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Abstract

Tendon tissue engineering is a field that arose as there became a need for alternatives for surgical grafts utilized in injured tendon treatments. This is due to either underperforming current technology of off the shelf materials or the fact that there is a limited supply of autografts, which is the gold standard for tendon grafting. Tendon tissue engineering utilizes all the aspects of the broader tissue engineering field: an appropriate scaffold, a cell source, and stimulation through mechanical and chemical means. This approach adopted herein utilizes the human umbilical vein (HUV) as a scaffold and mesenchymal stem cells (MSCs) for a cell source as a tendon construct. Previous studies have utilized a custom bioreactor to provide mechanical stimulation to this construct in the form of cyclical stretching. Two week cultures increased the mechanical properties, cellularity, and ECM quality of the umbilical vein, improving the construct as a tissue engineered tendon. The following studies built upon the foundation of the previous research to elucidate the importance of the different parameters comprising the mechanical and chemical stimulation.

The first study presented investigated altering the duration and frequency of the mechanical stimulation. This was done to improve early construct quality by varying the two previously mentioned parameters. By improving early time point culture, the future development of the tissue could be greatly improved. The durations investigated included 0.5, 1, and 2 hours/day and the frequencies investigated were 0.5, 1, and 2 cycles/minute. All dynamic stimulations increased cellular proliferation except for 2 cycles/min and 2 hours/day. A 1 cycle/minute frequency resulted in the greatest cellular proliferation (170% increase) while the 0.5 hour/day duration was best (203% increase).

Extracellular matrix quality had a direct relation to significant increases in cellularity, those groups showing the highest matrix deposition and alignment of the matrix. Gene expression indicated cellular activity consistent with remodeling the scaffold to be more tendon-like in terms of biglycan and elastin. In addition, scleraxis, tenascin-C, and tenomodulin were upregulated in certain stimulations after at most 7 days, with non-tendon phenotypes depressed. The stimulation parameters investigated in this study indicated that slower frequencies and shorter durations such as 0.5 cycles/min and 0.5 hours/day were best for construct quality when short term (up to 1 week) culture are employed.

The second study involved chemical stimulation of the construct. This study aimed to find if an extract of tenocytes can positively affect the development of the HUV/MSC construct that also underwent cyclical mechanical stimulation. This extract possesses soluble factors and genetic material from tendons that could potentially influence MSC behavior. By supplementing with tenocytic extract, a synergistic effect could be obtained with the aforementioned improved method of mechanical stimulation of the construct. After 14 days of dynamic culture, extract supplemented constructs possessed higher cellularity (37% and 150%) and tensile strengths (33% and 45%) when compared to non-supplemented MSC or tenocyte groups respectively. In addition, histological images indicated the extract supplemented constructs cultured dynamically possessed a dense connective tissue structure similar to native tendon. Gene expression profiles indicated that the dynamic construct initially expressed higher amounts of collagen type I rather than specific tenocytic markers after 7 days. However, after 14 days of dynamic culture, tenocytic marker and tendon development

genes such as elastin, scleraxis, and tenomodulin were upregulated, indicating a tendency towards a tendon phenotype greater than the non-supplemented group, which only received mechanical stimulation. This indicated that chemical stimulation, in addition to the improved upon mechanical stimulation in the previous study, allowed for further improvements in the culture compared to either of the two individual stimuli.

Finally the third study investigated the effects of long term culture (up to 4 weeks) on the construct. This was important, due to the fact that the previous two studies were limited in culture duration due to the bioreactor and construct design, which in turn limited the improvements in the overall construct. In this study, the HUV/MSC construct was modified to be suitable for long term culture within the bioreactor by opening the construct into a flat sheet to overcome nutrient transport limitations observed in previous studies. By providing a longer culture time, the construct properties could be improved by allowing for more cell and tissue growth. Opening the construct initially into a flat sheet increased cell numbers by 15.3 fold along with an increased tensile strength of 3.7 ± 0.7 MPa. However, analysis by RT-PCR showed upregulation of the osteoblastic markers osterix in the MSCs after 21 days and osteocalcin after 28 days, along with delayed tenocytic development. In contrast, by culturing the construct in its original cylindrical form for 2 weeks, MSCs are protected from the shear forces in their early developmental stages that may have increased the osteoblastic tendencies of the MSCs. After 2 weeks, the HUV is cut open into a flat sheet to allow for direct exposure to circulating media. By doing this, cell numbers did increase throughout the culture time but were 71% less than the initially open construct. However, the ultimate tensile strength after 28 days was 5.6 ± 0.7 MPa,

50% higher than the initially open construct. Gene expression analysis showed earlier tenocytic differentiation with the initially closed construct while osterix and osteocalcin expression was continually downregulated throughout the 28 days.

Overall, with the improvements provided by this study, the construct increased its ultimate tensile strength by 37% compared to previous studies and the MSCs utilized in this study have shown to differentiate towards a tenocytic linage and remodel the scaffold with more tendon-like characteristics and an overall higher quality ECM. These properties approached some of the properties of native tendons in the body, indicating that the HUV/MSC construct in its current form, with further improvements can be a viable alternative to current technologies for treating tendon injuries.

Chapter 1: Introduction

Tendon ailments are an increasing prevalent pathology observed within clinical settings. These can be chronic injuries which include tendinosis, tendinitis, and tenosynovitis. These maladies can cause pain, loss of motion, loss of stability, or loss of mechanical strength at the injury site. If left untreated or if these issues reoccur frequently, rupture of the tendon can occur. Tendon ruptures can also occur acutely if high enough tensile loads are applied to the tendon.

The tendon possesses a poor healing capacity due to its sparse cellularity, metabolic activity, vascularity, and lymphatic system.[1–4] Treatment for chronic issues can include rest and rehabilitation. If a rupture occurs or chronic issues cause enough damage, a replacement is needed to maintain the prior tendon's function. These replacements take on the form of grafts (including allografts and autografts) and synthetic materials.[5–10] However, these are not without issues, depending on the type of replacement, it can cause donor site morbidity, poor integration, loss of mechanical integrity, or rejection by the patient's immune system.[11,12] With these issues, an alternative to conventional grafts has been desired.

Tissue engineering aims to provide graft alternatives through development of artificial tissues. Tissue engineering accomplishes this by creating a construct from any or all of the following: a scaffold, a cell source, and mechanical/chemical stimuli. These are usually applied *in vitro* within custom bioreactors. By providing some or all of these aspects of tissue engineering, tissue growth *in vitro* can occur and the properties of the construct can approach the native tissue if developed properly. As the construct approaches these tissue characteristics, it can be implanted into the patient to

promote regeneration and integration of surrounding tissue or replace the entire tissue entirely. Tendon tissue engineering, a specific area of tissue engineering, applies the broad concepts of tissue engineering to the specific application of tendon replacements.

As can be surmised, there are many variables which go into creating a viable tissue engineered tendon construct. These can include scaffold type, cell source, seeding densities, the type, timing, and duration of mechanical stimulation, what types of chemical stimulation to apply, length of construct culture, and configuration of the construct within bioreactors.

The work presented in this manuscript investigates many of these variables while building upon previous initial studies into an artificial tendon construct created out of a human umbilical vein (HUV) and mesenchymal stem cells (MSCs). Successful tissue engineering requires a thorough understanding of the native tissue, therefore chapter 2 will investigate the tendon biology, physical properties, and pathologies. Chapter 3 will describe techniques used for tendon tissue engineering. Chapter 4 will investigate how changing the parameters of mechanical stretching affects initial culture of the tendon construct. By improving the initial construct cultures, future culture times can be more beneficial also, and the overall result creates a higher quality tissue-engineered construct. As a supplement to mechanical stimulation, chapter 5 will describe the use of tenocytic extract as a chemical stimuli for tendon development. By combining a mechanical and chemical stimulus together, beneficial effect from both stimuli at the same time can occur, resulting in improved properties compared than the stimuli alone. Chapter 6 will then describe the ability of the existing system to culture for periods of up to 4 weeks, whereas, only 2 weeks of culture had been previously

allowed Long term culture allows for greater improvements in cellularity, ECM creation and remodeling, mechanical properties, and differentiation into a tenocytic lineage. The sum of these three chapters will result in a construct that has been not only improved from previous studies, but allow for future development into a viable and improved upon alternative to current surgical grafts for treating tendon injuries. Finally chapter 7 will provide some conclusions for the entire body of work along with some future directions the project can take. By combining previous research with this specific system, along with the current manuscript, and the suggested future directions, a viable graft replacement can become closer to reality.

Chapter 2: Tendon Properties and Pathologies

2.1 Introduction

Tendons are primarily collagenous tissues that are responsible for connecting bone and muscle and transmitting forces between the two. Tendons can vary in shape and size depending on the location in the body and amount of forces being transferred. These shapes range from flat to ribbon to cylindrical.[13] To illustrate the complete muscle/tendon/bone unit, the Achilles tendon, the strongest and largest tendon in the body, is pictured in figure 2.1.

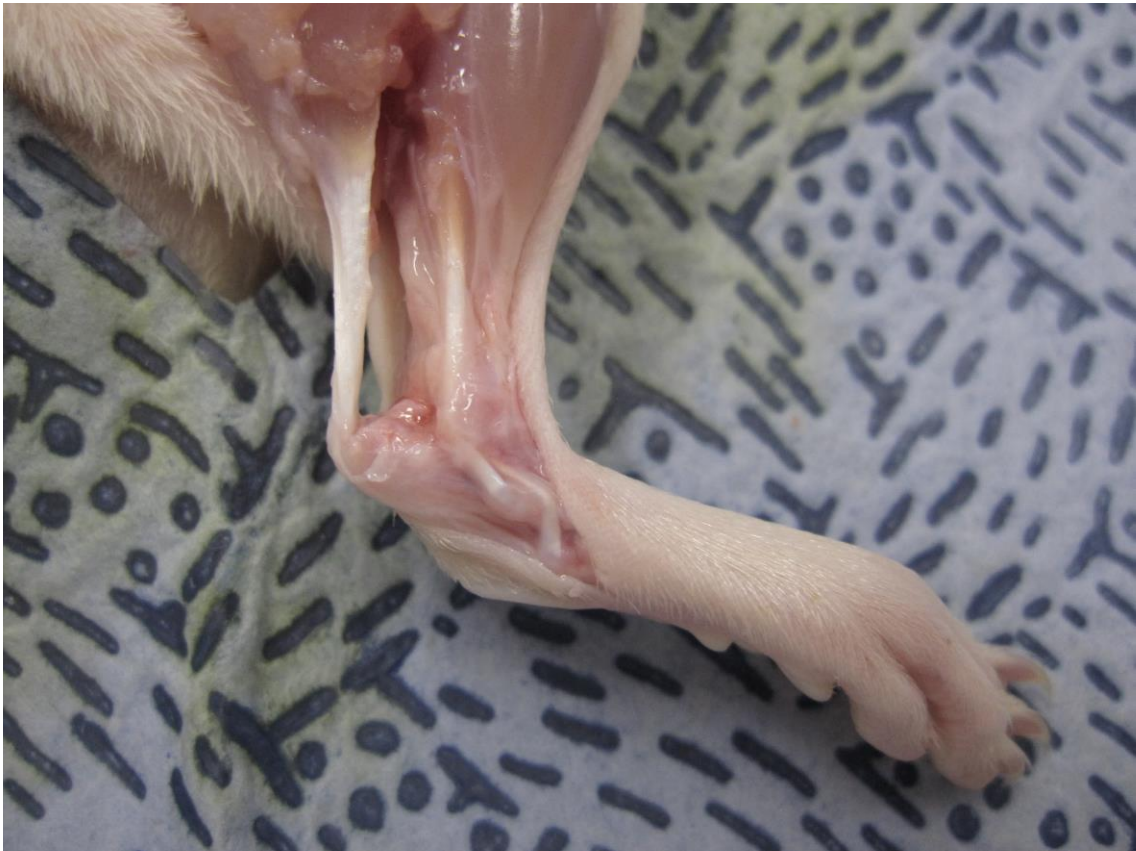


Figure 2.1: Image of the Achilles Tendon connecting the muscles of the calf to the calcaneus bone in a rat.

The tendon is designed to transmit and withstand high amounts of force. However, with continuous, long term overuse or a high, instantaneous, and unfamiliar stress, injury can occur, which may eventually lead to a tendon rupture which requires surgical treatment.[15–17] With chronic injuries, inflammation may occur around the injured site (tendinitis) or the tendon may suffer microtears within the matrix (tendinosis).[17] Although these pathologies are commonly associated with athletes and repetitive, high stress activities, they can also occur with common, day to day activities.[17,18] One hypothesis for recent increases in tendon injuries in the general population is due to the increase in sedentary lifestyle for industrialized countries, which leads to weaker tendons when actually in use.[19] A different population, athletes, which are more prone to tendon injuries, experienced 232,000 injuries in 2002.[20] On average, days of work lost due to tendon injuries averaged 16 days per year.[21] When considering only tendon ruptures, time lost from work can depend on treatment. Time lost from surgery averaged 13 weeks, while patients who opted for non-surgical treatment still missed 9 weeks of work recovering from a tendon rupture.[22] Taken together, tendon injuries cause a disruption in day to day life and advances in treatment of these pathologies can be extremely beneficial.

This chapter will first describe the tendon structure, composition, and organization, along with its mechanical properties. It will then conclude with a description of tendon pathologies and current clinical treatments for both chronic and acute injuries.

2.2 Tendon Organization

Tendons are soft connective tissues that connect and transmit forces from the muscle to the bone.[14] They are composed primarily of collagen along with other extracellular matrix (ECM) molecules and cells that will be discussed in sections 2.3 and 2.4.[23]

The tendon can be thought of as many well organized subunits combined together to function as a cohesive tissue. The smallest unit of the tendon is the collagen molecule, which in itself is comprised of smaller subunits. It is a triple helical protein that has a diameter of about 1.5 nm, a functional length of 300 nm, and is comprised of two subunits, two $\alpha 1$ chains and one $\alpha 2$ chain.[25] These chains follow a Gly-X-Y amino acid pattern where every third residue is glycine and also are rich in proline.[26] These molecules are then arranged into fibrils, which are comprised of overlapping collagen molecules with a 64 nm periodicity.[27] These fibrils are not linear, but are crimped, which is a result of a periodic triangular arrangement.[28]

The resulting fibrils are then organized into fibers, which are further organized into bundles called fascicles. Within the fascicles reside the cells of the tendon, of which types will be discussed later.[29] These fascicles also show a crimp pattern, due to the amplification of the crimping present in the fibrils.[28] Both bundles of collagen fibers and fibrils are wrapped together by a dense connective tissue called the endotendon.[30] Bundles of fascicles are grouped together by the epitenon and form the macrostructure of the tendon, this tissue is similar in form and function to the endotenon.[30] Finally, there is another outer layer of the tendon, the paratenon. This

consists of both type I and type III collagens along with elastin and synovial cells. This layer provides lubrication and protection from surrounding tissues.[24,30]

The junctions of the tendon to the bone and muscle have slightly different anatomies. The myotendinous junction is a region where the muscle merges into the tendon, creating a relatively smooth transition. Muscle cell membrane and actin filaments form finger like appendages that extend into the tendon as shown in figure 2.2.[31] These appendages are responsible for transmitting force from the muscle to the tendon. They are also very stable, as many of the muscle and tendon ruptures around this area occur near the transition zone, but not actually within it.[32]

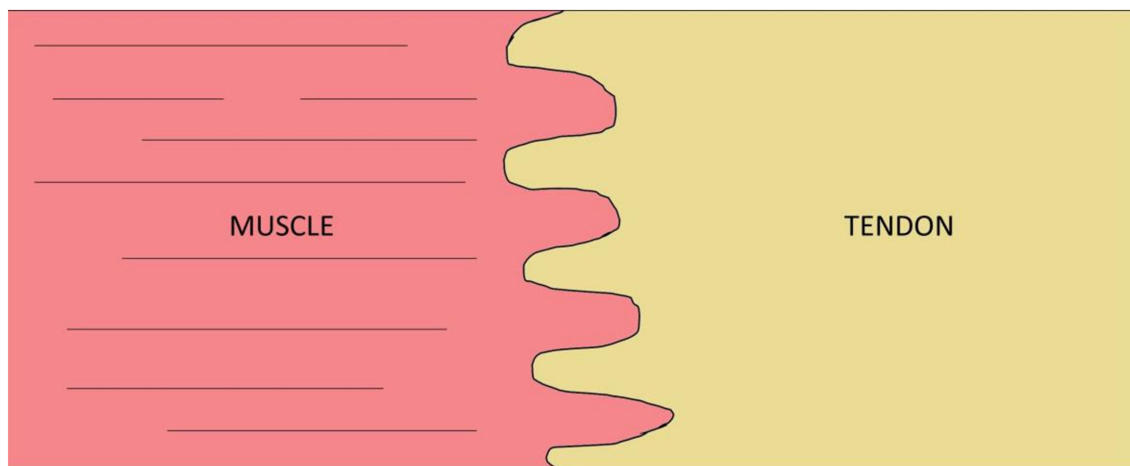


Figure 2.2: Schematic of the myotendinous junction. Muscle processes can be seen protruding into the tendon to form a connection of the two tissues.

