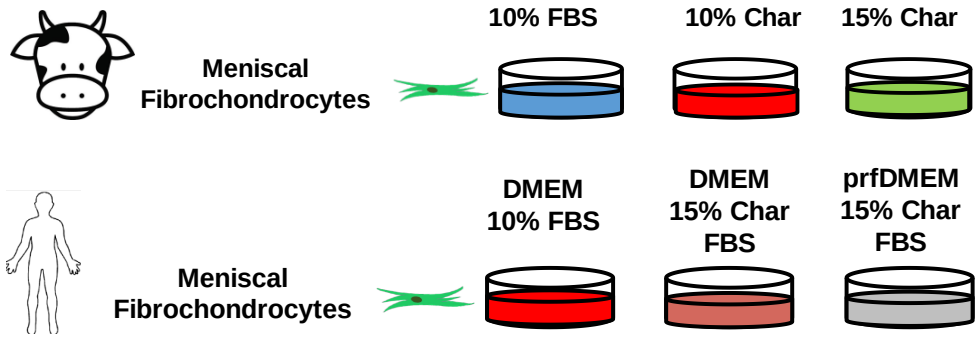

Figure 10 *Experimental design for media composition study.*

During **Experiment 1,** optimization studies were performed to assess the role of basal media components on cell viability and proliferation prior to estrogen dosing studies. In order to determine the effect of passaging on MFCs, a visual analysis was performed. **Figure 10** has the experimental groups in this experiment. Images in **Figure 11** display the morphological changes

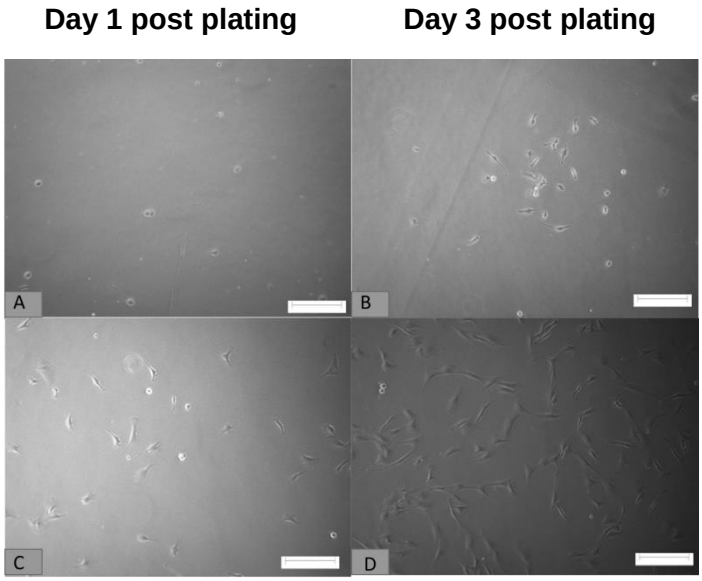

Figure 11 *Cell morphology of MFCs post plating at P0 and P1, cells were plated at a density of 10,000 cells/cm^2 morphology differences were observed from P0 (A, B) to P1 (C,D) at 1 and 7 days.*

of the MFCs; these changes could be due to the change in substrate or passaging. The cells in the figure were plated at the same initial cell density. **Figure 11A** and **11B** are cells post-harvest at days one and three, respectively. By passage one these cells start to quickly adhere and stretch in a more spindle-like morphology as seen in **Figure 11C** and **11D** at days one and three, respectively. Moving forward an experiment was carried out to determine the effects of media composition on MFCs. The groups of the experiment are summarized in **Figure 10**. To determine the effect of charcoal/dextran treated FBS (Char FBS) on proliferation, the meniscus

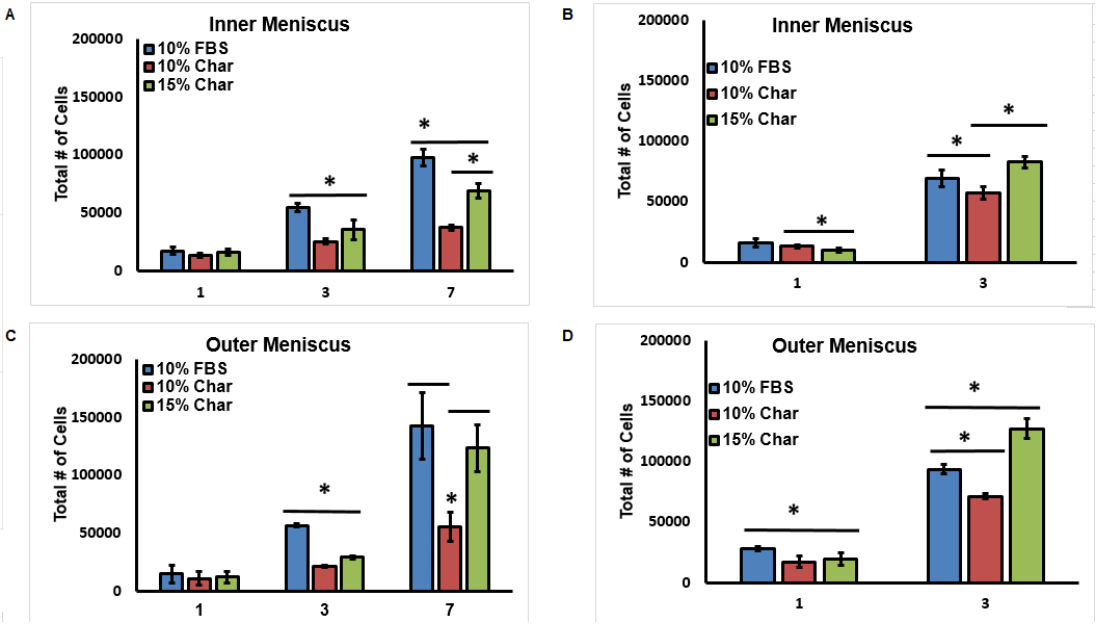

Figure 12 *Effect of Char FBS on male bovine MFCs proliferation during* ***Experiment 1****.(A) (B) PicoGreen assay data showing the proliferation of the inner MFCs over time and with different media compositions during two different iterations *p<0.05. (C) (D) PicoGreen Assay data of the outer MFCs over time with different media compositions *p<0.05.*

fibrochondrocytes treated with media that had 10 % FBS, 10 % Char FBS, or 15 % Char FBS. The groups included inner and outer meniscus cell populations. Cells were lysed at one, three, and seven days and proliferation determined using the PicoGreen assay as shown in **Figure 12.** Proliferation at day one in both 10 % and 15 % Char FBS was not statistically different from the control group. At day three, the control group had a significantly higher cell count compared to