The osteotendinous junction or enthesis has a length of nearly 1 mm and serves as a transition from the soft connective tissue of the tendon to the calcified bone.[33] There are 4 zones within the enthesis: the tendon, non-mineralized fibrocartilage, mineralized fibrocartilage, and then the bone.[13] The enthesis can occur either directly

where the tendon directly contacts the bone at a right angle or whether it has an indirect insertion where it blends with the periosteum of the bone.[33]

Internal vascularization of the tendon is typically formed longitudinally through the tendon around the fibers in the endotenon.[35,36] The origination of these vessels occur in three locations: the muscle/tendon junction, the bone/tendon junction, and through the paratenon.[37,38] However, even with all of these supplies, the vascularization of the tendon is poor, which helps contribute to the poor healing capacity of the tendon.[39]

2.3 Tendon ECM Composition

Tendon ECM is comprised mostly of collagen and water. Water is about 60-80% of the wet weight of the tendon.[40] Furthermore, collagen type I composes nearly 80%-95% of the dry weight of the tendon.[24] Collagen type III, found mainly in the epitenon and endotendon, and collagen type V, found in the core of collagen type I fibrils, are the other collagen types found in substantial amount (nearly 5% total).[30,41,42] Collagen type III and type V are both thought to regulate fibril diameter within the tendon[41,43] There are also small amounts of collagen types II, VI, IX, X, and XI present mostly at bone insertion sites to improve the bone/tendon connection strength.[41,44]

Other than collagen, there are other proteins and ECM molecules present that provide stability, elasticity, lubrication, and maturation of development to the tendon. Elastic fibers consist of 1-2% of the tendon dry weight.[45] These fibers are thought to allow for recovery of the collagen fibers after the load on the tendon has stopped, providing elasticity.[46] Besides the elastic fibers, there is the ground substance, which

includes proteoglycans, glycosaminoglycans (GAGs), and glycoproteins. Some of these include biglycan and decorin, proteoglycans which help regulate the formation of collagen fibrils in development and is also though to assist in early and late stage healing.[47,48] Tenascin-C, a glycoprotein is often found in developing or healing tendons, although not as often in normal tendons.[49] Tenomodulin, another glycoprotein, is highly expressed by tendon cells, and is involved in the recruitment of tenocytes, maturation of collagen fibrils, and also a marker of tenocytic differentiation of stem cells.[50,51] GAGs such as chondroitin and dermatan sulfate are present in the tendon, and are thought to potentially assist in mechanical integrity of the tendon.[52]

The previous description was the composition of the main portion of the tendon, and although the junctions where the tendon meets the bone and muscle are similar, they also have some differences in makeup. For example, tendons near the myotendinous junction sre still composed mainly of collagen type I but also possess more collagen type VI and tenascin C compared to the main body of the tendon.[31] The osteotendinous junction, which is comprised of the four zones, has a spatial variance in composition. The tendon at the junction contains mostly collagen type I and decorin. While the fibrocartilage contains collagen types II and III, aggrecan, and decorin. The third mineralized cartilage zone is made up of collagen type II and X, and aggrecan. Finally, the bone portion of the junction is composed mostly of mineralized matrix.[53]

2.4 Cells of the Tendon

Cells of a developed tendon are comprised of 90-95% tenocytes.[13] The remaining cells are related to the neighboring tissues at the junctions of the tendon,

synovial cells, and vascular-related cells.[30] The tenocyte arise from the tenoblast, which is the immature terminal cell of the tendon found mainly during devleopment.[54] These cells are metabolically more active than tenocytes and as they mature, they lose much of their activity and become tenocytes.[54] Tenocytes are elongated and also possess elongated nuclei, often spindle shaped. They are anywhere from 20-70 μm long and 8-20 μm wide.[55,56] These cells are more dense in developing and young tendons, while they decrease in amount as the tendon matures.[13,55] Tendons of a newborn can have as many as 200,000 cells/mm^3 and decrease to 50,000 cells/mm^3 as an adult.[57] As they mature, they develop longer cell processes to maintain cell-to-cell contact, due to their decreasing number.[30]

Tenocytes express genes that are related to proteins found throughout the ECM of the tendon and produce the related molecules. This infers that these cells are at least responsible for normal tendon maintenance and also play a part in tendon regeneration and healing.[30] These genes include, but are not limited to: collagen type I, collagen type III, collagen type V, decorin, biglycan, elastin, scleraxis, tenascin C, tenomodulin, and growth factors such as bone morphogenic protein (BMP) 4 and isoforms of transforming growth factor beta (TGF-β).[30,58–64] As will be discussed later, these genes can therefore be utilized to signify differentiation of MSCs into tenocytes.

The metabolism of tenocytes relies on both aerobic and anaerobic pathways, however, as tenocytes age, glycolysis and anaerobic energy production is preferred.[65,66] Matrix metabolism follows a similar trend, where matrix production is high in early development and slows in the mature tendon, including collagen turnover.[30] However, this slow metabolism also inhibits healing of injured tendons.[1,67]

Within the past few years, another niche of cells within the tendon has been discovered. The cells, tendon derived stem cells (TDSCs), are found within the tendon, are multi-potent and possess similar characteristics to MSCs.[68–71] However, these cells actually possessed greater proliferation, higher differentiation potency, and possessed more BMP receptors, indicating a different phenotype than MSCs.[72] Even then, it is thought that both of these cell lines originated from the same precursor cells during development. These cells are found throughout the tendon, as they have been isolated from the vascular-rich paratenon and also between collagen fibrils.[72] These cells are also thought to play a role in tendon healing, as they were found at tendon wound sites after initial healing began with traditional MSCs.[73] Their function and fate are most likely determined by numerous biological factors, physiological factors, tenocyte communication, and mechanical stimulation.[74]

2.5 Mechanotransduction of Tendon Cells

Tendons are a highly mechanical tissue, transferring forces from muscle to tendon. Previously, cell processes were described as a way for tendon cells to communicate. In addition, tenocytes also utilize gap junctions, especially in response to mechanical loading of the tendon.[75] The process of converting mechanical stimulation to a biochemical response is called mechanotransduction.

The process of mechanotransduction is still not fully understood, applies also to tendon cells.[44,76] Studies involving several cell types have shown that mechanotransduction can include integrins, ion channels, focal adhesions, and growth factors and their receptors.[77] This in turn, activates the cell signaling cascade, which

can involve any of the Rho-dependent kinase, nuclear factor kappa B (NFκβ), mitogen-activated protein kinase (MAPK), and protein kinase C (PKC) pathways.[77,78] These pathways then affect gene transcription and expression. A general schematic of these processes is shown in figure 2.3.

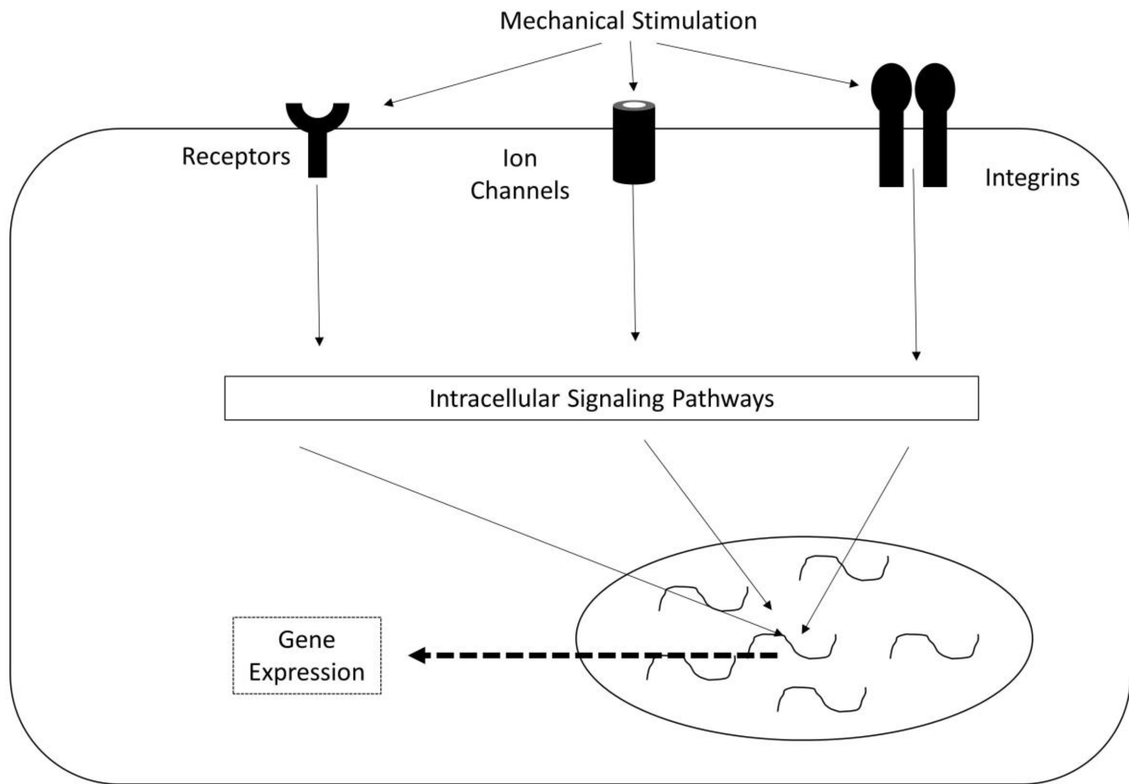


Figure 2.3: Diagram showing mechanotransduction in response to mechanical loading.

The gene expression changes due to mechanical stress on the tendon cell is a complex cascade dependent on the nature of the stress in many cases. Positive mechanical stress can increase collagen production of tenocytes, proliferation rates, and expression of growth factors such as TGF-β, platelet-derived growth factor (PDGF), and fibroblast growth factor 2 (FGF-2) .[79–81] In addition, stretching also promotes the expression of vascular endothelial growth factor (VEGF) within tenocytes.[82] This

factor promotes vascularization, allowing for increased blood flow and healing at an injured site.[83]

However, mechanical loading can also have drawbacks if the stress is large enough. After 12% stress it has been shown that tenocytes increase the production of cyclooxygenase 1 and 2, along with prostaglandin E2, which are related to inflammation of the tendon.[84] This is also true for other inflammatory cytokines.[85–88] It has also been shown that these lead to increased expression of matrix metalloproteinases (MMPs) which can be responsible for breakdown of the tendon ECM if expressed in high levels.[85,89] In addition increasing mechanical strains of up to 9% have shown to increase activation of the c-Jun N-terminal kinase pathway, which when persistently activated, can lead to apoptosis.[90]

As tendon stem cells were discovered relatively recently, little has been known about their response to mechanical stimulation *in vivo*. However, *in vitro*, at low strains (4%), mechanical stimulation induces the tendon stem cells to become tenocytes, while larger strains induced differentiation into osteogenic, chondrogenic, and adipogenic lineages.[91,92] This may provide insight into part of the reason why controlled rehabilitation encourages tendon repair, while high mechanical loads and subsequent tendon injuries cause irregular tendon tissue formation, such as calcification and lipid formation.[3,91,93]

2.5 Mechanical Properties of the Tendon

Since various tendons throughout the body have different functions and mechanical loads to transfer, the mechanical properties of the different tendons can vary

significantly. Table 2.1 gives mechanical properties for some of the tendons in the body.

	Ultimate Tensile Strength (MPa)	Young's Modulus (MPa)	Strain at Maximum Stress (%)
Achilles	819 ± 208	870 ± 200	8.8 ± 1.9
Patellar (29-50 years old)	64.7 ± 15.0	660 ± 266	14 ± 6
Patellar (64-93 years old)	53.6 ± 10.0	504 ± 222	15 ± 5
Anterior Supraspinatus (Rotator Cuff)	16.5 ± 7.1	165 ± 20	N/A
Middle Supraspinatus (Rotator Cuff)	6.0 ± 26	70 ± 20	N/A
Posterior Supraspinatus (Rotator Cuff)	4.1 ± 1.3	40 ± 15	N/A

Table 2-1: Mechanical properties for selected tendons throughout the body.[94–97]

Also as seen in the table, there is a difference in mechanical properties of the tendon due to age. A healthy adult patellar tendon had superior mechanical properties compared to an older patient.[96] This is also seen in newborns, where the tendons of a newborn, which is not finished developing, are weaker than developed tendons.[98] Finally, there can also be a difference within the tendon, when it is heterogeneous, as is seen in the rotator cuff tendon.[97] Even though the ultimate tensile strengths of the various tendons can approach nearly 1 GPa, actual physiological forces are much lower. For example, the strongest tendon in the body, the Achilles tendon, will experience a 53 MPa stress during human running, only 1/15th the ultimate tensile strength of the Achilles tendon.[94,99]

Between the previously mentioned crimp of the collagen fibers, its water content, and other proteins found in the ECM of the tendon, tendons exhibit viscoelastic behaivior.[100] Viscoelastic materials have a transient relationship between stress and strain, shown in figure 2.4. When a viscoelastic material is exposed to a constant stress for certain amount of time, creep will occur. This phenomenon is observed when the strain of the material increases to a certain threshold to accommodate the applied stress by rearranging its individual subunits, in this case, fibrils and fascicles of the tendon.[102] When the stress is unloaded, the material recovers to its original configuration. On the other hand, when a constant strain is applied, the viscoelastic material undergoes stress relaxation. When the strain is initially applied, the stress reaches a maximum and then decreases until the strain is released. Afterwards, the viscoelastic material will recover.[102] Another property of viscoelastic materials is hysteresis. This is represented by a loop in the stress-strain curve when a material is loaded and relaxed. This loop represented energy that is dissipated by the viscoelastic material during loading and unloading, usually in the form of heat.[103] This energy is wasted energy when transmitting forces from the muscle to the bone. Finally, because the tendon is viscoelastic, its stiffness depends on the velocity of the stretching applied, with it becoming more stiff at higher velocities.[104] Therefore, a higher amount of force will be required to rupture a tendon with a fast change in mechanical load, protecting the tendon. Summarized, low strain rates allow for the tendon to absorb more energy but transfer less mechanical load, while high strain rates allow the tendon to become stiff and transfer higher mechanical loads with less deformation.[13]

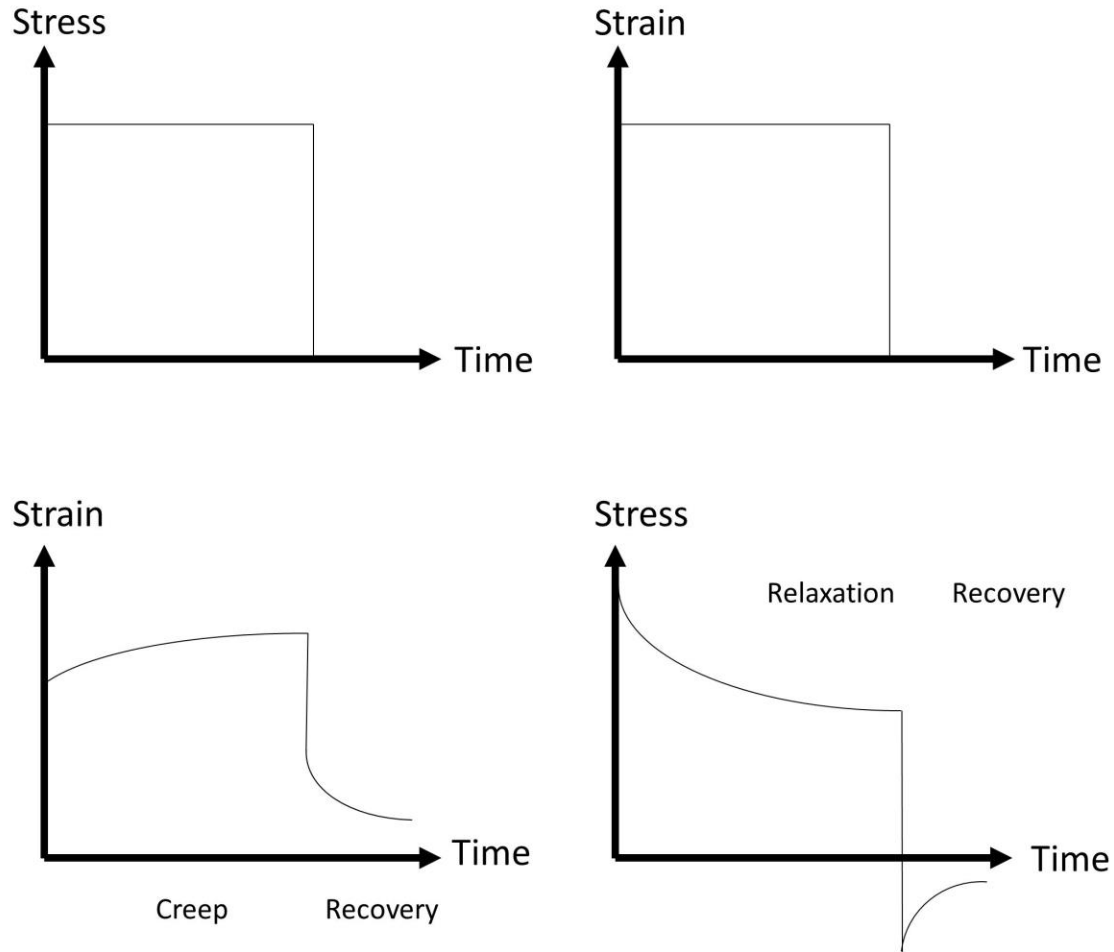


Figure 2.4: Stress-strain transient relationships for viscoelastic material. Creep is demonstrated on the left while stress relaxation is shown on the right.

Figure 2.5 shows a typical stress-strain curve of the tendon. It consists of four regions. The first region is the toe region. Generally, up until about 2% strain, the crimp within the fibers straighten out with initial elongation.[44] If there is a sudden, large initial loading, this protects the tendon from early failure.[105] The linear region occurs until nearly 4% strain. This is where fibers are still being elongated, and fully lose their crimp.[44] Up until 4%, any elongation and deformation can be fully recovered by the tendon.[13,106] After this point, failure begins to occur. However, most normal physiological forces only exert up to a 4% strain on the tendon, allowing for recovery in

most situations.[13,42,106] If enough load is applied, microscopic failure or tears within collagen fibrils appear first, up to 8-10% strain.[44] This area is where many of the tendinopathies can occur, and will be discussed in the next section. After about 10% strain, rupture, or macro-failure of the tendon occurs, and at this point, surgical intervention is usually required.[13,44]

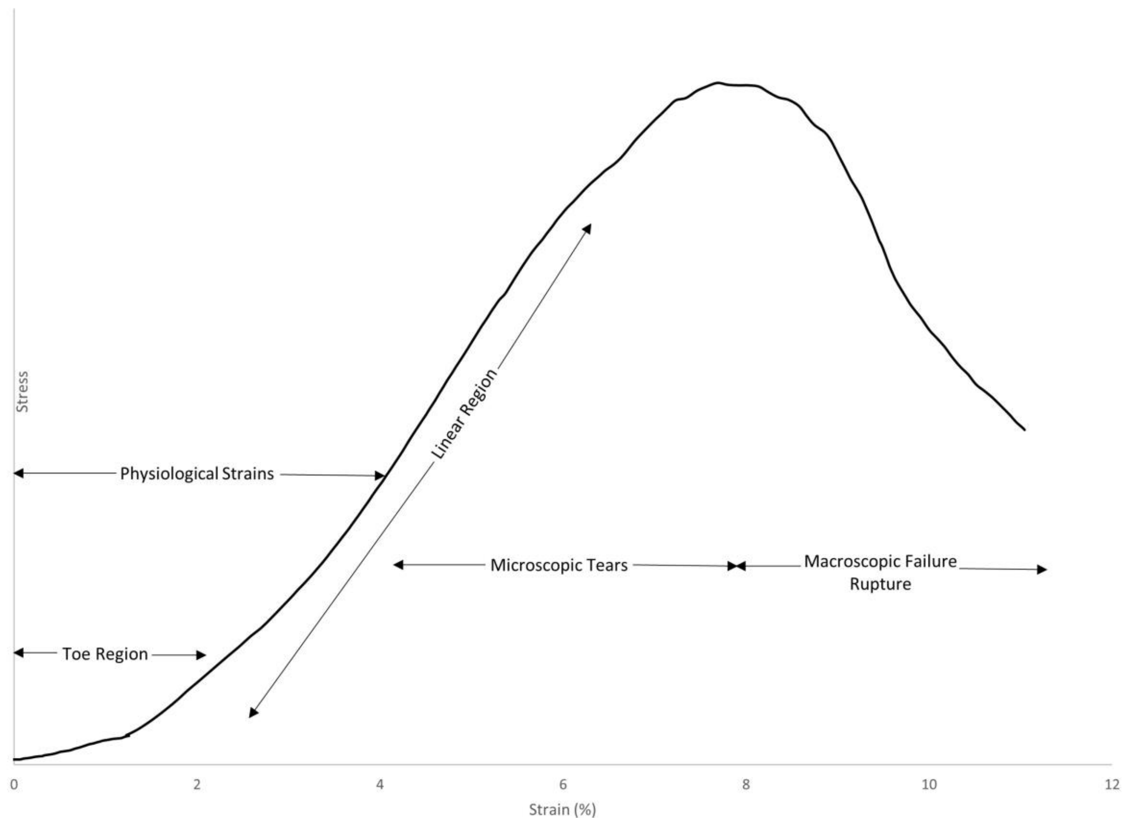


Figure 2.5: Stress strain curve of a tendon, indicating tendon fiber pattern for each of the four regions: toe, linear, microscopic failure, and macroscopic failure.

2.6 Tendon Pathologies

Tendons as discussed previously possess poor healing capacity. This is due to sparse cellularity, low vascularity, and low metabolism in normal tendons.[1,2,4,13] For example, tendons have about a 1/3 of the blood supply of muscles, that are considered highly vascularized tissues.[107] Therefore, if not treated properly, even common tendon

injuries can lead into more serious tendon pathologies such as rupture, which in most cases requires surgery and a potential need for a graft. As was discussed in the introduction, the statistics point to many work hours lost to tendon injuries and for the patient, a loss of quality of life due to acute and chronic tendinopathies is a common outcome. This section will cover the various typical tendon pathologies, their symptoms, causes, and common treatment methods.

2.6.1 Tendinitis and Paratendinitis

Tendinitis is the occurrence of inflammation within the tendon. Its symptoms include pain and tenderness, which can increase at night or with activity.[108] Other symptoms related to inflammation may occur such as warmth and swelling.[13] It is often caused by overuse and repeated injury to the tendon.[109] It includes common injuries such as tennis elbow and jumper's knee.

Often the inflammation phase lasts for 2-5 days and then remodeling occurs.[13] This involves recruitment of tendon cells and remodeling of the ECM of the tendon at the injured site. However, this ECM is still immature and disorganized, and without proper protection and rest, reinjury can occur fairly easily, leading to a more chronic injury.[110] As such, rest and support is important in clinical treatment of the injury. If rest and braces are not enough, non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids can help alleviate symptoms and recovery.[111]

Paratendinitis is similar to tendonitis, but is limited in location to the outer layer of the tendon, the paratenon.[13] As such, the symptoms and treatments for paratendinitis are the same as for general tendinitis.

2.6.2 Tendinosis

Tendinosis is the occurrence of microscopic tears within the collagen fibrils.[13,112,113] These tears are accompanied with an increase in cellular density and there is little to no inflammation present.[112] The tendon itself when healthy is glistening white, but is described as dull and brown when inflicted with tendinosis.[114] Tendinosis is separate from tendinitis, but tendinitis may accompany tendinosis.[13]

On an ECM level, tendinosis often is accompanied by an increase of collagen type III fibers. These fibers are immature and disorganized, which leads to a weaker tendon.[115] Along with this, the existing collagen type I is often denatured or damaged.[116] Furthermore, MMP activity is higher along with low pentosidine levels (signifying new matrix) indicating that remodeling and recovery is occurring at the injury site.[116] In addition, ground substance of the tendon is increased. [113,117] Ground substance gives an appearance of disorganized matrix, but is also found to assist in the assembly of mature collagen, potentially providing a benefit to the recovering tendon.[118,119]

As long as tendinosis is not severe enough to warrant surgical intervention, treatment suggested includes rest, rehabilitation, supports, and proper nutrition when applicable.[115] Oftentimes physicians will misdiagnose or treat tendinosis as tendinitis and treat with NSAIDs and other anti-inflammatory treatments.[114] However, as there is no inflammation occurring directly with tendinosis, these are not effective in the recovery process. Whereas tendonitis recovery can occur within a couple of weeks as long as it is not chronic, tendinosis has a longer recovery time. This recovery time can

range from 6 weeks to 9 months depending on severity and whether it is acute or chronic.[114,120]

2.6.3 Tendon Rupture

Many tendon ruptures occur spontaneously when the tendon experiences a large, abnormal force through a physical activity.[13] However, chronic and overuse injuries can also exacerbate and cause tendon ruptures.[66,121] There are two types of tears: partial tears where the muscle/tendon/bone unit are still connected with significant macrotearing of the tendon, or a complete rupture where there is separation somewhere within the muscle/tendon/bone unit.[121]

Ruptured tendons display less collagen content than normal tendons along with reduced mechanical properties.[122] Similarly to tendinosis, collagen type III content was increased compared to the normal tendon, as part of the healing process. However, this also decreases the tensile strength and collagen quality of the tendon.[123] Also MMP activity is higher at the rupture site, possibly leading to further degradation after the initial injury, further reducing mechanical integrity.[124,125] It is thought that these MMPs are initially utilized to repair and turnover damaged collagen, however, regulation may be compromised with chronic injury or additional strain on the injury site.[125]

Surgical treatment of both partial and complete ruptures is the most common form of treatment.[13,121] However, recently more conservative treatment methods such as rest and rehabilitation has been used in some cases with success, especially with patients that are poor surgical candidates such as the elderly.[126–130] However, rerupture rates are four times greater with non-surgery than surgical treatment of the rupture.[127]

If the rupture is small enough, the gap may be sutured together, utilizing techniques such as the modified Kessler suture.[127] If the defect is large enough (2 cm) a graft may be needed.[131] Surgeons have many different graft options such as: xenografts, allografts, autografts, and synthetic materials. Table 2.2 gives a listing of some of the common grafts used in tendon treatment. Xenografts are often times porcine in nature. They and allografts are mostly decellularized to reduce the chance of an immune reaction.[132,133] Autografts come from locations that have redundant function and good healing capacity, such as the semitendinosus tendon connecting the hamstring to the back of the knee.[149] Finally, synthetic grafts are often polymeric in nature, possessing mechanical properties and degradation rates to support regeneration of tissue while providing adequate support.[140] However, they are not without issue, as these grafts can also have complications. Xenografts and allografts can still elicit an immune response.[12,150] Autografts have the complication of donor site morbidity along with a second surgical site and accompanying pain.[151] Synthetic grafts can cause poor healing of regenerated tissue and mechanical properties can be degraded with long-term usage.[11,152]

Surgery currently provides the best chance of full recovery in tendon rupture as long as there is not complications.[22,153] Rerupture rates for surgical treatment of Achilles tendon ruptures was 1.54% while rates for non-surgery treatments were 17.7%.[22] However, even with successful surgical intervention of a rupture, some tendon functionality may be missing up to 2 years after treatment.[154] Additionally, even though surgical treatment may give the best odds for recovery, rerupture can still

	Company	Material	Size	Approval
Xenograft				
CuffPatch	BioArthro (USA)	Porcine SIS (97% collagen, 2% elastin)	6.5 cm x 9 cm x 0.6 mm	FDA
Restore	DePuy Orthopedics (USA)	Porcine SIS (90% collagen, 5-10% lipids)	6.3 cm x 1 mm	FDA
TissueMend	Stryker Orthopedics (USA)	Fetal bovine dermis (No lipids or carbohydrates)	5 cm x 6 cm x 1.2 mm	FDA
Allografts				
GraftJacket	Wright Medical (USA)	Tissue bank dermis (Collagen, elastin, GAGs, proteoglycans, FGF, vascular channels)	Varies: Up to 5 cm x 10 cm x 2mm	FDA
Anthrex Tendon Allograft	Arthrex (USA)	Tendon tissue	Varies depending on tendon	FDA
Autografts				
Patellar Tendon Repair		Semintendinous Tendon	N/A	FDA
Biceps Tendon Repair		Semintendinous Tendon	N/A	FDA
Synthetic				
Sportmesh	Arthrotek (USA)	Polyurethane urea	4 x 6 cm x 0.8 mm	FDA
GORE-TEX Soft Tissue	Gore (USA)	Expanded polytetrafluoroethylene	Varies: Up to 26 cm x 34 cm x 2 mm	FDA

Table 2-2: Table of available grafts for use in tendon rupture treatment. SIS - Small intestinal submucosa, FGF - Fibroblast growth factor.[134–148]

occur.[154] Ideally, as will be discussed in chapter 3, a tissue engineered tendon would be able to reduce or eliminate this time of reduced function.

2.7 Conclusions

Tendons are relatively inert soft tissues that transmit mechanical forces from the muscle to the bone. They are comprised mostly of collagen type I fibrils along with small amounts of other proteins that provide support, elasticity, and hydration. Normal tendons are sparsely cellularized and its cells have low metabolism. The tendon's unique mechanical properties are due to its viscoelastic nature. This allows it to adjust the type and rate of mechanical load and recover fully. Since tendons are poorly vascularized and have low cellularity with low cell metabolism, healing capacity is poor compared to other tissues of the body. This can result in chronic or acute tendinopathies. These tendinopathies can include tendinitis (inflammation), tendinosis (microtears), or rupture. In some cases, rest, rehabilitation, and medication is all that is needed for recovery. However, in severe enough cases, such as rupture, surgery is needed to either clean out damaged tissue, suture a small gap, less than critical defect size of the tendon, or apply a graft to supplement the native tissue. Grafts can come from xenografts, allografts, autografts, or synthetic grafts. All of these have benefits and drawbacks, some or all of which could be remedied with a tissue engineered construct. Chapter 3 will discuss the current state of tendon tissue engineering.

Chapter 3: Tendon Tissue Engineering

3.1 Introduction

Tissue engineering is the application of engineering and biology among other fields to create an artificial neotissue that can be utilized as a graft *in vivo* to either replace or assist the regeneration of injured native tissue.[155] The field arose as a need for alternatives for grafts during surgical treatment of injuries developed. This could be due to the lack of availability of grafts, inadequate healing, or immune responses among other reasons.[156,157] A successful tissue engineered construct will support cell growth, tissue formation, and when implanted, not elicit an immune response and integrate with natural tissue.[24,155] The following sections will discuss each aspect of tendon tissue engineering in more detail and their importance to the overall construct.

3.2 Functional Tendon Tissue Engineering

Functional tissue engineering refers to the creation of an artificial neotissue that resembles or has the same biological and biomechanical properties of the native tissue it is replacing[155] For this to occur, most or all of these three aspects should be utilized: a biocompatible scaffold, a cell source, and stimulation via biochemical or mechanical means either *in vitro* or *in vivo*, or both. [24,46,155,158–162] The following sections will discuss the important aspects of each and how to relate to each other to form a functional tendon tissue engineered construct.