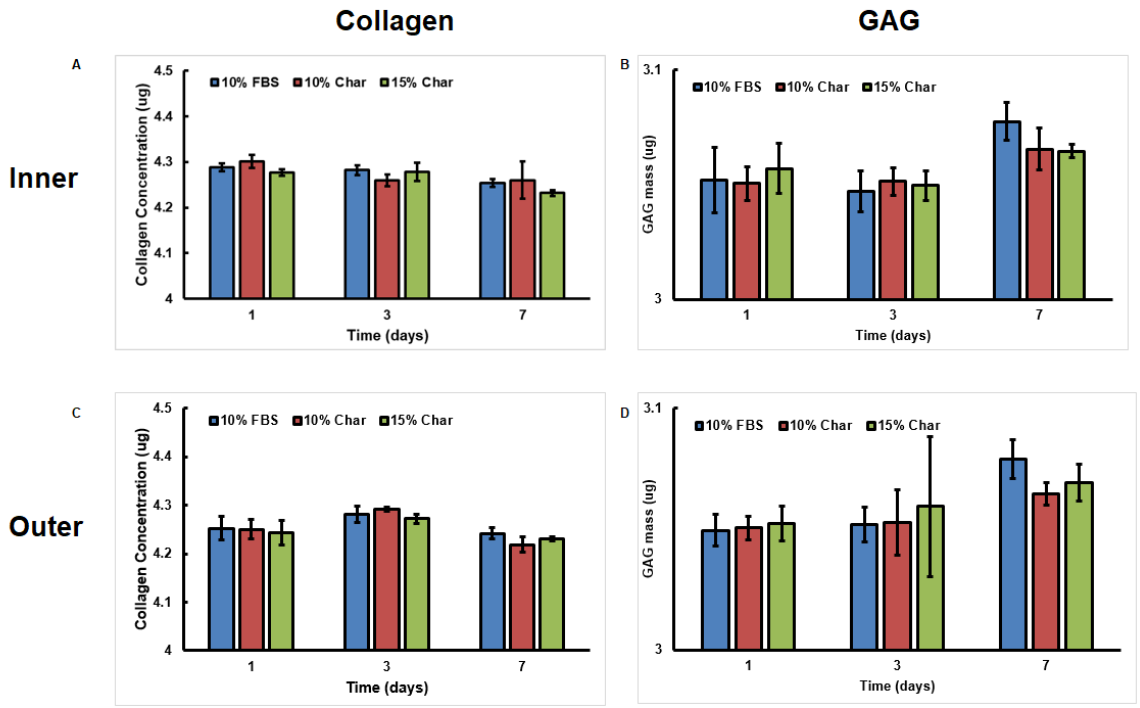

Figure 13 *Presence of extracellular material in MFCs with different Media compositions during* ***Experiment 1****. (A)(C) Collagen assay data showing no significant differences or changes in quantity of collagen due to time or media composition in either inner or outer meniscus. (B)(D) DMMB assay data showing no change in GAG concentration for either time or culture conditions.*

the experimental groups with the Char FBS. Similarly, at day seven for the inner meniscus the control group had a significant difference compared to both groups. The outer meniscus at 15% Char FBS did not have a statistically significant difference from the control group. No statistically significant changes to total GAG and collagen content were observed due to media composition or time as seen in **Figure 13.** The effect of charcoal FBS and phenol red-free DMEM (prfDMEM) on human MFC growth kinetics was determined. The proliferation analysis shows a significant difference in cell proliferation as a function of charcoal FBS and prfDMEM in Days 5 and 7-10

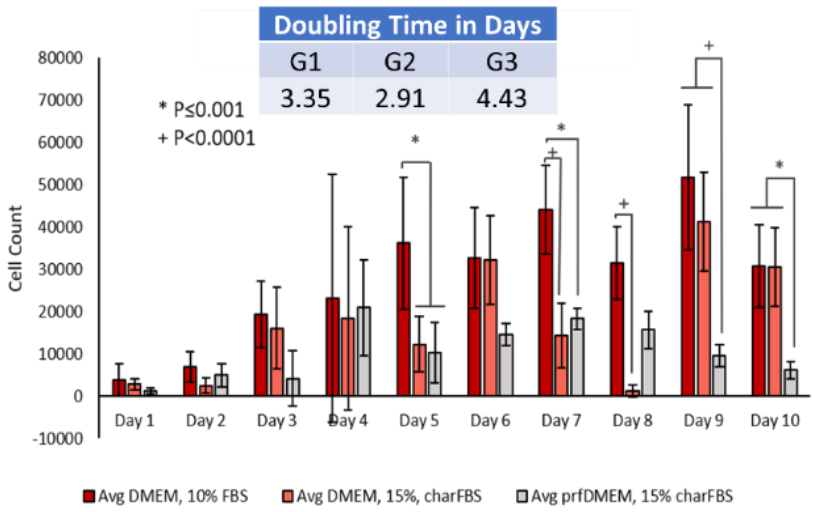

Figure 14 *Doubling rate of human meniscal fibrochondrocytes with different media and FBS/Char FBS concentrations. *p<0.01 +p<0.001.* ***Experiment 1.***

shown in **Figure 14**. Overall, prfDMEM significantly reduced cell proliferation compared to cells in charcoal FBS and normal FBS medium with doubling times of 4.43 days compared to 2.91 in

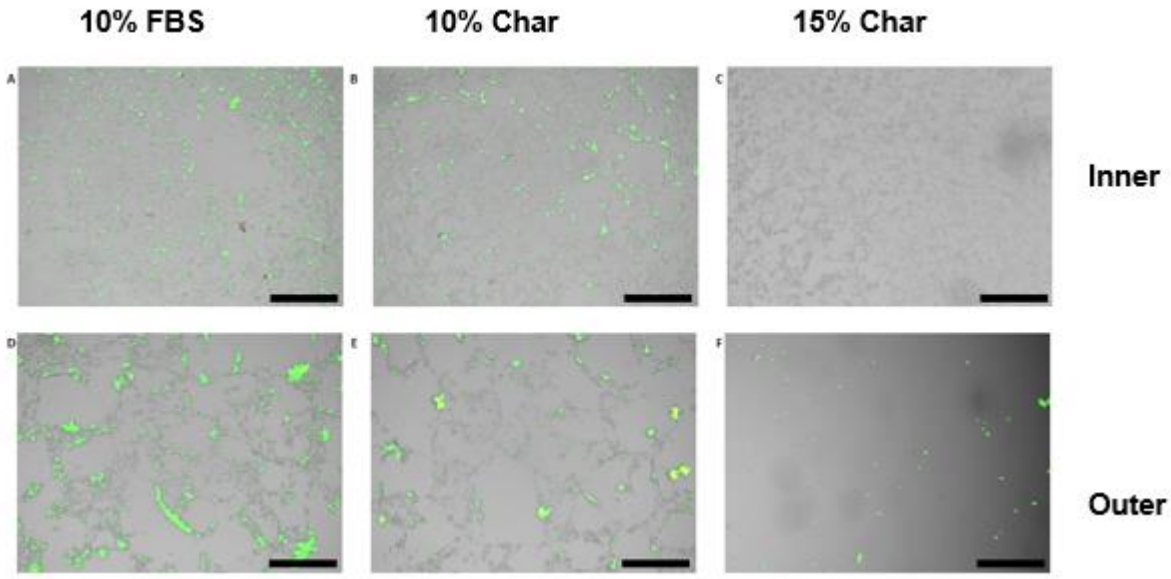

Figure 15 *LIVE/DEAD Cell viability assay. Representative figures at different media compositions of medial meniscus MFCs. Live cells are shown in green and dead cells are shown in red. (A)(B)(C) Inner meniscus cells did not have an observable difference in cell viability due to media conditions (C) Fluorescence was not detected at 15% Char when imaged but there was a reported value for its intensity that did not significantly differ from the other conditions. (D)(E)(F) Outer meniscus cells were also not affected in their viability due to media composition during* ***Experiment 1****. Scale bar 400 µm.*

charcoal FBS and 3.35 in normal FBS. To determine if the media composition had any negative effects on cell viability, a live/dead assay was performed. The results showed no observable differences between conditions at 24 and 72 hours from inner and outer menisci. **Figure 15 A-C** are the inner meniscus cells at 72 hours. The pseudo color failed to show in the last image but its registered intensity values did not differ significantly from the other two conditions. **Figure 15 D-F** are the outer meniscus cell representative images. As observed in the inner meniscus fibrochondrocytes, there were no significant differences between treatments.