3.2.1 Scaffolds

An initial requirement for a scaffold utilized in tendon tissue engineering is the ability to initially support (or be modified to allow) cell attachment, allow for nutrient transport within the tissue, and allow for integration of new tissue both *in vitro* and *in vivo*.[158,163,164] This scaffold should also ideally have mechanical properties that approach the native tissue's.[164] In addition the scaffold needs to not elicit an immune response when implanted.[165] Furthermore, the degradation rate must be tailored to support tissue growth initially while providing sufficient mechanical support.[164] Degradation rates can depend on structure, molecular weight, morphology, porosity, and where it is implanted.[166] When degrading, the materials must be bioresorbable so that the degradation products are eliminated through natural means within the body.[163]

Natural material based scaffolds consist mostly of collagen type I based gels for tendon tissue engineering.[167–170] This is a popular choice as collagen type I composes a significant portion of the dry weight of tendons.[11] These gels seeded with MSCs have shown increased healing and function *in vivo* in rabbit Achilles tendon injuries.[167] However, these collagen gels do not replicate the aligned fibril structure of the tendon and their mechanical strength is usually lacking compared to mature tendons.[11] To remedy the mechanical strength issues of collagen gels while maintaining some of the natural material characteristics, some researchers have created hybrid collagen gels with synthetic polymers or silk.[171–173] Small intestine submucosa (SIS), chitosan, alginate, and hyaluronic acid have also been used as natural materials in tendon tissue engineering scaffolds, but to a much lesser extent than collagen hydrogels.[174–177]

The natural material utilized in the research presented in this dissertation is the HUV. The vein is obtained from umbilical cords which are otherwise discarded after birth. It has many of the benefits of being a natural material like collagen, however, it also possesses a higher initial mechanical strength than collagen hydrogels.[178–182] In fact, the decellularized HUV is only an order of magnitude weaker than some of the tendons in the body.[13] The HUV has been utilized in various tissue engineering strategies from blood vessels to the vocal fold.[183,184] The HUV and its other umbilical vessel, the human umbilical artery (HUA) possess similar mechanical strengths: 1.47 ± 1.08 MPa and 1.37 ± 0.80 MPa respectively for the HUV and HUA.[185,186] In addition, it has also been utilized in previous studies for tendon tissue engineering by Abousleiman, et al upon which these studies were based.[178–180] The HUV supported cell growth and ECM deposition, and when cyclically stretched, the ECM became aligned and MSCs became more tenocytic-like in appearance.[178,179] Furthermore, the mechanical strength of the HUV was increased by 3 fold to 4.1 ± 0.5 MPa when supplemented with MSCs and stimulated for 2 weeks.[180]

Another benefit to the HUV is its performance and immunological characteristics to xenografts, or tissues from another species. To create a high quality graft, cellular incorporation into the scaffold must be at high levels. It has been shown that human fibroblasts infiltrate more efficiently and further on human dermis allografts compared to porcine dermis xenografts.[187] In terms of use in clinical settings, xenografts have shown to cause local and systemic immune responses, even after the typical surgical recovery time.[188–190] This has also been seen in tendon specific xenografts

through both non-specific and specific (potential allergic reaction) immune responses.[191–193]

Ideally, a decellularized healthy tendon (ether from the patient or another donor) would be one of the best scaffolds for a tissue engineered tendon replacement as it would have tendon specific biomolecules and possess a mechanical strength near the native tissue. If decellularized completely, immune responses should be limited.[166] However, the supply of such a scaffold is scarce and still carries the chance for disease transmission.[194] In addition, the decellularization process can negatively affect the structure and mechanical strength of the tendon and remove some of the beneficial biomolecules.[195] There have been some studies utilizing the decellularized tendon as a scaffold in the literature.[196–198] Decellularized tendons have shown the ability to support reseeding of cells along with increased ECM deposition and increased mechanical strength.[198] However, histology has shown that the scaffold doesn't facilitate easy migration and incorporation of the cells as they primarily reside on the surface of the scaffold.[196,197]

One of the biggest drawbacks of natural materials is their initial mechanical strength. Synthetic polymers tend to have higher mechanical strength, but are accompanied by poor cell attachment (due to their hydrophobic nature) and sometimes immune responses (in some cases).[163,164,199] Synthetic polymers used in tendon tissue engineering include but are not limited to: polyglycolic acid (PGA), polylactic acid (PDLA and PLLA), its l-lactide form poly-l-lactic acid (PLLA), poly(lactic-co-glycolic) acid (PLGA), polycaprolactone (PCL), and polydioxanone.[131,200–208] In the literature, PLGA is most commonly used among these options.[131,200–202,205] A reason for this is

due to its ability to be tuned based on ratios of PLA and PGA. Based on the ratios, properties such as degradation rates and mechanical strength can be changed based on need.[209,210] Braided PLGA has been utilized due to its high tensile strengths compared to other PLGA preparations.[211] However, even though cells did attach on the surface of braided scaffolds, infiltration of these cells and tissue is poor due to the architechture.[203,211] As a comparison, knitted PLGA supplemented with MSCs were implanted within rabbit Achilles defects. After 12 weeks, the scaffold allowed for native tissue ingrowth along with nearly a tensile strength that was 60% of the original tendon.[212] Finally, with electrospinning, PLGA can be supplemented with other molecules, such as the growth factor FGF-2 during scaffold production. This supplemented nanofiber mesh increased cell growth, tendon gene expression, and collagen deposition when compared to the mesh without supplementation.[201]

As seen, there are many characteristics of a scaffold that must be considered when choosing an appropriate material for tendon tissue engineering. Furthermore, often times, when choosing a scaffold, there are trade-offs between the characteristics such as: mechanical strength, cell attachment, and degradation rates among others. As will be seen in future sections regarding mechanical stimulation, the scaffold stiffness can also play a large part of how the cells and tissue develops. The material stiffness can influence cell development and proliferation directly, or it can also affect how the cell senses and translates the mechanical signals into biochemical signals influencing the cell's fate.[213]

3.2.2 Cells

While scaffolds provide the support and base for a tissue engineered construct, cells are what make the construct a living tissue. The cells are what create, deposit, and modify ECM along with adapting the tissue during the culture and regeneration process.[162] They guide the tissue towards its ultimate result, an artificial tissue that can replace and regenerate an injured tissue. They are what make the tissue alive.

Primarily, the two cell types utilized in tendon tissue engineering are mature tenocytes and stromal stem cells. However, stem cells provide a better option for tissue engineering of an artificial tendon. Research has shown that tenocytes can remodel and create tissue similarly to adult stem cells, however they proliferate at a slower rate than adult stem cells.[214] This is a critical aspect of the cell source as a decellularized tissue or polymer must have and support a robust cell population at least initially to create sufficient high quality tissue to provide adequate mechanical properties and future tissue growth necessary for successful implantation. In addition, without consistent stimulation, tenocytes have a tendency to drift phenotypically *in vitro* at later passages.[215] Finally, it is much easier to acquire an aspirate of bone marrow for MSCs or biopsy of adipose tissue for adipose derived stem cells (ADSCs) from a patient as a cell source than it is to get a patient-specific source of tenocytes.

However, these drawback have not stopped research into tenocytes as a tissue engineering stem cell source.[175,216] Cao, et al. seeded PGA fibers with hen's tenocytes and reported high amounts of tissue deposition after 6 and 10 weeks, however tensile strength was lacking (1.3 MPa).[131] Tenocytes have also been used to repopulate

decellularized tendon tissue, but actually showed a decrease in ultimate tensile strength of the tissue.[197]

Chong, et al. repopulated the tendon tissue with tenocytes and implanted them into rabbits. *In vivo*, the donor tenocytes were eventually replaced by the body's tenocytes after 12 weeks.[217] The other previously mentioned group that utilized a tenocyte-seeded PGA construct also implanted their artificial tissues to test *in vivo* responses. After 14 weeks, tissue deposition, alignment, and tenocyte population began to resemble natural tendon, but it was found that an immune response was elicited by the PGA.[205]

As demonstrated by the previous results, tenocytes are utilized with mixed results. Various stem cell types are by far more popular in the literature for tendon tissue engineering when compared to tenocytes.[204,218–221] As the tenocyte originates from MSCs, they are a viable cell source to use for tendon tissue engineering. MSCs may differentiate into many mesenchymal linages which include, but are not limited to: muscle, bone, cartilage, ligament, and tendon.[222] This differentiation can be due to many different cues, either physical (ECM stiffness, mechanical stretching, fluid shear, etc.) or chemical (cytokines, other biological factors).[180,223–230] Once committed, MSCs primarily progress to their terminal lineage state, however, there is some opportunity to switch lineages after initial commitment if early enough in the hierarchy of progression.[231] They are found in many locations throughout the body, although primarily, and most plentiful, in the bone marrow.[232] In addition to their capacity to differentiate, they are also therapeutic in their immature state with their ability to secrete

cytokines which can influence local cells and recruit distant ones for the healing process.[233]

Tendon tissue engineering with MSCs can range from suspending them within a collagen gel and immediately implanting in a defect site *in vivo* to a mechanically stimulated PLGA scaffold seeded with MSCs *in vitro*.[170,234] The MSC-seeded collagen gel did increase the mechanical properties of the defect, however, most of the cells were still rounded and non-aligned with the ECM, indicating little tenocytic integration or differention of the MSCs.[170] This indicates that *in vitro* culture and stimulation (mechanical and/or chemical) may provide even further benefits to a MSC-seeded construct that allows for even better healing of a grafted defect. This will be discussed in further detail in section 3.3. As an example, a collagen gel seeded with MSCs and subjected to dynamic mechanical stimulation after 7 days did demonstrate increased ECM alignment, cell elongation, and increased expression of scleraxis, a marker of early tendon development.[235]

Another niche of the stromal stem cell population is ADSCs. These cells are isolated from adipose tissue through isolation of the stromal fraction from the adipose fraction of digested tissue and then further selection of ADSCs from the stromal fraction.[236] This stem cell population has also be recently used in tendon tissue engineering and tendon repair.[200,237,238] When cultured on a PLGA fiber scaffold in the presence of growth differentiation factor 5 (GDF-5), cell proliferation increased along with an increase in tendon related genes such as collagen type I, scleraxis, and tenomodulin.[200] In addition, ADSCs have also been differentiated utilizing a decellularized, powdered form of tendon matrix as a supplement in culture.[239]

Finally, as discussed before, a population of stromal stem cells, TDSCs, is found within the tendon. However, as their discovery has been only recent (2007), their cell density low compared to other stromal stem cell sources, and their autologous harvesting difficult, few studies specifically for tendon tissue engineering have been shown in the literature.[69,74] Ni, et al. placed TDSCs into patellar tendon wound sites with fibrin glue to improve healing.[240] Their presence was gone within four weeks, however mechanical properties did improve compared to the negative controls. The same group then attempted to make tissue engineered constructs out of TDSC cell sheets produced within culture flasks.[241] The cells within the sheets did express genes characteristic of a tendon and did show improvement *in vivo* compared to the previouse fibrin glue/TDSC method as mechanical properties were more improved.

3.2.3 Stimulation Techniques

The healing process of tendons gives some insights on the importance of mechanical stimulation of tenocytes. Lengthy immobilization adversely effects healing while controlled motion in rehabilitation can increase and hasten healing of the injured tendon.[193,242,243] When strained in cultured dishes *in vitro*, tenocytes showed increased proliferation rates after 15 and 60 minute 5% strains at a 1 Hz frequency.[244] In another study these stimulations also increased production of TGF-β, PDGF, and FGF-2, growth factors involved in tendon development and tenocyte growth.[81] Furthermore, tendons that were sliced 90% of their depth were cyclically stretched at 2 cycles/min continuously for 21 days. After 14 days, new tenocytes were present mostly at the surface of the injured site, aligned in the direction of stretching at much higher rate than

the control tendons that were not stretched. After 21 days, cell penetration was seen in the stimulated tendon while new tenocytes were only seen at the surface of the non-stimulated tendons.[245] In addition to increased proliferation and cellular activity, light strains (4%) are also shown to modulate and reduce inflammation responses in tenocytes.[89] However, higher strains (8%) actually exacerbate the response.[89] Finally, initial responses to high enough strain stresses can induce apoptosis, however, tenocytes have shown the ability to develop stress tolerance, as further stretching at the same parameters showed a decline in apoptosis rates.[246]

Mechanical stimulation of MSCs also has beneficial outcomes. 2D stretching with strains between 2-8% can increase proliferation rates of MSCs.[247,248] As an example, strains between 2% and 8% also increased proliferation rates in 2D cultures at 1 Hz for up to 60 minutes.[247] However, strains above 7.5% have also been shown to increase rates of apoptosis in 2D cultures.[249] Therefore, there appears to be an upper limit to the benefit of strain in respect to MSC growth, which is near 8% (for 2D cultures). When investigating morphology and migration, MSCs can align and elongate with the direction of stretching, characteristic of tenocytes within a tendon. [250] Cyclical stretching has also been shown to increase collagen types I and III gene expression along with increased collagen production. [250–252] Furthermore, tenascin C, a marker used for tenocytic differentiation of MSCs has been upregulated in 2D stretching studies. [248,252,253] Zhang et al. demonstrated that collagen production and tenascin C gene expression is increased after only 24 hours of stretching at 10% strain and 1 Hz.[250] Finally, cyclical stretching can also inhibit adipogenesis of MSCs when strained at 2%

for 10 cycles/min for 6 hours, preventing an undesirable lineage for tendon tissue engineering.[254]

In addition to mechanical stimulation, chemical stimulation also plays a vital role in cell fate and activity. Typically *in vivo* this is done through cytokines, hormones, growth factors, and other small molecules.[255–258] In tissue engineering, growth factors are often used *in vitro* to provide chemical stimulation to constructs to direct and promote growth of the artificial tissue. Tendon tissue engineering has its own set of growth factors that influence development.

GDFs -5, -6, and -7 are signaling molecules that have been shown to significantly participate in and regulate tendon and ligament development during embryogenesis.[259] These factors are part of the TGF-β superfamily. This family progresses the signaling through both Smad and non-Smad pathways.[260] Multiple FGFs also participate in the embryogenesis of tendons.[261] FGF-2 also participates in early stages of healing to increase cell proliferation and collagen production.[262] Therefore, it would be expected that these growth factors would also influence MSC and tenocyte activity *in vitro* implicating their use in tendon tissue engineering.

Rat ADSCs were treated with GDF-5 in 2D culture to induce expression of tendon-related genes. A 100 ng/mL dose increased ECM genes such as collagen type I and aggrecan along with early tendon markers such as tenascin-C and scleraxis, among others.[236] Haddad-Weber, et al. investigated the effect of increasing BMP-12 and BMP-13 (also known as GDF-7 and GDF-6 respectively) expression in MSC cultures through gene transfer with a focus on ligament differentiation. However, ligaments and tendons share many similar characteristics and their results demonstrated many tendon

characteristics also. After 21 days, immunohistochemistry showed presence of collagen, fibronectin, decorin, elastin, scleraxis, tenascin, and tenomodulin, all ECM components of tendons.[263] Another study also confirmed the ability of GDF-7 to induce tenocytic differentiation in MSCs.[264] In addition, GDF-6, also known as cartilage derived morphogenic protein-2, has shown to increase the mechanical strength (35%) and healing rates of a rabbit Achilles tendon after 14 days, indicating an increase in healing capacity of tendon cells.[265]

In addition to GDF family, other growth factors also promote tenocytic differentiation of stem cells along with increased activity in tenocytes themselves. A primary one is FGF-2. Chen, et al. transduced MSCs with genes to overexpress FGF-2. These cells proliferated at a faster rate and expressed collagen type I, collagen type III, and scleraxis at higher rates than negative controls. It was also found that the MAPK signaling pathway played an important role in the FGF-2 signaling cascade.[266] Another member of the FGF family, FGF-5 has shown to increase tenocytic differentiation in equine ADSC along with FGF-2. Both FGFs increased proliferation along with early tendon markers tenascin C and scleraxis.[267] FGF-2 also can influence tenocyte development, it has been shown to increase proliferation and influence migration *in vitro*.[268] *In vivo*, acellularized dermal matrix treated with FGF-2 was placed into rats. After 6 weeks, the FGF-2 treated defects had significantly higher tensile strengths than negative controls and showed improved neotissue deposition, indicating increased migration and activity of tendon cells to the injury site.[269] Insulin like growth factor 1 (IGF-1) is thought to play a large part in tendon healing as it is expressed more strongly shortly after the initiation of tendinosis. *In vitro*, IGF-1 has shown to increase tenocyte

proliferation and collagen production (both types I and III).[270,271] In another study, PDGF-BB is proven to be beneficial to tenocytes. Its application increased proteoglycan production, cell growth, and viability after administration of dexamethasone, which was shown to decrease these characteristics.[272,273] Finally, a synergistic effect was seen when culturing tenocytes with IGF-1, PDGF-BB, and FGF-2. Tenocytes from the synovium were increased by 251% while a 100% increase was seen in epitenon and endotendon cells.[274]

3.3 Tendon Tissue Engineering Bioreactors and Construct Stimulation

A tissue engineered construct can be greatly improved upon if it is *cultured in vitro* as similar as possible to its physiological environment. Bioreactors can provide much of this mimicry of the physiological environment by supplying the construct with any or all of the following: mechanical stimulation, biochemical stimulation, and media flow to improve mass transport properties. The following sections will delve further into these aspects.

3.3.1 Tendon Tissue Engineering Bioreactors

Bioreactors aim to provide stimuli *in vitro* that mimic the physiological environment (mechanical and chemical) of native tissue to improve the properties of tissue engineered constructs. By mimicking this environment, mature cells can retain their phenotype, stem cells can be differentiated, and growth of ECM and the cell population can be encouraged.[160] In addition to encouraging tissue development, mass transport within the tissue can also be improved compared to a static culture, also

increasing the efficacy of the tissue culture.[161] Some of these include spinner flasks, rotating wall vessels, and flow perfusion bioreactors.[161]

The previous examples of bioreactors often introduce fluid shear to the construct. This stimulus is mostly effective for bone and sometimes cartilage formation, and not necessarily tendon tissue engineering.[275–277] In the body, tendons are most often stretched and relaxed as they transfer loads from the muscle to the bone.[13] To mimic this *in vitro*, most often tendon tissue engineering bioreactors are designed with a mechanical strain/stretch in the longitudinal direction.[161] This has been introduced as either a constant tension of the construct or as a dynamic stretching and relaxing of the construct.[46,218,219,278,279] Furthermore, the dynamic stimulation can be tuned further by its frequency, duration, and strain applied. Short term culture effects by modifying some of these variables is the subject of chapter 4. Sections 3.3.2 and 3.3.3 will discuss in more detail the effects of mechanical and chemical stimulation on tissue engineered tendon constructs.

3.3.2 Mechanical Stimulation

As with tendon treatments, it has been shown that immobilization can actual deter the healing process.[242,243] Therefore, it stands to reason that mechanical stimulation in the form of stretching (mimicking the natural tendon environment) would be also beneficial in tendon tissue engineering. Indeed, this is the case with both static and dynamic stretching.

However, there is a difference in efficacy between the static and dynamic stimulation. In two separate studies, collagen fascicles were subjected to either static or

cyclical loads. In both cases, loads near 40-50% of the peak stress of natural tendons encouraged the greatest increase in the mechanical properties of the fascicles such as tensile strength and elastic modulus. [280,281] It was concluded that that cyclical stretching caused larger increases compared to the static stretching. This was also seen in a MSC-seeded PLGA constructs. A 6.7 N static load did not show any significant differences in collagen synthesis or cellular proliferation between loaded and non-loaded scaffolds[234] In addition, it was seen that the timing of the strain initiation (during seeding, after seeding, or two days after seeding) had no sign effects on those parameters either.[234]

Indeed, these results that favor low intensity cyclic stimulation also seem to relate to other tendon tissue engineering studies utilizing mechanical stimulation. Strains between 1% and 5% have been shown to increase cell proliferation, gene expression of tendon related genes, tissue formation, and increase mechanical properties such as tensile strength, stiffness, and elastic modulus.[179,180,235,282–285] However, many of these studies also vary other parameters such as frequency which can range up to 1 Hz and duration of stimulation which ranges from 30 minutes per day to 8 hours per day. Although many studies opt for shorter duration of stimulations of up to an hour/day to allow for recovery. [179,180,235,285] In terms of frequency, Joshi and Webb investigated on how frequency affects the construct's ultimate tensile strength and elastic modulus. They found that higher frequencies (1 Hz) were less beneficial and possessed lower ultimate tensile strengths and elastic moduli than their lower frequency stimulated counterparts. Depending on the stimulation pattern, it was found that 0.1 Hz (resting periods at maximum tension) and 0.25 Hz (resting periods at no tension) were most beneficial.[283]

When utilizing a MSC-seeded HUV as a tissue engineered tendon construct, the aforementioned low intensity stretching (2% strain at a frequency of 1 cycle/min and 1 hour per day) has been utilized to improve its properties.[178–180] After 2 weeks of culture, tensile strength increased by 300% and a 20-fold increase in cell proliferation rates was observed.[180] In addition, the use of cells and mechanical stimulation increased tissue formation and alignment of the new and existing ECM in the direction of the uniaxial stretching, making the construct more tendon-like in nature.[179] Chapter 4 will investigate how changing the stimulation duration and frequency affects the short term culture of cell seeded constructs.

As discussed previously, high intensity stretching can be damaging to cells, inducing apoptosis.[246,249] This phenomenon occurrence has also been shown in fibroblasts cultured in synthetic gels. Cell alignment occurred in fibroblasts stretched at 1 Hz for 24 hours in a biosynthetic polymer ProNectin, which contained RGD ligand to increase cell attachment. However, above 4% strain, few cells survived (8, 12%) and cell orientation began within 3 hours.[286,287]

In other studies where high intensity stimulation was used some beneficial effects were observed. However, not as great as lower intensity stimulation. MSCs seeded on oligo(poly(ethylene glycol) fumarate) (OPF) were cultured for three weeks and subjected to a 10% strain at 1 Hz. This strain was applied for 3 hours, then removed for 3 hours and this cycle was repeated throughout the culture duration. This stimulation didn't increase the cellularity on the construct as opposed to other stimulations, but the cells remained viable, and upregulated collagen type I, collagen type III, and tenascin C gene expression indicating that the cells weren't actively

growing but were differentiating towards a tenocytic lineage.[288] In another study, SIS membranes seeded with tenocytes were loaded with a cyclical 9% strain at 0.1 Hz for 16 days. The cyclical stimulation increased stiffness by 125% where the other groups with no cells or a static 9% strain did not significantly change the construct siffness.[177] However, cellularity did increase over the 16 days for all groups, but no significant difference was seen between the unloaded, static, and dynamic stimulation groups. Finally, the same Joshi and Webb study that investigated the effect of frequency of a polyurethane/fibroblast construct also looked at varying the strain up to 10%. They found that 2.5% was the most beneficial in terms of increasing tensile strength and the elastic modulus of the construct while higher strains provided moderate improvements when compared to the static control.[283]

3.3.3 Chemical Stimulation

Although the effects of growth factors in tenocyte development have been shown both *in vitro* and *in vivo* (discussed in section 3.2.3), fewer studies have been performed specifically for enhancing a tissue engineered tendon construct.

One of the main tenocytic growth factors, FGF-2 has been incorporated directly into the scaffold for delivery to MSCs on polymer scaffolds.[201] A FGF-2 coated silk/PLGA scaffold demonstrated a positive effect on MSC activity and differentiation. The addition of FGF-2 maintained proliferation of the MSCs initially, and after two weeks, tenocytic differentiation was observed with increased tendon gene expression (collagen types I and III, fibronectin, and biglycan) along with mechanical properties

that had tendon-like characteristics (more viscoelastic and a more pronounced toe region) and increased tensile strength.[202]

GDF-5 has also been utilized in tendon tissue engineering. In one study, PCL was coated with collagen containing GDF-5 and seeded with stem cells. The construct was then subjected to cyclic strain (10% at 0.33 Hz) for 2 days. Collagen type I and scleraxis were increased by both stimuli, however no synergism was observed except the case of the the proliferation of the cells which was increased by 70% when both stimuli were applied.[289] In another study, Basile, et al. utilized a tendon allograft coated with GDF-5 gene transfection vectors to induce healing *in vivo*. These scaffolds induce more cell migration and proliferation along with enhanced cell healing compared to a control vector coated allograft.[290]

No studies were found in the literature on angiogenesis and/or VEGF specifically for *in vitro* tendon tissue engineering. However, it must be considered as a strategy in certain tendons if a construct is to be viable long term. Even though tenocytes possess relatively low metabolism, they must have a consistent nutrient and oxygen source provided by a blood vessel network.[13] For oxygen diffusion, cells must reside within few 100 μm of a blood vessel for viability.[291] VEGF encourages blood vessel formation, and can also be enhanced further by other growth factors, such as PDGF-BB.[292]

3.4 Conclusions

Tissue engineering provides a future remedy for the need of an alternative graft solution in surgical treatments of tendon injuries. The field can remedy many of the

issues with current graft treatments such as immune response, incomplete healing, and degraded function and integrity. By creating a substitute artificial tissue, these issues need to be considered and addressed in the construct.

These constructs are created using a combination of scaffolds, cells, bioreactors, and mechanical/biochemical stimulation. Scaffold choices can vary among natural and synthetic materials, with pros and cons for each. Both tenocytes and stem cells (MSCs and ADSCs) have been used in tendon tissue engineering, and with stem cells it is possible to obtain an autologous source of tenocyte inducible cells from the patient. However, even with a scaffold and cell source, the addition of stimulation either by mechanical or biochemical means, can generate improved neotissue.

Both mechanical and biochemical stimulation have proven beneficial in tendon tissue engineering, with mechanical stretching within a bioreactor being the preferred method of stimulation. It has been shown that a wide variety of stimulation is capable of increasing construct quality, however, lower intensity stimulations prove more beneficial. Ultimately, even though the literature trends towards low intensity stimulation, every tendon tissue engineering system will be different with various scaffold materials, culture techniques, and mechanical variables. Therefore individual systems should be investigated to find the best stimulation parameter. Chapter 4 will discuss how varying frequency and duration of the dynamic stretching can influence construct properties. Unlike bone tissue engineering, where there is one key biochemical signal to assist in osteogenic differentiation (BMP-2 or dexamethasone), there is not a specific growth factor or combination of factors that has the same result in tissue engineering. However, many growth factors have proven beneficial for tenocytic

development and differentiation such as FGF-2, GDF-5, GDF-6, and GDF-7 among others. Chapter 5 will discuss biochemical influence on tenocytic differentiation of MSCs on a construct and an alternative source of biochemical stimulation.

Chapter 4: The Effects of Varying Frequency and Duration of Mechanical Stimulation on a Tissue-Engineered Tendon Construct

4.1 Introduction

Tissue engineering requires a three-pronged approach: a cell source (mature or stem cells), a suitable scaffold to support cell and ECM growth, and cues provided by mechanical or chemical means to regulate cellular activity.[293,294] The mechanical stimulation in terms of tendon tissue engineering is typically a uniaxial strain applied on the scaffold.[218] This stimulation has been utilized with various magnitudes of strains, frequencies, and durations across research groups. It is understood that beneficial stimulation can enhance cellular proliferation, promote tendon-like ECM production, and assist in the differentiation of stem cells towards a tenocytic lineage.[247,295–297] However, if improper stretching is applied, the construct can be degraded, due to cell death or declining ECM quality.[249,298] Since mechanical properties and stimulation response can vary from construct to construct depending on system and techniques, it is important to elucidate which stimulation parameters are important for each system and how those parameters result in the most improved construct.

Previous research has investigated a decellularized HUV as a scaffold seeded with MSCs in a custom bioreactor that provides cyclical mechanical stimulation. This stimulation was provided for 1 hour/day, at a 2% strain and 1 cycle/minute frequency.[179] Compared to other studies, these conditions provide relatively low magnitude stimulation. Although many studies have frequencies on the magnitude of 1 cycle/minute, some studies do approach 1 Hz (60 cycles/min). [179,180,235,282–285] In

addition, durations of the stretching can range from 1 hour/day to 8 hours/day to the entire culture duration. However, in chapter 3, it was discussed that shorter durations and lower frequencies tended to have more beneficial effects than their higher counterparts.[179,180,235,283,285] The previous stimulations utilized in the HUV/MSC construct already resided in these lower ranges. Ideally, the stimulation would approach the natural environment of a tendon, where stretching frequency and durations can vary.[218] However, it has been shown that tendon ruptures can begin to occur at 6% strains.[299,300] This corresponds with tendon tissue engineering data that reports that strains up to 5% were most beneficial for construct development. [179,180,235,282–285]

This study was done to determine the effects of changing the frequency and duration of stimulation on the HUV/MSC construct. It is hypothesized that varying the frequency and duration of stretching will affect the construct's properties. In addition, for early culture time points, mechanical stimulation is hypothesized to be more beneficial since the cells are still adapting to their new environment. To test this, a slower and faster frequency compared to our previous studies along with a shorter and longer duration of stimulation was investigated.

4.2 Materials and Methods

4.2.1 Scaffold Preparation

Human umbilical cords were obtained from Norman Regional Hospital (Norman, OK). The HUV was then extracted utilizing established protocols.[178,179] Briefly, the umbilical cord is mounted onto a steel mandrel and frozen at -80°C. The

HUV was then extracted by a computerized lathe. The resulting HUV had a diameter of 6.75 ± 0.25 mm and a wall thickness of 0.75 mm.

After extraction the HUV was inverted so that the remaining Wharton's jelly region is on the interior of the scaffold. The HUV is then decellularized, washed, and sterilized using previous procedures utilizing 1% sodium dodecyl sulfate (JT Baker, Center Valley, PA) and 0.2% peracetic acid solutions (Sigma Aldrich, St. Louis, MO). After adjusting the pH of the HUVs to physiological levels (7.2-7.4) utilizing phosphate buffered saline (Life Technologies, Grand Island, NY), they were stored at most for 1 week at 4°C prior to use.

4.2.2 MSC Extraction

Bone marrow MSCs were obtained from the femur and tibia of male Wistar rats (Harlan, Indianapolis, IN) using established procedures.[301,302] After harvest, the bone marrow MSCs were cultured on 75 cm^2 culture plates. After 80% confluency, cells were lifted and further passages were cultured on 75 cm^2 flasks until ready for use. Cells were cultured with supplemented α-MEM media in a humidified incubator at 37°C and 5% CO_2. The α-MEM (Life Technologies) was supplemented with 10% FBS (Gemini Bio-Products, West Sacramento, CA) and 1% Antibiotic:Antimycotic (Gemini Bio-Products). Passage 2 cells were utilized for all experiments.

4.2.3 Experimental Design

The tissue constructs were prepared for placement into the bioreactor utilizing previous procedures.[180] Briefly, MSCs were mixed with 2 mg/mL of collagen type I (BD Biosciences, San Jose, CA) at a density of 1 million cells/mL. 0.6 mL of this

mixture was injected into the HUV and custom stainless steel adapters were attached to seal the interior of the HUV. The construct was then incubated at 37°C to allow for gelation of the collagen. The constructs were then placed into the bioreactor pictured in figure 4.1. These were then cultured for 3 or 7 days.

At this point, mechanical stimulation was applied daily to the constructs according to the specific experimental group described in table 4-1. Regular stimulation was performed at 2% strain for 1 hour/day at 1 cycle/minute meaning the construct was strained and relaxed 1 time per minute.[180] Using this as a starting point, the stimulation frequency was varied under two different conditions: 1 cycle/2 minutes (slow) or 2 cycles/minute (fast). In addition, the duration of the stimulation was also changed; constructs were subjected to either 0.5 hour/day (brief) or 2 hours/day (extended) durations at 1 cycle/minute. The regular stimulation was based upon previous studies which were shown to be beneficial to the HUV/MSC construct. The variations were chosen to create a difference in mechanical stimulation without exposing the construct to too much stimulation, which has been shown to be detrimental to the tendon tissue engineered constructs.[178–180,249,283]

Figure 4.1: Depiction of the bioreactor. The tissue constructs are attached via specialized adapters to the actuator generating the cyclical stretching. Double reservoirs allow for continuous media circulation even during media replacement. Inset: View of the bioreactor containing tissue constructs in triplicate.

	Strain	Frequency	Duration
Static	None	None	None
Regular	2%	1 cycle/minute	1 hour/day
Slow	2%	1 cycle/2 minutes	1 hour/day
Fast	2%	2 cycles/minute	1 hour/day
Brief	2%	1 cycle/minute	0.5 hour/day
Extended	2%	1 cycle/minute	2 hours/day

Table 4-1: Description of stimulation parameters for the various experimental groups.

Controls included static culturing of the construct for 3 or 7 days. This consisted of placing the MSC-seeded construct into the bioreactor, but not subjecting it to any dynamic stimulation. After culturing, constructs were removed from the bioreactors and prepared according to the specific analysis being performed.

4.2.4 Cellularity

A 0.5 cm section was obtained from the top, middle, and bottom of the construct for analysis. These sections were then incubated overnight in collagenase type I (Life Technologies) dissolved in water to digest the ECM. This resulting solution was then sonicated for 15 seconds and subjected to three freeze-thaw cycles to release the cells' DNA content. A Quant-iT™ PicoGreen dsDNA Assay kit (Life Technologies) was utilized to measure the DNA concentration by measuring the DNA/PicoGreen dye mixture's fluorescence at 480/520 nm excitation/emission wavelengths on a Synergy

HT Multi-Mode Microplate Reader. The resulting DNA concentration was then converted to a cell number using the measured DNA content in each cell (7 pg/cell).

4.2.5 Mechanical Analysis

A uniaxial tensile testing frame (Untied Testing Systems, model SSTM-2K, Flint, MI) was used for mechanical testing. Whole constructs were utilized for analysis. Samples were preconditioned for 5 cycles to remove hysteresis and then underwent failure analysis at 1%/s. Extensions and force data were recorded utilizing accompanying software and were used to calculate stress and strain values for the construct. To avoid end effects of the construct, analysis was only conducted on samples that failed away from the end regions.

4.2.6 Histology

0.5 cm sections were fixed in 10% formalin (Azer Scientific, Morgantown, PA) and then embedded in paraffin (VWR, Radnor, PA). The samples were then sectioned into 8 μm slices and mounted onto Fisherbrand Selectfrost slides (Fisher Scientific). The slides were then stained with hematoxylin and eosin (Thermo Scientific) to observe ECM quality and orientation along with cell migration and shape. Lateral sections of the construct were analyzed with ImageJ software and the FibrilTool plug-in to determine the fiber alignment.[303] This was reported in terms of anisotropy of the fibrils where 0 indicates no directional dependence of fibrils while 1 indicates a complete alignment in one direction. Cell penetration distances were also measured with ImageJ.