A significant difference in the expression of Col1a1 was observed between regions and media composition. **Figure 16** shows that MFCs in 10% FBS condition experienced a significant increase in Col1a1 gene expression, while the 10% Char FBS treatment resulted in significantly lower Col1a1 expression in both inner (IM) and outer meniscus (OM). In addition, the fibrochondrocytes from the inner region experienced significantly lower expression of Col1a1 in comparison to cells from the outer meniscus when given the same treatment. Col1a1 gene transcripts were not detected with cells in 15% Char. Overall, MFCs from OM had a significantly larger expression of Col1a1, and both IM and OM had an increased expression in traditional

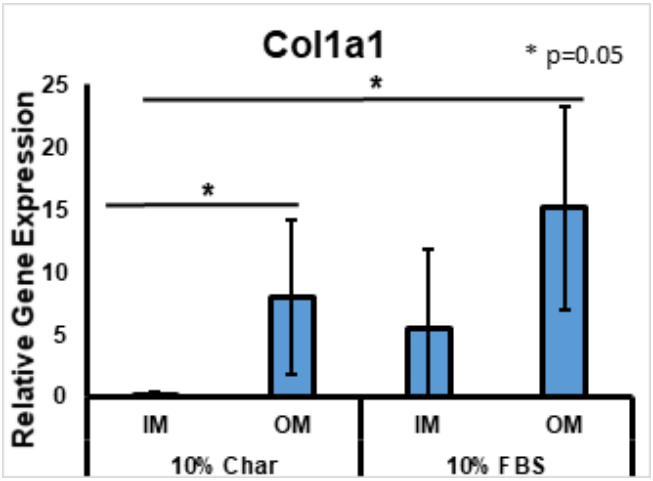

Figure 16 *Gene expression differences of col1a1 in* ***Experiment 1*** *while treating the meniscus with different media compositions. *p<0.05*

10%FBS compared to 10% Char FBS. The results from the media optimization studies determined that 15% Char FBS was adequate for estrogen dosing studies.

4.2. Effects of estrogen on bovine meniscal fibrochondrocyte proliferation and extracellular matrix production

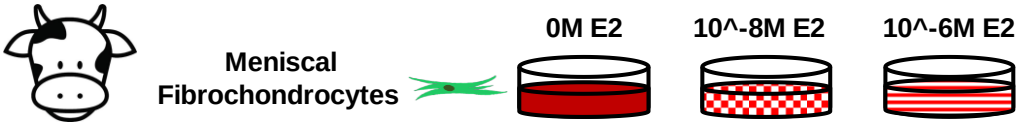

Figure 17 *Experimental design for estrogen treatment study (**Experiment 2**). Male bovine MFC were isolated and treated with 17β Estradiol (E2) at different concentrations.*

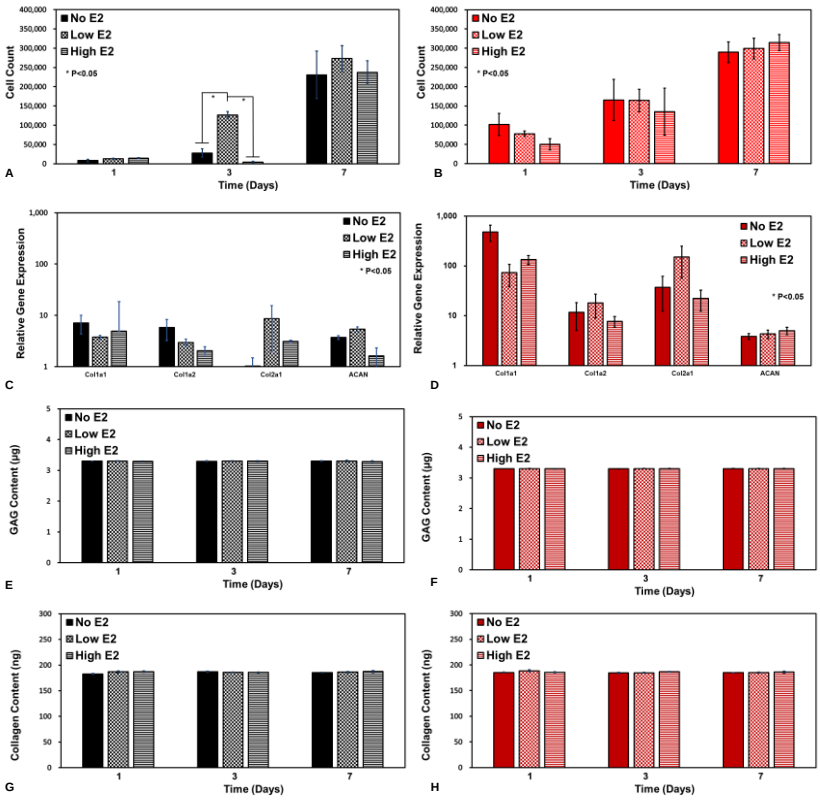

Figure 18 *Proliferation, expression of ECM markers, and the presence of extracellular material in male and female meniscal fibrochondrocytes with different estradiol treatments for **Experiment 2**. (A) (B) PicoGreen assay data showing the proliferation of the cells over time and with E2 treatment. *p<0.05. (C)(D) Quantitative PCR analysis showing relative Gene expression of Col1a1, Col1a2, Col2a1, and ACAN in male and female bovine meniscal fibrochondrocytes. (E) (F) DMMB assay data showing no change of GAG concentration for either time or E2 treatment conditions. (G)(H) Collagen assay data showing no change in quantity of collagen due to time or E2 treatment.*

During **Experiment 2** the effect of estrogen on male and female bovine MFC was investigated, the experiment design is seen in **Figure 17**. The effect on proliferation is displayed in **Figure 18A**

and **18B**. Overall, E2 did not affect proliferation except for male cells at Day three. This effect was not observed at seven days. Relative gene expression values are shown in **Figure 18C** and **18D**. Both male and female cells experienced a trend of reduced Col1a1 gene expression and an increased trend in Col2 and ACAN expression with low estradiol treatment. In this study, estradiol treatment did not significantly affect total GAG (**Figure 18E** and **18F**) or collagen content (**Figure 18G** and **18H**). In all of the analysis, there was evidence that estradiol exhibited dose-dependent effects. However, due to small sample sizes, the data was not statistically significant.

4.3. Effects of estrogen on human meniscal fibrochondrocyte proliferation and extracellular matrix production

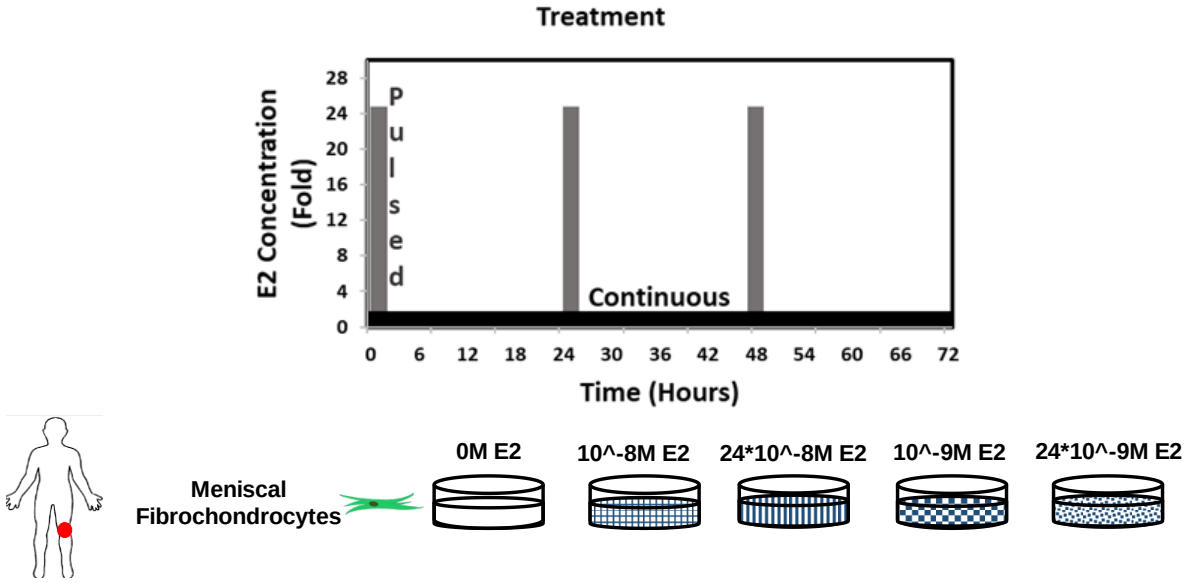

Figure 19 *Dosage scheme for pulsed versus continuous treatment of MFCs. Experimental design for* ***Experiment 3****, human MFCs were treated with different dosages and kinetics as seen at the bottom of the figure. Grey lines represent the pulsed treatment, black line represents the continuous treatment. Treatment was administered during the first hour and removed, replenishing with the samples respective E2 concentration.*

During **Experiments 1** and **2** studies were performed using bovine meniscal cells to optimize the parameters for treatment of human meniscal cells. Using the optimized media conditions, estrogen-dosing studies were then conducted as part of our **Experiment 3** on human male and female MFCs harvested from the tissue of 47-year old patients. In order to compare the effects of E2 treatment at pulsed versus continuous dosing, the MFCs were exposed to the same total

amount of E2, either provided as a 1-hour dosage or administered continuously for 24 hours. The treatment was performed at concentrations where a significant response was previously observed. A schematic of the dosage and treatment groups and parameters is displayed in **Figure 19.** At 24 hours, male LM cells treated with E2 experience a significant increase in cell proliferation when compared to the No E2 group as seen in **Figure 20**. This was not observed at the medial

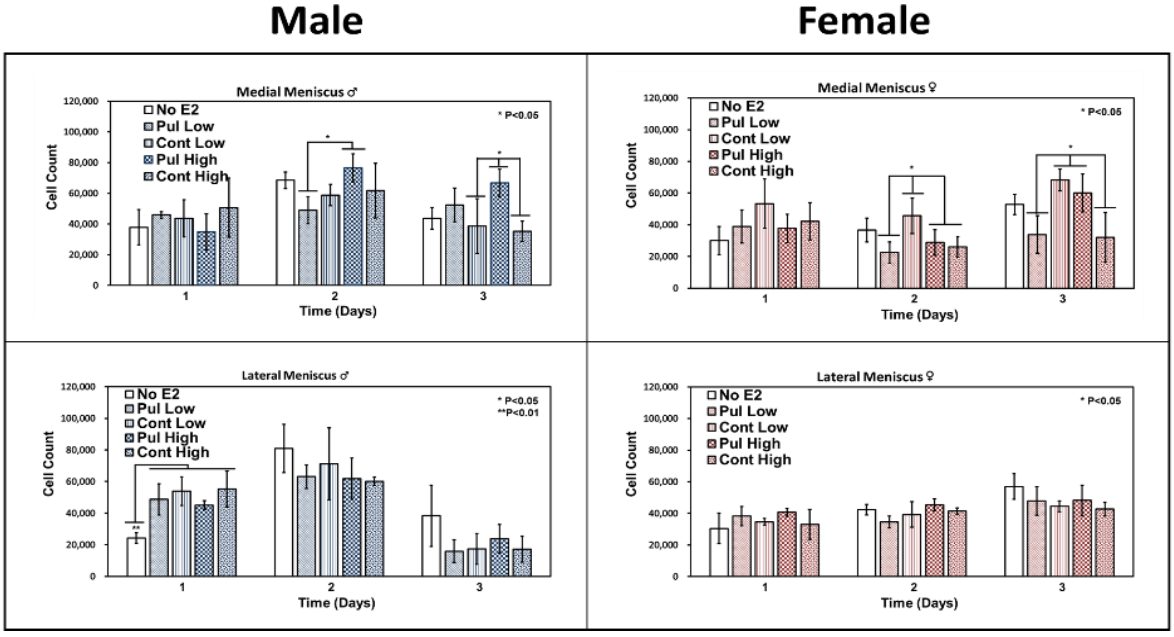

Figure 20 *Proliferation over time of MFC with different dosage kinetics for* ***Experiment 3****. Male MFCs experienced a significant increase in proliferation at 24 hours shown in the right side *p<0.05. Female MFCs were significantly more proliferative at con Low condition in the medial meniscus *p<0.05.*

meniscus or at the same time with female samples. At 48 hours, pulsed high condition elicited a significant difference in male medial meniscus. Meanwhile the pulsed high E2 condition elicited a significantly higher response than other treatment groups for female cell samples. At 24 and 48 hours, male samples of all treatment groups had a significant difference when compared to female samples where the male cells had a larger cell proliferation.

Gene expression was determined by performing quantitative polymerase chain reaction (qPCR). No significant differences were observed in the gene expression of matrix macromolecules

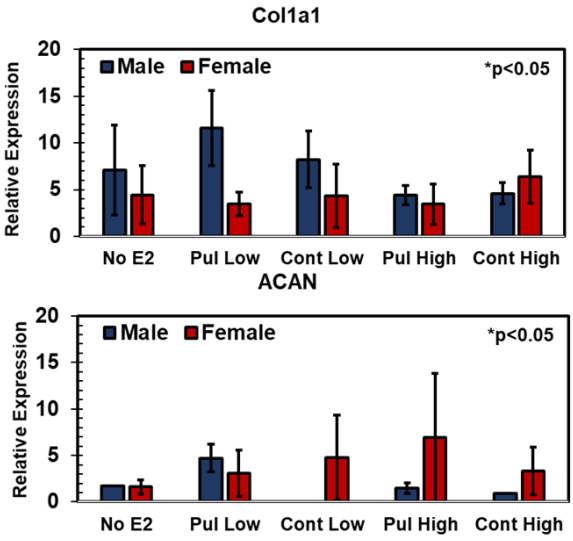

Figure 21 *Gene expression profile of MFCs after treatment with E2 during* ***Experiment 3*** *at different dosage kinetics. Relative expression data demonstrated no significant differences due to treatment or sex in the expression of Col1 and ACAN.*

comparing male and female lateral meniscus at 24 hours of treatment. **Figure 21** shows the gene expression of ACAN, and Col1a1, which did not experience a significant increase with treatment.

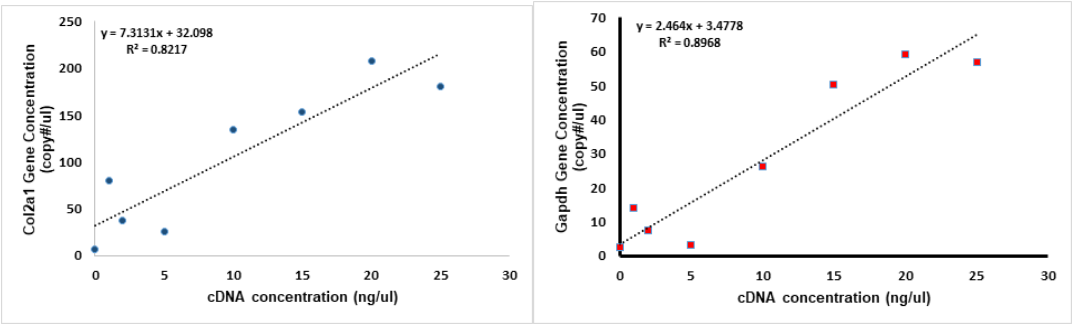

Figure 22 *Standard curves for GAPDH and Col2a1 for gene expression studies using ddPCR for* ***Experiment 3***

Col2, ESR1, ESR2, and GPER failed to amplify during qPCR. Due to low amplicon levels, droplet digital PCR (ddPCR) was used. To optimize the ddPCR reaction due to its high sensitivity and the recommendations of the supplier (BioRad), a study was performed using the endogenous