4.2.7 Gene Expression

Gene expression was measured utilizing real time polymerase chain reaction (RT-PCR). Sections of the construct were homogenized utilizing a tenbroeck tissue grinder (Wheaton, Millville, NJ) and Trizol reagent (Life Technologies). The mRNA was isolated from the homogenized Trizol solution per the manufacturer's instructions. The RNA was then treated with DNase (Life Technologies) prior to reverse transcription. Reverse transcription to DNA was performed utilizing a RNA-to-cDNA kit (Life Technologies) and Mastercycler ep realplex4 (Eppendorf, Hauppauge, NY). The resulting DNA was then amplified utilizing SYBR Green PCR Master Mix (Life Technologies) and specific genes were detected utilizing the primers (Integrated DNA Technologies, Coralville, IA) listed in table 2.[236,304–310] Genes in the various experimental groups were normalized utilizing the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and comparison between controls and the experimental groups were done utilizing the $2^{-\Delta\Delta Ct}$ method of comparing experimental gene targets to the GAPDH housekeeping gene (ΔC_t) and then comparing that change to a control experiment ($\Delta\Delta C_t$).[311]

	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
GAPDH	CCATTCTTCCACCTTTGATGCT	TGTTGCTGTAGCCATATTCATTGT
Biglycan	CTGAGGGAACTTCACTTGGA	CAGATAGACAACCTGGAGGAG
Decorin	TGGCAGTCTGGCTAATGT	ACTCACGGCAGTGTAGGA
COMP	GTTTCCCGGACGAGAAGCTT	ATCCTCCTGCCCTGAATTGG
Collagen Type I	ATCAGCCCAAACCCCAAGGAGA	CGCAGGAAGGTCAGCTGGATAG
Collagen Type III	AGGCTTTGATGGACGCAATG	GCGGCTCCAGGAAGACC
MMP-3	TCCCAGGAAAATAGCTGAGAACTT	GAAACCCAAATGCTTCAAAGACA
Elastin	TAAATACGGAGCAGCAGGTG	GCACCATATTTGGCAGCCTTAG
Scleraxis	CGAAGTTAGAAGGAGGAGGGT	CGCTCAGATCAGGTCCAAAG
Tenascin C	GCTACTCCAGACGGTTTC	TTCCACGGCTTATTCCAT
Tenomodulin	GGACTTTGAGGAGGATGG	CGCTTGCTTGTCTGGTGC
PPAR-γ	CGGTTGATTTCTCCAGCATT	AGCAAGGCACTTCTGAAACC
Osterix	CTTTCCCCACTCATTTCCTG	CTAGGCAGGCAGTCAGAAG
Collagen II	CTCCAGGTGTGAAGGGTGAG	GAACCTTGAGCACCTTCAGG

Table 4-2: Primers for gene expression analysis utilizing RT-PCR.

4.2.8 Statistical Analysis

For all analysis, at least three tissue constructs were utilized ($n \geq 3$). For each construct, a sample from the top, middle, and bottom of the tissue was collected to determine a representative sample. All results are reported as mean ± standard deviation. Statistical significance was determined by applying two-way ANOVA methods with Bonferroni post tests to determine significance between individual groups and controls as appropriate.

4.3 Results

4.3.1 Cellularity

Figure 4.2 shows the cellularity of the MSC/HUV constructs after 7 days of culturing for the stimulation frequency and duration changes. The dashed line indicates the initial cell seeding density of 600,000 cells per construct. Prior research has shown that the decellularization processes utilized does remove all existing cells, therefore, no additional contribution in cellularity is from the HUV itself.[178,179]

In most cases for the MSC/HUV construct, there was a significant increase in cell number compared to static controls, with the exception of the fast frequency (4.26 $\pm$ 0.32 million cells/construct) and the extended duration (3.98 $\pm$ 0.39 million cells/construct). However, there was no significant difference between the various stimulation groups. The regular frequency of stimulation was shown to have the greatest increase in the number of cells (figure 4.2a) with 5.98 $\pm$ 1.04 million cells/construct, compared to the static culture, which only had 2.21 $\pm$ 0.41 million cells/construct, a 170% increase. The brief duration (figure 4.2b) was the best group investigated in terms of cellularity, with 6.71 $\pm$ 0.41 million cells/construct, a 300% increase.

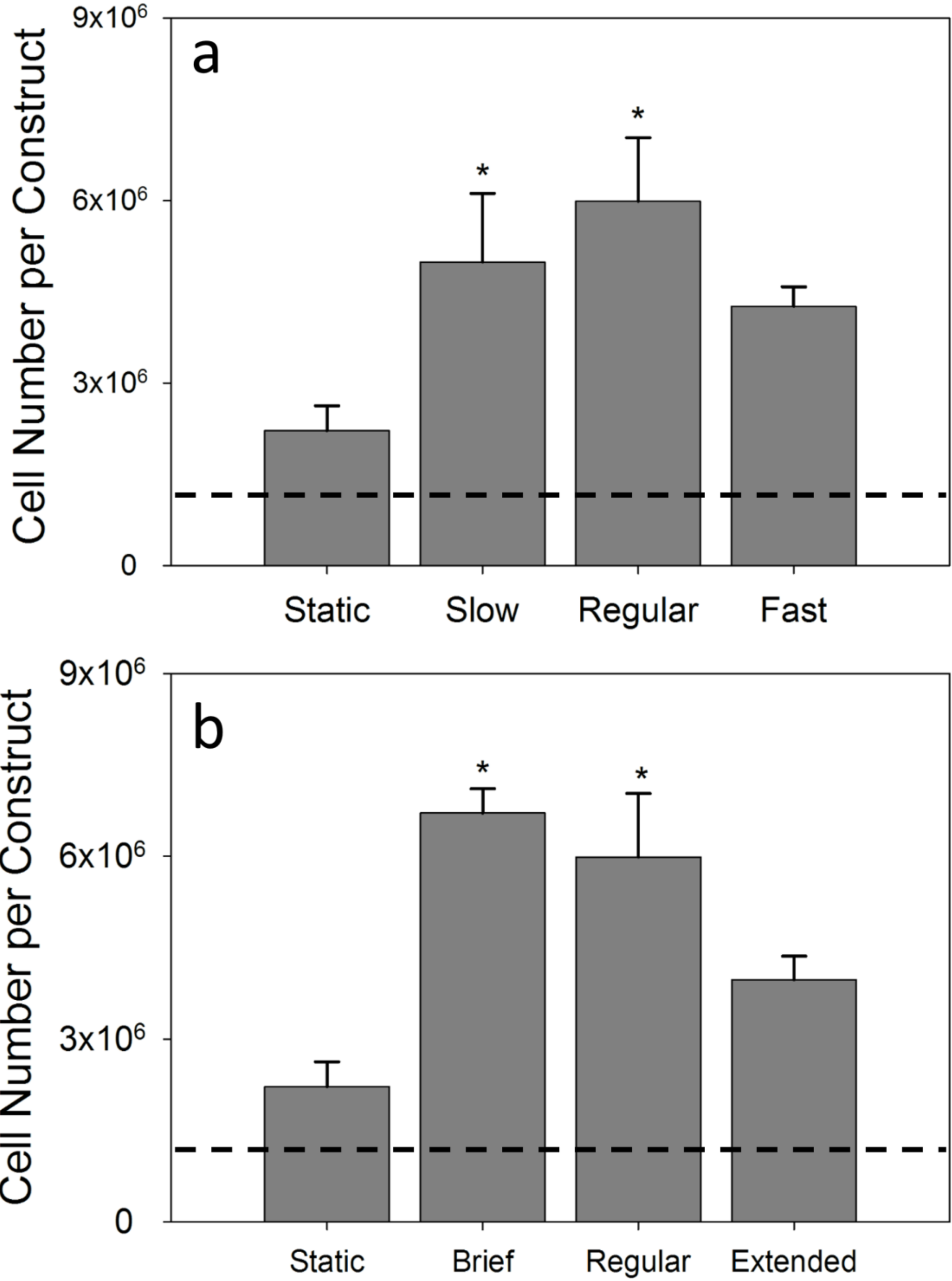


Figure 4.2: (a) Cellularity of the HUV/MSC construct as a function of the (a) frequency or the duration (b) of the stimulation. * indicates $p < 0.05$ compared to the static control. Data represented as mean ± standard deviation. A sample size of $n \geq 3$ was used. Dashed line indicates initial seeding density of 600,000 cells/construct.

4.3.2 Mechanical Analysis

Mechanical testing at 7 days (not pictured) revealed that the various stimulation groups did not result in significant changes compared to the static group. In addition, varying the type of stimulation also did not have a significant effect. Values ranged from 1.06 ± 0.34 MPa, which was the fast group to 1.58 ± 0.35 MPa, corresponding to the slow group. The ultimate tensile strengths were similar to values reported previously for 1 week stimulation of the MSC/HUV construct.[179]

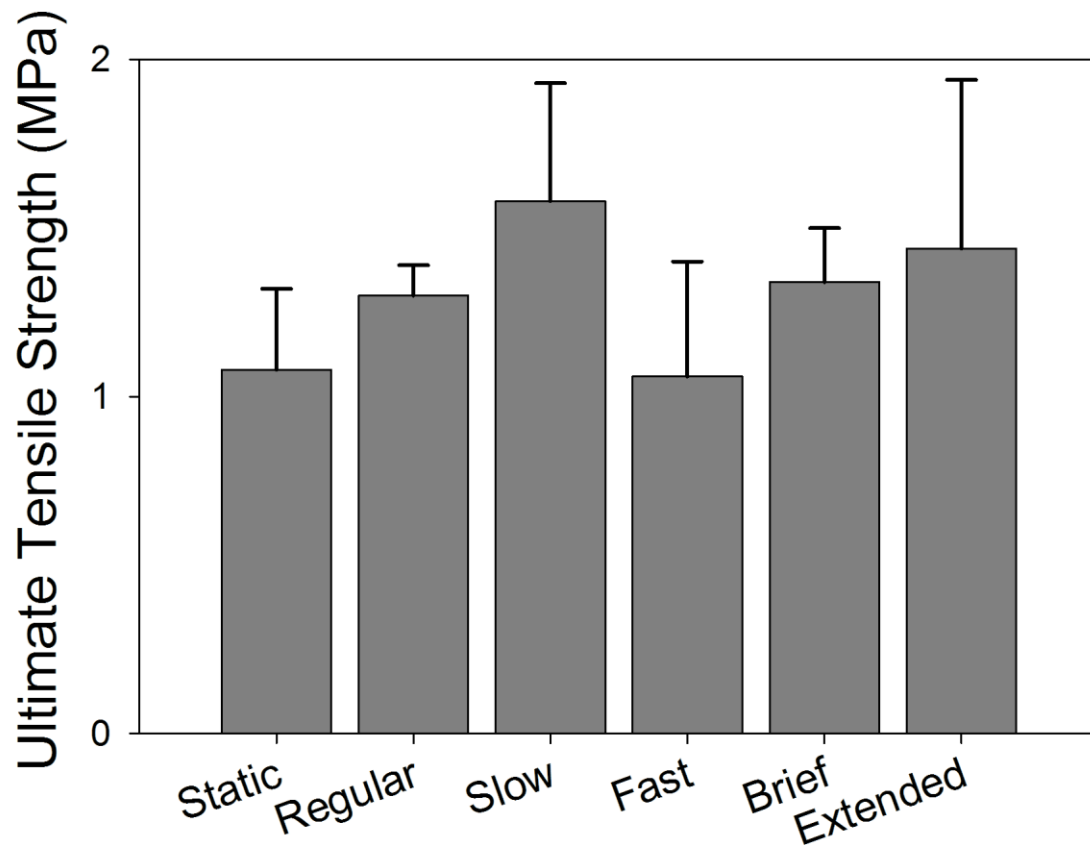


Figure 4.3: Ultimate Tensile Strengths for the various experimental groups after 7 days of culture. Data represented as mean ± standard deviation. A sample size of n ≥ 3 was used.

4.3.3 Histology

Figure 4.4 shows lateral sections of the HUV after 7 days, demonstrating the alignment, or lack thereof, of the ECM fibers with the direction of stretching, indicated by the arrow. In figure 4.2a, the fast frequency was shown to have a non-significant increase in cell number compared to static controls, and in figure 4.4d, the fibrils are disorganized and random, similar to the static control. The other stimulation groups demonstrated fiber alignment in the direction of stretching as indicated in table 4-3. Cross sections of the construct are given in figure 4.5. Cells in all cases are primarily located in the interior wall of the constructs, with some migration into the walls, reaching a maximum distance of about 29% of the wall thickness (table 4-4).

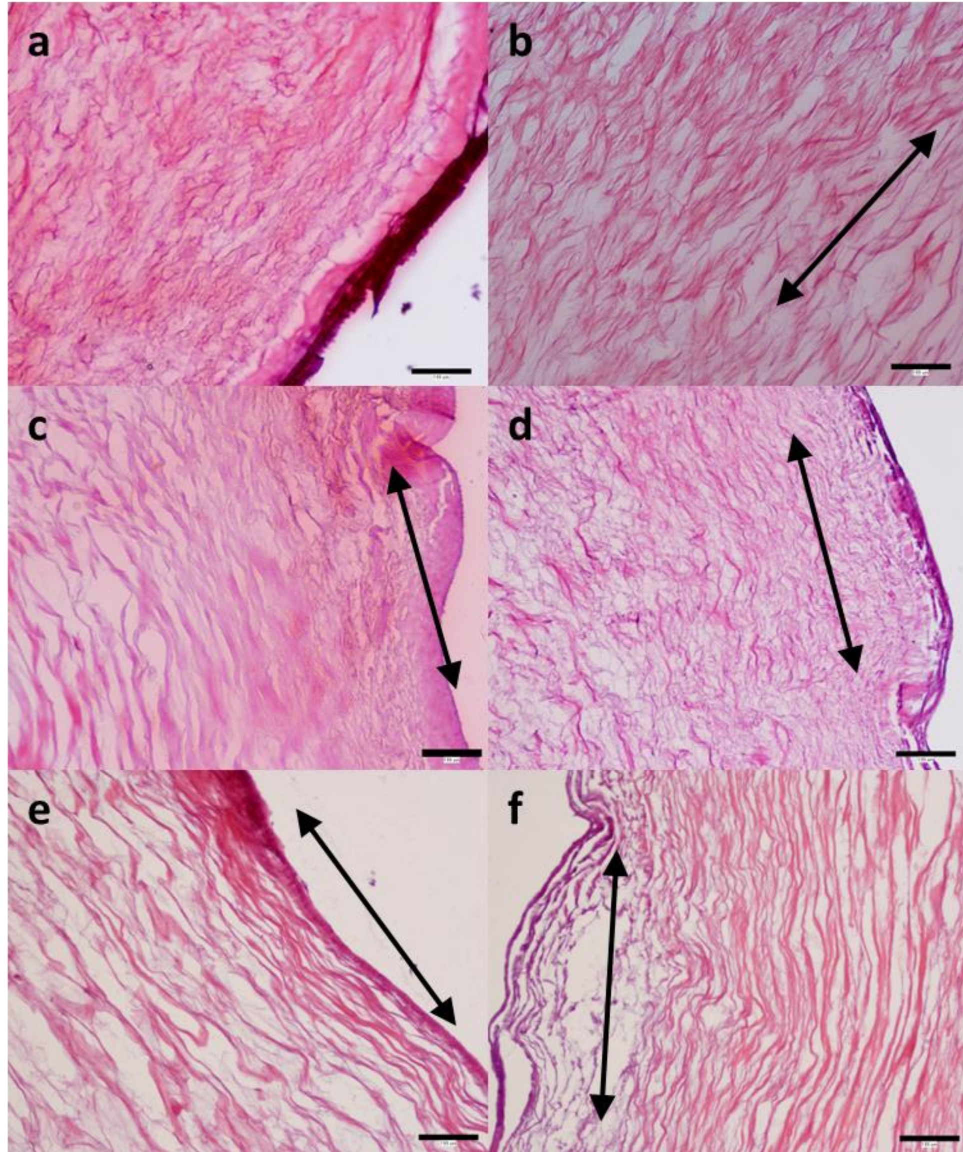


Figure 4.4: Histological longitudinal sections of the construct after 7 days for (a) static control (b) regular stimulation, (c) slow frequency, (d) fast frequency, (e) brief duration, and (f) extended duration. Arrows indicate direction of mechanical stretching. Scale bar = 200 μm.