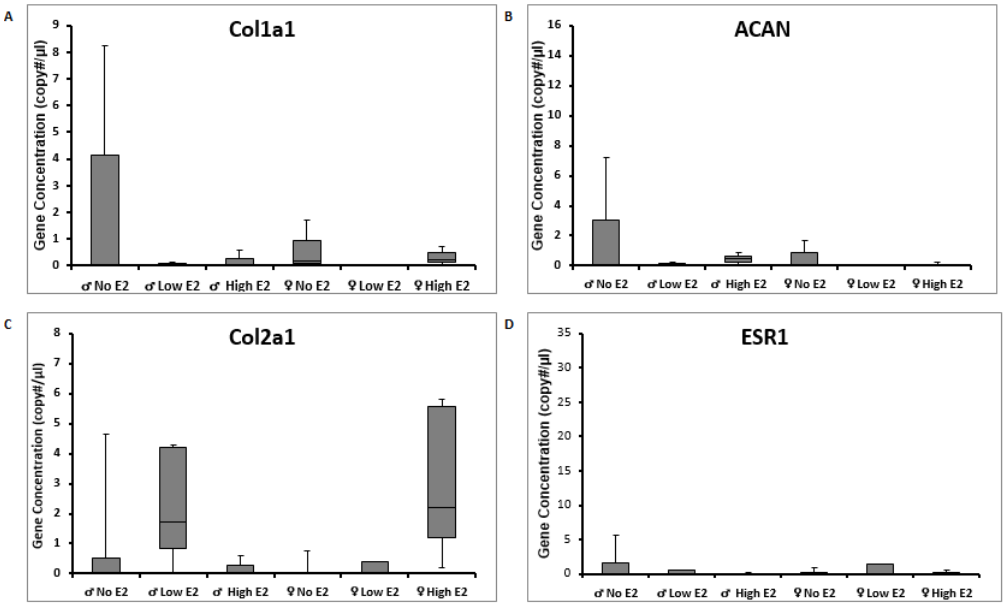

Figure 23 *Gene expression profile of MFCs after 24 hour treatment with E2 at high (10^{-8}M) and low (10^{-9}M) continuous dosages during* ***Experiment 3****.*

control (GAPDH) as well as Col2 to determine the appropriate cDNA concentration to use to observe a reading without having a large cluster of positive results. Both runs had a linear growth with increased cDNA concentration. The standard curve is shown in **Figure 22**. **Figure 23** and **Figure 24** illustrate ddPCR results for 24 and 48 hours, respectively. There were no observed

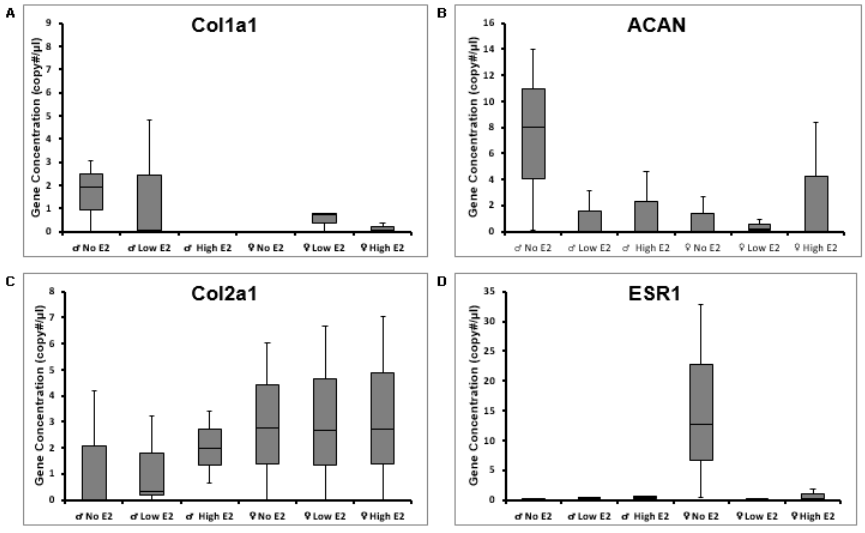

Figure 24 *Gene expression profile of MFCs after treatment for 48 hours of E2 at high (10^{-8}M) and low (10^{-9}M) continuous dosages during* ***Experiment 3***

significant differences between sex and treatment in the combined regions of the lateral meniscus samples from both 24 and 48 hours.

4.4. Regional separation of young male meniscus

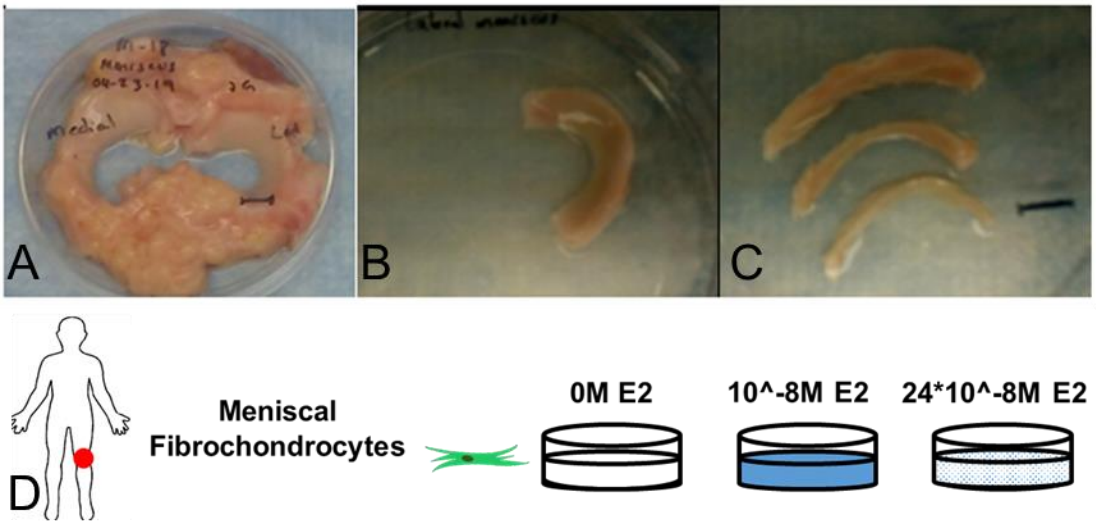

Figure 25 *Regional separation of the meniscus. (A) Meniscus as received from NDRI. (B) Meniscus after fine dissection was performed. (C) Meniscus separated by regions. (D) Study treatment conditions for* ***Experiment 4****.*

Experiment 4 was carried out to determine if there were regional differences in proliferation with estrogen treatment. The cell isolation was performed per region of the meniscus in a male sample (18 years old) as seen in **Figure 25(A-C)**, the cells post isolation were then treated with the dosages noted in **25D.** Cells from both the medial and lateral disc were combined. The groups for this experiment are summarized in **Figure 25 D**. Proliferation of the initial run did not have statistically significant differences due to treatment within regions except for day 1 where the no E2 was significantly larger than the pulsed and continuous treatment groups in the inner region during the initial run. Differences were significant when groups were compared per region. A second run of the experiment was performed to evaluate reproducibility. As the outer and middle regions of the meniscus did not have a significant difference in the initial study, the repeat study

was only conducted with inner and outer fibrochondrocytes. During this run, the cells did not experience a significant increase as seen in the right side of **Figure 26.**

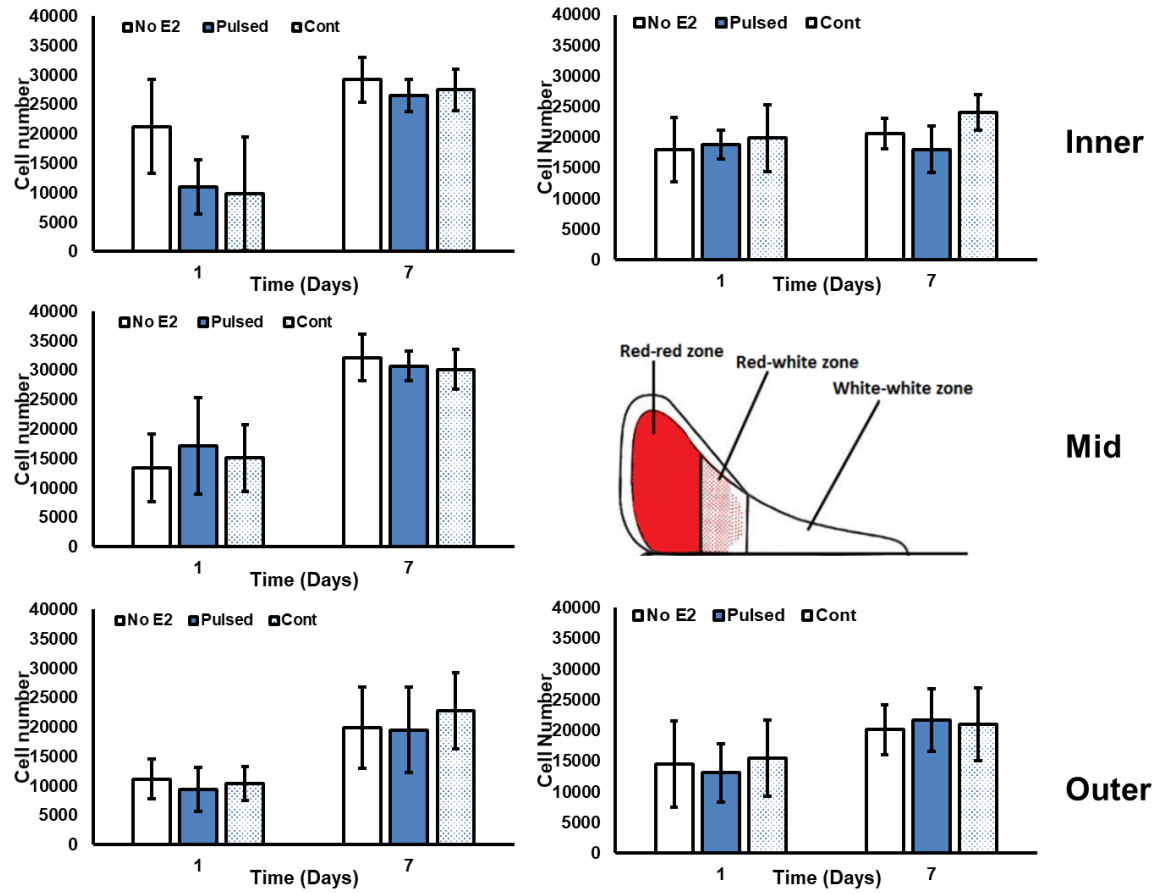

Figure 26 *Proliferation of young adult human male (18 years) MFCs in* ***Experiment 4*** *separated per region and treated with NoE2 (0M) pulsed (24*10^{-8} M) and continuous dosages (10^{-8}M) of Estrogen. On the Left are the results of the initial run, while the right has the results of the second run. *p<0.05*

4.5. Spheroid formation

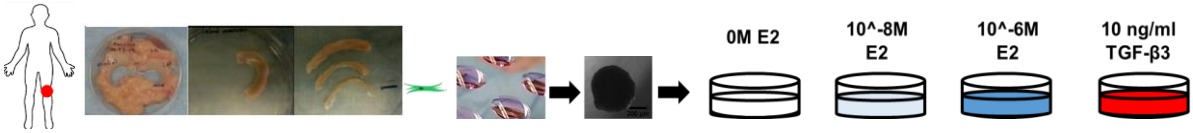

Figure 27 *Preparation of MFCs scaffoldless 3D culture model. Human MFCs were isolated and cultured to P2. They were formed over a 3-day period using hang-drop method at 50,000 cells and treated with different estrogen concentrations with 0M as a negative control and TGF-β3 as a positive control.*

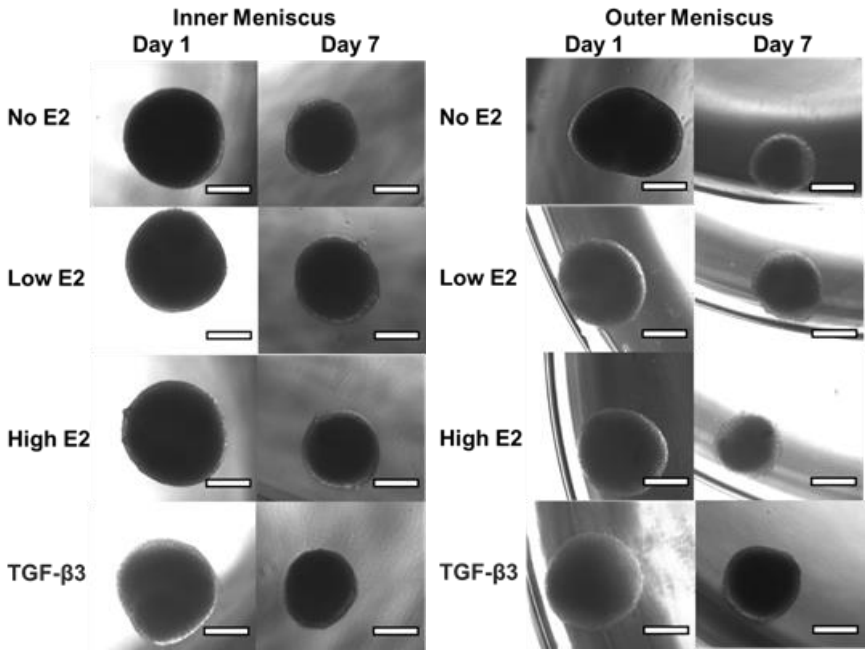

Figure 28 *Spheroids from human male MFCs (18 years) formed at cell density of 50K cells. Inner and outer meniscus spheroids experience a significant decrease in size from fabrication (day 1) to last day of treatment. Low E2 in the inner meniscus experience a significantly lower decrease in size.* ***Experiment 5*** *Scale bar 200μm.*

To determine the effects of E2 treatment in 3D cell culture, a pilot study **Experiment 5** was carried using spheroids formed at a density of 50,000 cells and treated with No E2, Low E2, High E2, or TGF-β3 as shown in **Figure 27**. The spheroids were imaged at days 1 and 7 of treatment and

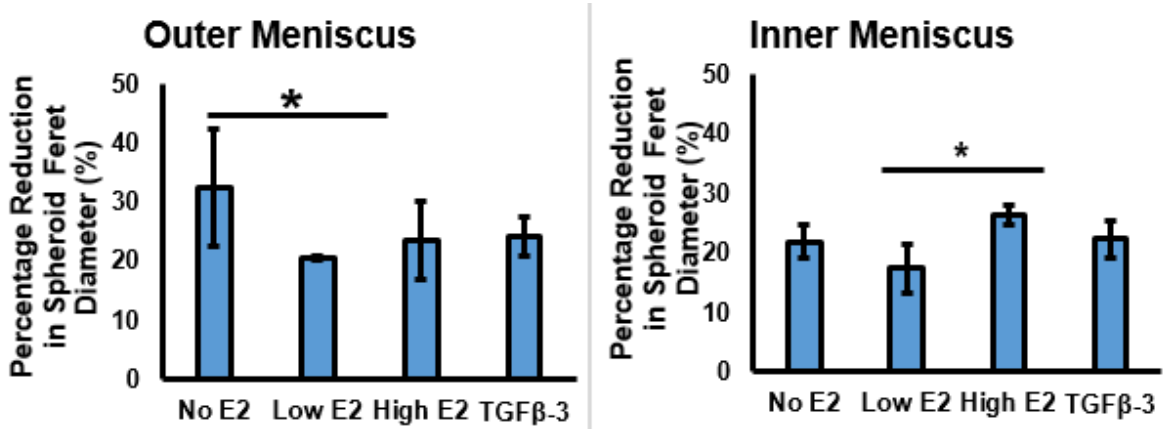

Figure 29 *Percentage reduction of spheroids in different culture media in* ***Experiment 5****. Overall, the outer meniscus spheroids in the Low E2 treatment group experience a significantly lower decrease in size when compared to the No E2 treated samples.*

measured using ImageJ. Representative images are shown in **Figure 28** and an analysis in the

decrease of Feret Diameter is shown in **Figure 29.** The averages of the spheroid sizes is as well as the changes observed are summarized in **Table 9**. This information provided a visual means to determine potential effects of the treatment. Size differences due to time were significantly different, and a significant difference due to treatment was observed in the LowE2 treatment group compared to No E2 in the outer meniscus spheroids while the inner meniscus spheroids had the LowE2 group significantly different to the HighE2 group. Spheroids of inner meniscus had an initial size larger than that of outer meniscus spheroids, but the size change was not significantly different between regions when compared as a whole. The differences due to treatment provide focus for future studies.