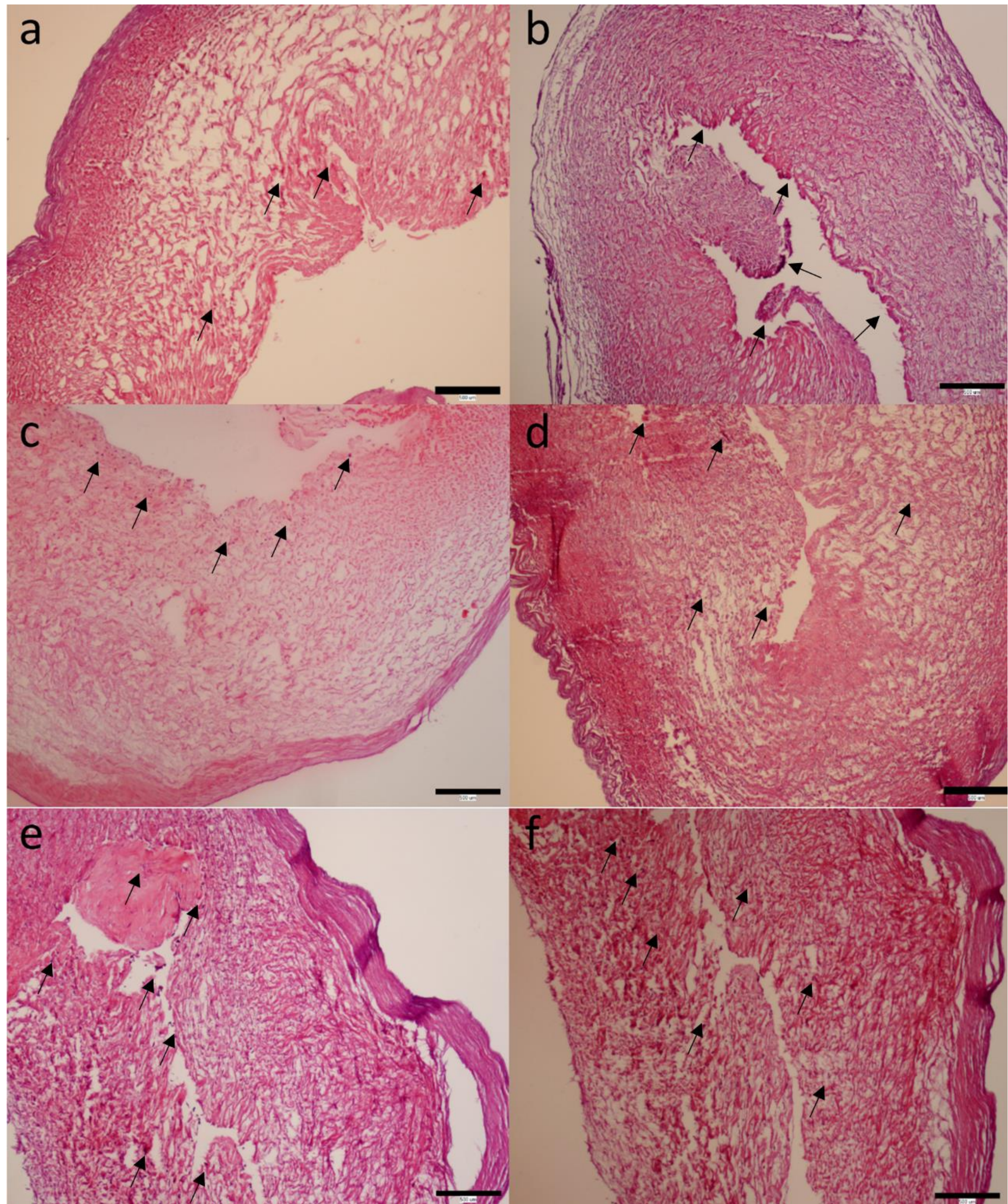


Figure 4.5: Histological cross-sections of the construct after 7 days for (a) static control (b) regular stimulation, (c) slow frequency, (d) fast frequency, (e) brief duration, and (f) extended duration. Scale bar = 200 μm. Arrows indicate cells within the scaffold.

	Average	Std. Dev
Static	0.20	0.01
Regular	0.44	0.01
Slow	0.39	0.04
Fast	0.19	0.01
Brief	0.44	0.02
Extended	0.46	0.07

Table 4-3: Anisotropy values for the lateral histological sections of the frequency and duration groups.

	Average	Std. Dev
Static	24%	5%
Regular	16%	4%
Slow	13%	4%
Fast	29%	10%
Brief	29%	8%
Extended	29%	14%

Table 4-4: Maximum cell penetration depth as determined by histological cross sections of the frequency and duration groups.

4.3.4 Gene Expression

Figures 4.6-4.9 show gene expression relative to a 3 and 7 day static control, utilizing the $2^{-\Delta\Delta Ct}$ method. The star indicates a significant difference to the static control. Figure 4.6a shows significant biglycan upregulation in all stimulation situations except the regular stimulation, while an extended duration produced downregulation of decorin. Cartilage oligomeric matrix protein (COMP) was relatively unaffected by various frequencies and durations of stimulation. Figure 4.7a shows that collagen production (both types I and III) was significantly upregulated (up to 10 fold magnitudes) in all situations, once again, with the exception of regular stimulation. Matrix metalloproteinase-3 (MMP-3) did not show any significant increases, and in all cases, elastin was downregulated or at similar levels to a static control. Tendon marker expression is shown in figure 4.8a and the only stimulations that significantly

upregulates gene expression is the regular and slow frequencies for tenascin C, at a 1.6- and 2.23-fold level respectively.

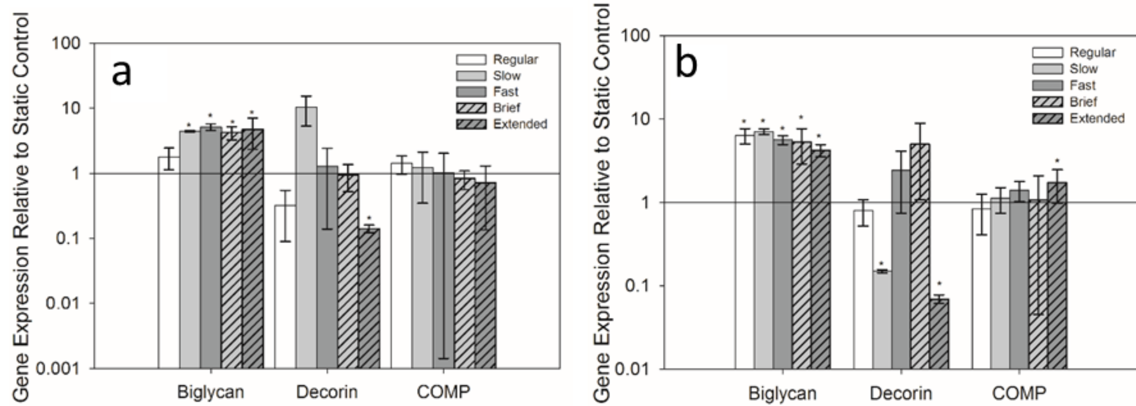


Figure 4.6: RT-PCR results for tendon-related ECM proteins at (a) 3 days culture time and (b) 7 days culture time compared to a static MSC/HUV construct control. * indicates $p < 0.05$ between experimental group and the control. Data represented as mean ± standard deviation. A sample size of $n \geq 3$ was used.

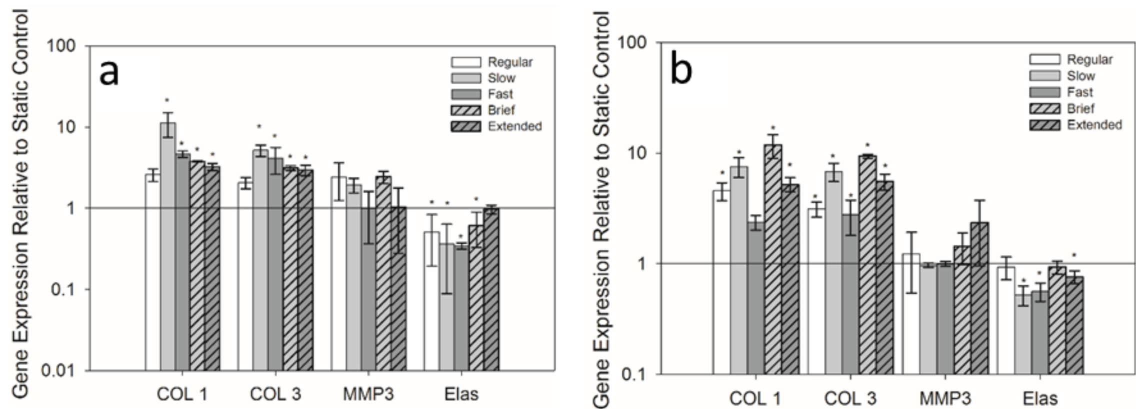


Figure 4.7: RT-PCR results for tendon-related ECM components at (a) 3 days culture time and (b) 7 days culture time compared to a static MSC/HUV construct control. * indicates $p < 0.05$ between experimental group and the control. Data represented as mean ± standard deviation. A sample size of $n \geq 3$ was used.

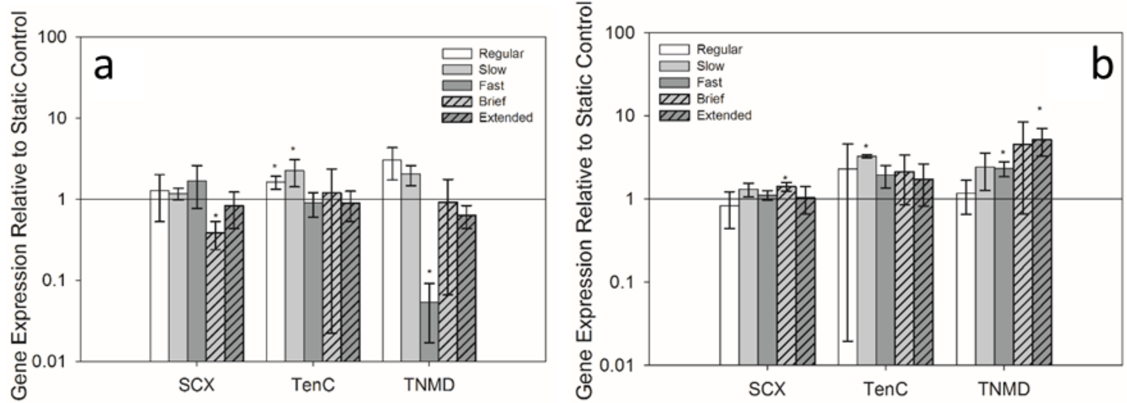


Figure 4.8: RT-PCR results for tendon-related differentiation markers at (a) 3 days culture time and (b) 7 days culture time compared to a static MSC/HUV construct control. * indicates $p < 0.05$ between experimental group and the control. Data represented as mean ± standard deviation. A sample size of $n \geq 3$ was used.

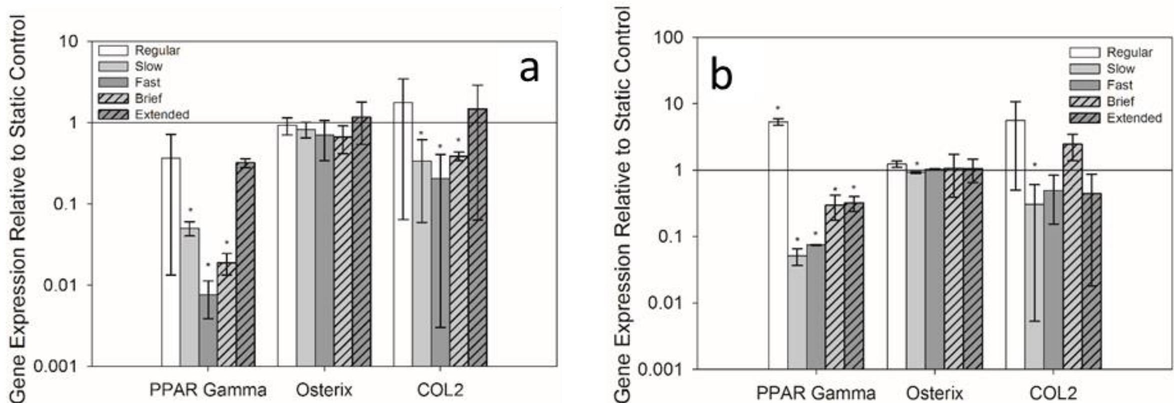


Figure 4.9: RT-PCR results for non-tendon related differentiation markers at (a) 3 days culture time and (b) 7 days culture time compared to a static MSC/HUV construct control. * indicates $p < 0.05$ between experimental group and the control. Data represented as mean ± standard deviation. A sample size of $n \geq 3$ was used.

Fast stimulations resulted in a significant decrease (95% decrease) of tenomodulin compared to the static control. Figure 4.9a demonstrates a mixture of upregulation and down regulation. Peroxisome proliferator-activated receptor gamma (PPAR-γ) and collagen type II both saw significant downregulation for 3 days for both frequency variations and the brief stimulation.

Similar upregulation profiles of biglycan are seen in figure 4.6b, compared to the 3 day results. In this case, all stimulations significantly upregulate biglycan expression. However, for decorin and COMP, the observed upregulation for the various stimulations is not statistically significant (with the exception of the extended duration), while slow frequencies and extended durations produce significant downregulation of decorin. The same trend continues between 3 and 7 days for collagen production. Figure 4.7b shows significant upregulation of collagen for all groups, with the exception of the fast frequency's effect on collagen type I. Once again, MMP-3 was near control levels and elastin was downregulated in all cases, significantly for slow and fast frequencies and extended stimulation durations. However, compared to 3 days, more significant upregulation of tendon-specific markers was seen (figure 4.8b). There was a significant increase in tenascin C for the slow frequency. Significant upregulation was also seen in scleraxis with a brief stimulation and also tenomodulin in the case of fast frequencies and an extended duration. Figure 4.9b shows that no non-tendon markers were significantly upregulated except for the regular stimulation, which significantly upregulated PPAR-γ expression. Interestingly, the slow frequency was the only stimulation to significantly downregulate all non-tendon markers at 7 days when compared to the static control.

4.4 Discussion

There are many variables in terms of providing mechanical stimulation via stretching to a tissue-engineered construct, which include frequency, duration, and strain. In this study we wanted to determine the sensitivity of the construct properties to frequency and duration changes from our previous studies, characterized by the regular stimulation present in this study. By assessing the cellular proliferation rate, ECM quality via histology, mechanical properties, and gene expression of the cells present in the construct, the best stimulation settings used in this study are determined.

It has been shown that mechanical stimulation has beneficial effects in many cases with regard to cellular proliferation rates.[247] However, in some of the experimental groups there are no significant benefits. In figure 4.2a, both slow and regular frequencies of stimulation showed significant increases in cellular proliferation compared to the static controls with 125% and 170% increases compared to the static group respectively. Comparatively, there is no significant change between the static and fast frequency groups. An explanation is that the stimulation is beyond the acceptable level for cells that are trying to adapt to their new microenvironment. Another explanation for the decreased proliferation for the fast frequency group could be due to early differentiation. As MSCs differentiate, cell proliferation typically slows down.[312] It has also been seen that MSCs can begin tenocytic differentiation after 7 days after providing mechanical stimulation.[235] In addition, figure 4.7c shows that tenomodulin, a later-term tendon marker is significantly increased at 7 days compared to the static MSC control group demonstrating a potential commitment to tenocytic differentiation.[62]

Figure 4.2b demonstrates a similar relationship with duration. In addition to the regular stimulation mentioned previously, the brief duration provided a significant increase in cellularity when compared to the static control with a 203% increase. However, the extended duration did not have as large of an effect on cellular proliferation. This agrees with other studies that greater than one hour of stimulation can limit the beneficial effects of stimulation for MSCs.[247] This may be due to differentiation, as was the case with the fast frequency, as after 7 days, compared to normal MSCs, a significant increase in tenomodulin gene expression was seen in the extended duration. The only other significant group to upregulate this gene was the fast frequency. Another explanation is that the cells are undergoing apoptosis. It has been demonstrated that cell death increases in a time-dependent manner for up to 6 hours with stretching immediately following the stimulation in periodontal ligament cells.[313]

The quality of fiber alignment appeared to be correlated with cellularity after 7 days of culture, according to figures 4.2 and 4.4. Significant increases in cellularity, as was the case with the regular, slow, and brief stimulations, resulted in ECM fibers aligned in the directions of stretching. These aligned fibrils also appeared thicker. However, staining on the static control and fast frequency groups showed a more random orientation of fibers. In addition, this fast frequency did not have a significant increase in cellularity compared to the static control. This could be explained by the expression of collagens type I and III, shown in figure 4.7b. Although MMP-3 activity was not significantly changed by the various experimental groups compared to a static control, collagen type I and III expression was increased, with the exception of collagen type I for fast stimulation frequency after 7 days. Taken together, it appeared that the

slow, regular, and brief groups were producing more mature type I collagen, with fibrils aligned in the direction of stretching, due to the increased cellularity. In the case of the fast frequency, ECM production was of lower quality, and the cells demonstrated significant increase in collagen type III expression compared to controls while collagen type I was not affected. In healing and developing tendons, collagen type III often precludes collagen type I, indicating that the fast frequency may stimulate more immature ECM compared to the other groups.[314] Although the extended duration did produce thicker and aligned fibers in the direction of stretching like the other groups, it did not have a significant increase in cellularity. This may be due in part to the significant increase in COMP gene expression after 7 days compared to static controls while no other experimental groups showed a significant increase. COMP is thought to catalyze collagen fibrillogenesis and assist in ECM organization.[315] This higher expression could possibly allow for the higher quality ECM without the higher cellular proliferation. Looking at cross-sections of the tissues in figure 4.5, cell penetration from the interior remains in the inner third to half of the construct. This is in agreement with previous studies, where cell migration was not able to fully penetrate the HUV scaffold until 14 days of culture.[178] Even though ECM quality did differ based on histology, this change is not represented by the ultimate tensile strength which was not significantly increased in any stimulation group. It is possible that after only 7 days, not enough time has passed to allow for significant variations in mechanical properties due to stimulation changes.