Table 9 *Spheroid sizes through experiment*

Region	**Treatment**	**Day 1 average (µm)**	**Day 7 average (µm)**	**Percent size Δ (%)**
Outer	No E2	370 +/- 65	245 +/- 12	32.7 +/- 9.9
Outer	Low E2	361 +/- 12	287 +/- 10	20.5 +/- 0.4 (*)
Outer	High E2	361 +/- 12	276 +/- 21	23.6 +/- 6.7
Outer	TGFB3	394 +/- 10	298 +/- 6	24.3 +/- 3.2
Inner	No E2	414 +/- 18	323 +/- 15	21.9 +/- 2.8
Inner	Low E2	417 +/- 20	344 +/- 2	17.4 +/- 4 (+)
Inner	High E2	429 +/- 12	316 +/- 8	26.5 +/- 1.7
Inner	TGFB3	407 +/- 12	315 +/- 3	22.4 +/- 3.1

5. Discussion

Through these experiments, the goal of our studies was to establish a platform for assessment of estrogens effects on the knee meniscus fibrochondrocytes. The effect of media composition, estrogen dosing and kinetics, 2D vs 3D culture were determined. Overall, E2 treatment did not

have a significant effect on the proliferation of female cells from bovine and human tissues, and the upregulation of ECM genes was observed to be upregulated with no significant differences. GAG and collagen production was not significantly different due to treatment or sex as seen in A **Table 10** were the overall findings of this thesis are summarized. Overall media composition had a significant effect on proliferation of MFCs, and there were trends that alluded to the effect of estrogen preserving the avascular phenotype of the MFCs in females but not in male MFCs. This was not what had been initially hypothesized.

Table 10 *Estrogen effects on meniscal fibrochondrocytes (↓ decrease, ↑ increase, ↔ no change, N/A not tested yet)*

Species	**Tissue**	**Gender**	**Age**	**Region**	**Proliferation**	**GAG**	**Collagen**	**Gene Expression**
Bovine	Medial Meniscus	Male	17 months	Whole	↔	↔	↔	↓ Col1a1 ↓ Col1a2 ↑ Col2a1 ↔ Acan
Bovine	Medial Meniscus	Female	17 months	Whole	↔	↔	↔	↓ Col1a1 ↓ Col1a2 ↑ Col2a1 ↔ Acan
Human	Medial meniscus	Male	47 years	Whole	↑	N/A	N/A	Used for Sequencing
Human	Lateral Meniscus	Male	47 years	Whole	↑	N/A	N/A	↔ Col1a1 ↑ Col2a1 ↔ Acan ↔ ESR1

(Continued)

Species	Tissue	Gender	Age	Region	Proliferation	GAG	Collagen	Gene Expression
Human	Medial Meniscus	Female	47 years	Whole	↔	N/A	N/A	Used for Sequencing
Human	Lateral Meniscus	Female	47 years	Whole	↔	N/A	N/A	↔ Col1a1 ↑ Col2a1 ↑ Acan ↓ ESR1
Human	Whole Meniscus	Male	18 years	Inner	↔	N/A	N/A	N/A
				Middle	↔	N/A	N/A	N/A
				Outer	↓	N/A	N/A	N/A
Human	Whole meniscus (Spheroid)	Male	18	Inner	N/A	N/A	N/A	N/A
				Outer	N/A	N/A	N/A	N/A

5.1. Effects of media composition on MFCs

Prior to estrogen dosing studies, it was critical to determine the effects of hormone-free media components on meniscal fibrochondrocytes(Welshons, Wolf, Murphy, & Jordan, 1988). In these studies, there was a different response due to region. Specifically due to media change between inner and outer menisci. This could be due to the differences in cell composition of the meniscus as others have noted the heterogeneity of the tissue cell population. It has been previously reported that the cells experience a doubling rate of .4X every day (Liang et al., 2017) contrasting the reported findings of this thesis in which cells doubled at a slower rate. These differences are likely due to media composition, and it was predicted to play a significant role in the proliferation of MFCs.

5.2. Effects of estrogen on bovine and human MFCs proliferation and extracellular matrix production

Estradiol treatment plays a role in maintenance of the fibrocartilaginous tissue. A study conducted by Robinson et.al has shown that exposure to estrogen in ovariectomized mice stimulated chondrogenesis and inhibited protease activity in temporomandibular fibrocartilage(Robinson et al., 2018). Additionally, it has been observed in a study done by Ham et al. in 2002 that the long-term use of estrogen can be used to slow the development of osteoarthritic lesions in the knee meniscus of primates.(Ham, Loeser, Lindgren, & Carlson, 2002) These animal models suggest that the sex hormone estrogen plays a role in maintaining the health and homeostasis of the meniscus. In this study, expression of Col1a1 decreased and expression of Col2a1 and ACAN increased in response to treatment. This is important as this could indicate potential regenerative properties of E2 in the avascular region of the meniscus. Similar findings were observed in murine growth plate chondrocytes at an intermediate dose of 17β-estradiol, further supporting the results of this study (Schwartz, 2013).

In general, studies in the literature show an increase in proliferation in female cells in response to E2 at the concentrations investigated in **Experiment 2.** This was not the case during the study. No significant differences were observed due to treatment in regards to proliferation and matrix production. In a rabbit animal model it was observed that the treatment of the knee meniscus with estrogen did not significantly have an effect in the degradation of GAG or collagen while other fibrocartilaginous tissues were(Hashem et al., 2006). Validating the current findings of this thesis, which indicates that these cells are likely not the target for treatment with estrogen to induce the repair of meniscal injuries. These events could be due to the lower expression and presence of estrogen receptors in the meniscus compared to other fibrocartilaginous tissues like the pubic symphysis and temporomandibular joint disc(W. Wang et al., 2009). While we saw, an increase in proliferation during **Experiment 2** this same effect was not detected through the remainder of the experiments when MFCs were treated with continuous dosage of the same concentration of E2 of 10^{-8}M. One potential reason for this effect is the relative levels of ERα in these cells was below

a level to be detected as seen in **Experiment 3** where only the female control group at 48 hours of treatment expressed some relative presence of ERα. Subsequent studies should look at the presence of ERβ as it has been shown in other fibrocartilaginous tissues to promote opposite effects to ERα(Ahmad, Chen, Wang, & Kapila, 2018). A summary to the effects observed through the experiments performed for this thesis are listed in **Table 10**.

The change of environment and short time in culture on a hard substrate could be the cause for low levels of total GAG and collagen observed, as it has been reported that MFCs, which possess fibroblastic and chondrogenic characteristics, exhibit phenotypic changes as they are passaged(Gunja & Athanasiou, 2007). These changes could elicit a lower production of the extracellular matrix macromolecules. We determined that, although no statistically significant changes were reported in proliferation or gene expression in both human and bovine, distinct changes were observed in male and female samples. For low E2, the expression of ACAN and Col2a1 changed from a few reported at 24 hours of treatment to every sample reporting at 48 hours, suggesting an upregulation of ACAN and Col2a1 while downregulating Col1a1Similarly, male samples expressed Col1a1 prominently at 24 hours in the low E2 treatment but decreased to almost zero by 48 hours. This could hint at the potential effects of 17β-estradiol to lead these cells to a more avascular meniscus phenotype.

In **Experiment 3,** it was observed that sex played a significant role in the proliferation of human MFCs of the same age. Male samples experienced an increase in proliferation, but they also had a higher expression of Col1a1. These findings suggest differences in response to estrogen treatment based on sex. This is different from other reports where it was observed that treatment of chondrocytes at similar conditions to the experiments in this thesis, demonstrated that female cells had a significant increase in proliferation compared to male chondrocytes (Chang, Kuo, Lin, & Yang, 2014; Hashem et al., 2006) in a rabbit model. Similarly, in costochondral cartilage it is observed that a difference in membrane estrogen receptors is the reason to the dimorphic

response to estrogen treatment(Elbaradie et al., 2013). Both of these studies ultimately point to differences in response due to sex. Another study performed by Brophy et al. showed distinct responses to trauma based on sex and age of the patients(Brophy, Rai, Zhang, Torgomyan, & Sandell, 2012). In this study, it was observed that the expression of inflammation markers was significantly higher in subjects under the age of 40. This indicates an increased catabolic response which can increase the degradation of the meniscus(Brophy et al., 2012). Overall, the results observed in the current studies of this thesis have similar responses in ECM formation. The differences observed due to sex is particularly different to the current understanding of E2 and MFCs in males and should be explored further particularly in the events of quiescence and apoptosis as these metabolic processes could elucidate further whether estrogen is acting as an arrestor in MFCs proliferation.

5.3. Understanding the effects of estrogen treatment in the meniscus at different regions

Understanding the cellular composition of the meniscus can help develop targeted treatments for the local environment. The meniscus is composed of a heterogeneous population of cells comparable to articular cartilage cells and fibroblast-like cells(Nakano, Dodd, & Scott, 1997). Recently, more studies have been aimed at defining the genetic markers associated to the regions of the meniscus in order to provide design criteria for the tissue engineering of the meniscus(Grogan, Pauli, Lotz, & D'Lima, 2017; Verdonk et al., 2005). The meniscus cells from vascular and avascular regions of the meniscus resemble those of hyaline cartilage(Grogan et al., 2017). Between these two regions, distinct markers that can help determine the population of the cells were found(Grogan et al., 2017; Verdonk et al., 2005). The outer meniscus has a higher incidence of CD90-positive cells, which is consistent with a more fibroblast-like phenotype. It has been reported that in the inner region of the meniscus the presence of Col2a1 and Acan make up the bulk of its ECM while the outer region of the meniscus expresses Col1a1 at a higher incidence (Verdonk et al., 2005). Both of these regions produced a higher amount of Col1a1 when compared

to articular chondrocytes(Nakano et al., 1997). This profile can be used in future studies to categorize the cell population in the meniscus.

There are mixed reviews regarding the plasticity of meniscal fibrochondrocytes and their ability to differentiate. There is evidence to support that, with optimized culture medium and appropriate cues, these cells have both chondrogenic and adipogenic differentiation potential (Cui, Hasegawa, Lotz, & D'Lima, 2012; Liang et al., 2017; Mauck, Martinez-Diaz, Yuan, & Tuan, 2007). Studies performed using MFCs of the inner and outer meniscus have both been shown to differentiate under hypoxic conditions with the addition of TGF-β3 to a chondrogenic phenotype(Liang et al., 2017) and, more importantly, that they were able to generate tissues with the functional matrix characteristics of avascular meniscus. Similarly, a study by Cui showed that a co-culture model with varying ratios of MFCs and mesenchymal stem cells (MSCs) was able to generate meniscus tissue constructs.

5.4. Importance of 3D Culture of MFCs

Since the early 1990s 3D culture studies have been performed demonstrating the importance to provide cells with an environment more like their native structure(B. M. Baker et al., 2015; Bao et al., 2018; Bhattarai, Aguilar, Park, & Kim, 2018; Cameron, Frith, & Cooper-White, 2011; Das, Gocheva, Hammink, Zouani, & Rowan, 2016; Elson & Genin, 2016; Galbraith & Sheetz, 1998; Gao, Peng, Feng, & Shuai, 2017; Gong et al., 2018; Papalazarou, Salmeron-Sanchez, & Machesky, 2018; Tan et al., 2013). In these studies, the cellular responses to their material surrounding played a significant role in expressed genes, as well as their potential to repair and remodel the ECM. We wanted to develop a 3D culture system that would allow cell expansion that more closely resembles those in vivo conditions. This was attempted since others have shown that the change in phenotype can be moderately reduced when these cells are cultured in 3D(Son & Levenston, 2017). To perform this we exploited the ability of meniscus cells to form small cell aggregates when forced together in hanging drops. The cells were suspended and the

cell aggregates formed within 3 days. Others have found that the use of a scaffold free environment when given the appropriate cues develop micro tissues that closely resemble those found in vivo(Klingelhutz et al., 2018). The size changes that occurred during the culture of these spheroids could be due to the media composition used for this experiment as the cells are experiencing a different environment in comparison to the previous experiments in monolayer culture. Kingelhultz et al performed a study with adipocytes using differentiating media and non-differentiating media where they saw that the spheroids in differentiating media grew through the length of the study while the volume decrease in the other condition. This is similar to what we observed in the meniscal fibrochondrocytes, which could be indicative that the effect of estrogen is not significantly aiding, but it is also not playing a detrimental role. To be able to determine more thoroughly the effects, subsequent studies analyzing the proliferation as well as ECM protein composition are necessary. Similarly as estrogen has been observed to induce apoptosis and enhancement of matrix biosynthesis in other fibrocartilage cells(H. Wang et al., 2014; Yang et al., 2018) determining the role of estrogen treatment in these cellular events in 3D culture of MFCs could provide valuable information.

6. Limitations

While the models and results produced in this thesis work provide a sound foundation for future studies, it is understood that every study has limitations. The fact that prfDMEM inhibited proliferation over time suggests that this media condition may promote cell quiescence. This change in cell cycle behavior could play a role in the response to estrogen observed in the presented *in vitro* studies. Variability between human subjects is another limitation in this study. Variability in biological systems encompasses differences from environmental factors to genetic differences, adding more nuance to determining effects for a global population based on three subjects (Wall et al., 2014; Zeise et al., 2013). The age of our subjects could have a significant role in the differentiation potential of the meniscal fibrochondrocytes it was shown that the effect

of estradiol does not play a significant role on jaw fibrochondrogenesis in mature mice(Robinson et al., 2018) . The estrogen treatment conditions for the studies presented in this thesis only included five concentrations, additional concentrations should be included in future studies to determine the cell response to treatment and to rule out any dosage dependency on the cells in question. The time points of collection in Experiment 3 and 4 should be expanded to determine the effects of dosage over time as an upregulation of ECM markers was observed to increase with time. Similarly a prolonged study in 3D culture should be performed to expand on the effects of estrogen in scaffoldless culture conditions as others usually treat for 21-28 days(Klingelhutz et al., 2018).

7. Conclusions and Future Directions

Development of 2D and 3D platforms for analysis of estrogen activity in the meniscus have been established in this thesis work. We hypothesized that the change from fully supplemented FBS to Char FBS would not affect the MFCs proliferation of significantly. What we observed was that hormone-free media components significantly affect fibrochondrocyte proliferation. In particular, the use of phenol red free DMEM can have a detrimental effect on the proliferation of meniscal fibrochondrocytes in human as well as bovine cells. Our hypothesis was that estrogen treatment would promote cell proliferation and ECM gene and protein production. E2 promotes the upregulation of ACAN and Col2a1 gene expression while decreasing Col1a1 expression, at concentrations of 10^{-8}M. Further, in the spheroid culture model, 10^{-8}M E2 decreased significantly less in diameter compared to the 0M E2 group. In part, our hypothesis that E2 would promote the upregulation of ECM proteins was partially validated, but the proliferation of cells was not. The experimental findings in this thesis supported by other findings indicate that the MFCs are likely not the primary target for E2 treatment in the pursuit of meniscus tissue regeneration. Current studies are focused on determining the role of estrogen on synovium progenitor cells, as these cells are known to regulate remodeling in the knee joint and likely play a role on new meniscus

tissue growth. These findings will continue to be explored to expand on our working knowledge and develop better strategies to probe the effects of E2 on other cells in the knee joint and determine their role in knee health and joint tissue homeostasis. As the knee joint is composed of multiple tissues co-culture models utilizing the findings of this work shall be developed to determine the individual role of chondrocytes, synovium derived progenitor cells, and MFCs as well as their crosstalk in the tissue homeostasis of the joint. Similarly, future studies will encompass the use of 3D culture models to probe the effects of E2 treatment and culture conditions.

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