In addition to collagen, other gene expression results also correlated with increased ECM production with mechanical stimulation. With the exception of the

regular stimulation at 3 days, biglycan expression was seen to be significantly increased for all groups and time points when compared to the static control. Biglycan is a small leucine-rich proteoglycan found in many different tissues, it has a significant presence in tendons with spatial variances within the tendon itself.[316] It provides structures and function to the tendon. Decorin is another proteoglycan that serves similar functions to biglycan.[317] However, it is not upregulated in any case, and during the 3 day extended and 7 day slow and extended groups, is actually downregulated as seen by figures 4.6a and 4.6b. This can be explained by the presence of Wharton's jelly matrix on the interior of the scaffold, as this ECM is rich in decorin compared to biglycan.[318] Therefore, the cells seeded on the scaffold are possibly synthesizing more biglycan to make up for this deficit as they create and remodel ECM.

In addition to those ECM components, elastin also possessed an overall trend regardless of experimental group or time point. However, in this case, it was a decrease compared to static MSC controls as seen in figures 4.7a and 4.7b. Elastin is present in tendons to provide its elastic properties and would typically be produced by cells. However, it is only present in about 0.8% of the tendon.[319] This is in comparison to the elastin content of the HUV, which is on the order of 8%.[320] As the elastin content of the vein is 10 times higher than a tendon, the cells do not have a need to produce much elastin for the ECM and their gene expression is reduced.

In addition to cellular proliferation and ECM production, mechanical stretching also provides cues to promote tenocytic differentiation. When investigating tenocyte markers as an indication of potential tenocytic differentiation, each experimental group saw a significant increase of one marker within 7 days. One common early tenocytic

marker is tenascin-C, a glycoprotein present in high levels in regenerating and developing tendons and less so in mature, healthy tendons.[3] After 3 days, the slow and regular stimulations show a significant increase in tenascin-C expression compared to static MSC experiments. After 7 days, only the slow stimulation increases its expression of this gene. Scleraxis is another early tendon markers. It is a transcription factor found in developing tendons during embryogenesis.[321] Scleraxis was shown to be upregulated compared to the static control after 7 days in the brief stimulation. This is in contrast to scleraxis being significantly downregulated after 3 days with the brief stimulation. Finally, tenomodulin is a glycoprotein that is responsible for regulation of proliferation and development of tendon fibrils, along with being a later-term tendon development marker as was mentioned previously.[50,62] Only the extended and fast stimulations after 7 days showed a significant increase in expression compared to static controls. As previously mentioned, these two groups were the only groups to not see a significant increase in cell growth, implying a possibly more tendon-committed cell type being present in these two stimulations stunting cellular proliferation.

Non-tendon genes were also investigated to determine if any other musculoskeletal lineages may be present within the MSCs. PPAR-γ was chosen to represent adipocytes, osterix for osteoblasts, and collagen type II for chondrocytes.[276,322,323] Figure 4.9a shows that expression levels for all genes and stimulations were up and downregulated to various degrees, indicating a lack of committed lineages and a potentially still immature MSC phenotype. However, after 7 days, as seen in figure 4.9b, most of the expression levels were either at baseline or downregulated, with significant decreases for PPAR-γ for most stimulations, with the

exception of a significant increase in expression for regular stimulation. Although these expression profiles do not prove tenocytic lineage, they demonstrate a tendency for the MSCs to move away from adipogenic, osteogenic, and chondrocytic characteristics. This was especially true for the slow stimulation which significantly downregulated all three genes.

4.5 Conclusions

Varying the frequency and duration of stimulation did cause significant effects on the overall construct. 2 hours/day and 2 cycles/minute stretching slowed cellular proliferation, while the other stimulations all improved cell growth compared to no stretching. This cell growth also was shown to directly contribute to ECM quality in terms of fibril alignment and diameter. Gene expression analysis showed that the MSCs were remodeling the ECM to have more tendon-like qualities such as biglycan and elastin content compared to HUVs. Tendon and non-tendon markers demonstrated little to no potential differentiation after 3 days of the MSCs, however at 7 days, most stimulations upregulated various tenocytic markers while inhibiting non-tendon phenotypes. Overall, shorter durations and slower frequencies such as the 0.5 hour/day and 0.5 cycles/min stimulations, allowed for increased cellular proliferation while maintaining the ability to promote tenocytic differentiation at 7 days.

Chapter 5: Improvement of a Tissue-engineered Tendon Construct by Supplementation with Mature Tencoytic Extract

5.1 Introduction

As discussed previously, typically in tendon tissue engineering mechanical stimulation is utilized in the form of cyclical stretching. This has proven to improve cellularity, construct quality, and mechanical properties.[179,180] In addition, there has been some investigation into the use of growth factors as an additional stimulation mechanism for cell development and differentiation into a tenocytic lineage. Some of the growth factors include FGF-2, GDF-5, GDF-6, and GDF-7 among others.[200,263,267,289,290]

However, there is not one definitive growth factor or mixture of growth factors found that is directly responsible for tenocytic differentiation of stem cells, such as BMP-2 for osteogenesis.[324] As an alternative to supplementing with one or a few specific growth factors, we propose the use of extract from mature tenocytes. Extract is the internal proteins and genetic material found inside the cell.[325,326] These molecules can be released into solution by lysing the mature cell and releasing the cell internals into solutoin.

Cell extract has been shown to possess growth factors stored within the cell in different cell types.[325,327] It also possesses nucleic acids and transcription factors contained within the cell.[326,328] All of these molecules can potentially affect stem cell differentiation. Previously, stem cells have been differentiated by being exposed to extract from cardiomyocytes and pneumocytes.[326,329] By supplementing the MSCs with tendon extract, it is hypothesized that cell fate can be influenced at an early stage, either

through interactions of the growth factors or incorporation of the transcription factors and genetic material. This extract could be obtained from the patient prior to tendon repair with the tissue-engineered tendon, as it has been shown that tendon biopsies can be performed with minimal donor site morbidity.[330]

This study aims to show that mature tendon cells should possess the proteins and genes necessary to influence stem cell development into a tendon lineage. By applying these molecules to the stem cell culture, it is hypothesized that they should significant increases cell growth and differentiation and construct properties, in addition to the benefits of mechanical stimulation.

5.2 Materials and Methods

5.2.1 MSC and Tenocyte Extraction

MSCs were obtained utilizing similar techniques as in chapter 4. During bone marrow MSC isolation, the Achilles tendon of the rats was also harvested to obtain mature tenocytes. This tendon was then digested overnight with 200 U/mL of Collagenase Type I (Life Technologies) dissolved in α-MEM (Life Technologies) supplemented with 10% fetal bovine serum and 100 U/mL antibiotic:antimicotic (Gemini Bio-Products, West Sacramento, CA). The solution was then transferred to 25 cm^2 flasks (Fisher Scientific, Hampton, NH) for further cell culture. After 80% confluency (between 1-2 weeks), cells were lifted and further passages were cultured on 75 cm^2 flasks until ready for use.

5.2.2 Scaffold Preparation

Scaffolds were prepared similarly to chapter 4. Figure 5.1a shows the vein being extracted utilizing the mechanical lathe and the seeded, decellularized HUV prior to bioreactor culture is pictured in figure 5.1b.

Figure 5.1: a) A partially lathed umbilical cord exposing the HUV. b) Image of an HUV seeded with collagen gel/MSCs. c) Constructs cultured within the bioreactor.

5.2.3 Extract Preparation

Confluent tenocytes (or up to 90% confluency) were used to obtain the tenocytic extract solution. The process followed modified previous protocols.[326] Cells were lifted from the flask utilizing 0.25% trypsin-EDTA (Life Technologies, Grand Island, NY) and spun down in α-MEM to neutralize and remove the trypsin. The resulting cell pellet was then washed twice with cell lysis buffer. This buffer consisted of 50 mM NaCl, 5 mM $MgCl_2$, 20 mM Hepes, and 1 mM dithiothreitol at a pH of 8.2 (Sigma Aldrich, St. Louis, MO).[326] After washing, cells were lysed in the buffer with liquid nitrogen snap freezing and sonication. The resulting solution was then centrifuged and the supernatant was transferred to a new centrifuge tube to remove cellular debris. A

Bradford assay was performed and a protein concentration of 38.2 ± 4.6 mg/mL was used for supplementation.

5.2.4 Experimental Design

The tissue constructs were prepared for placement into the bioreactor based previous procedures along with addition of tenocytic extract.[180] MSCs were mixed with 2 mg/mL of collagen type I at a density of 3 million cells/mL. Extract was added to this solution to obtain a concentration of 16.8 mg/mL according to a previous protocol.[326] 0.6 mL of this mixture was injected into the interior of the HUV and incubated at 37°C for two hours to allow for setting up of the collagen gel. The constructs were then placed into the bioreactor pictured in figure 1c. Within the bioreactor, constructs were subjected to either a static culture or cyclical stretching. This stretching was a 2% strain for 0.5 hours/day at 0.5 cycle/min. Culture times were for 7 and 14 days.

Experimental controls included MSC-seeded constructs without the extract supplementation and mature tenocytes cultured with the HUV. The constructs were subjected to the same culture times and stimulations as the extract-supplemented groups.

5.2.5 Cellularity

Cellularity analysis was done as described in chapter 4.

5.2.6 Mechanical Analysis

Mechanical analysis was done as described in chapter 4.

5.2.7 Histology

Histology was performed in the same manner as chapter 4.

5.2.8 Gene Expression

RNA and DNA were isolated and reverse transcribed respectively utilizing the same methods as chapter 4. The genes analyzed for this study were: GAPDH (housekeeping gene), collagen type I, collagen type III, elastin, scleraxis, COMP, and tenomodulin. These primers had the same sequences as in chapter 4. RT-PCR and cell preparation was also done the same as in chapter 4. The analysis was also done utilizing the $2^{-\Delta\Delta Ct}$ method with both tenocyte and non-supplemented MSC constructs as comparisons.[311]

5.2.9 Statistical Analysis

At least 3 samples were performed for each analysis. To get a representative sample, sections from the top, bottom, and middle of the constructs were analyzed. All results are reported as mean $\pm$ standard deviation. Statistical significance was determined by applying two-way ANOVA with Bonferroni post tests to determine significance between individual groups and controls as appropriate. A $p < 0.05$ was used to determine significance.

5.3 Results

5.3.1 Cellularity

As seen in figure 5.2, cell growth was significantly higher after 14 days for both types of MSC cultures when stimulated mechanically. After 14 days, MSCs not cultured with extract increased by 503%. This construct had 10.86 ± 1.12 million cells, a 212% increase over the 7 day dynamic culture (3.48 ± 0.19 million cells) and a 74% increase over the static 14 day culture (6.23 ± 2.21 million cells). Meanwhile, HUVs seeded with MSCs and supplemented with extract possessed 14.84 ± 1.36 million cells after 14 days dynamic culture, a 127% increase from the 7 day dynamic culture (6.52 ± 0.36 million cells). In addition, the extract supplemented experimental group possessed significantly higher cell numbers when cultured dynamically compared to the non-supplemented groups. Tenocytes cultured in the HUV did not show significant changes from 7 to 14 days or if cultured with stimulation, actually decreasing from 6.43 ± 0.42 to 5.94 ± 2.06 million cells in 7 to 14 day dynamic cultures. After 14 days of dynamic culture, both of the MSC experimental groups had a significantly higher amount of cells compared to the tenocyte group.

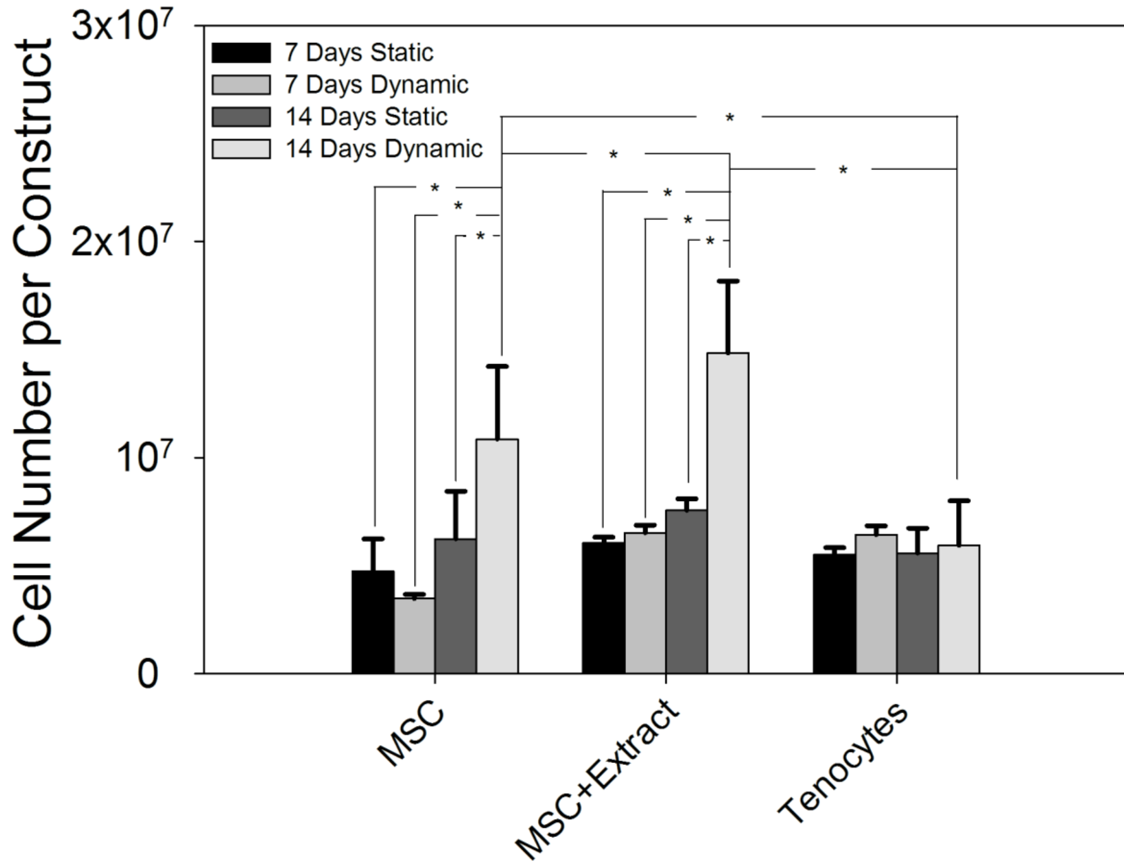


Figure 5.2: Cell density of experimental construct groups. * indicated $p < 0.05$ between indicated groups. Data represented as mean ± standard deviation. A sample size of $n \geq 3$ was used.

5.3.2 Histology

Figure 5.3 presents cross section views of the HUV construct during static culture. Figures 5.3a, 5.3c, and 5.3e show that the cells remain on the inner portion of the tissue where they were seeded after 7 days of culture for non-supplemented, extract supplemented, and tenocytic groups respectively. After 14 days of culture, the cells have shown to migrate through the construct towards the outer walls, penetrating the tissue (figures 5.3b, 5.3d, 5.3f) to varying degrees given in table 5-2.

Figure 5.4 shows the quality and alignment of the ECM of the statically cultured constructs and table 5-1 quantifies the fibril alignment in terms of anisotropy. The non-

supplemented group after 7 days (figure 5.4a) and 14 days (figure 5.4b) had little to no alignment of the fibers in the direction of stretching. And although the fibrils after 14 days do appear thicker, large amounts of void space are still present. Figures 5.4c and 5.4d show the extract supplemented groups after 7 and 14 days of static culture respectively. These groups did have increased amounts of ECM present, and after 14 days, some alignment was present, more so than the non-supplemented groups. However, the random structure was still present. The tenocyte static cultures are depicted in figures 5.4e (7 days) and 5.4f (14 days). These constructs had thinner fibrils than the supplemented group, however, alignment in the direction of stretching was greater than the non-supplemented group and similar to the supplemented group after 14 days.

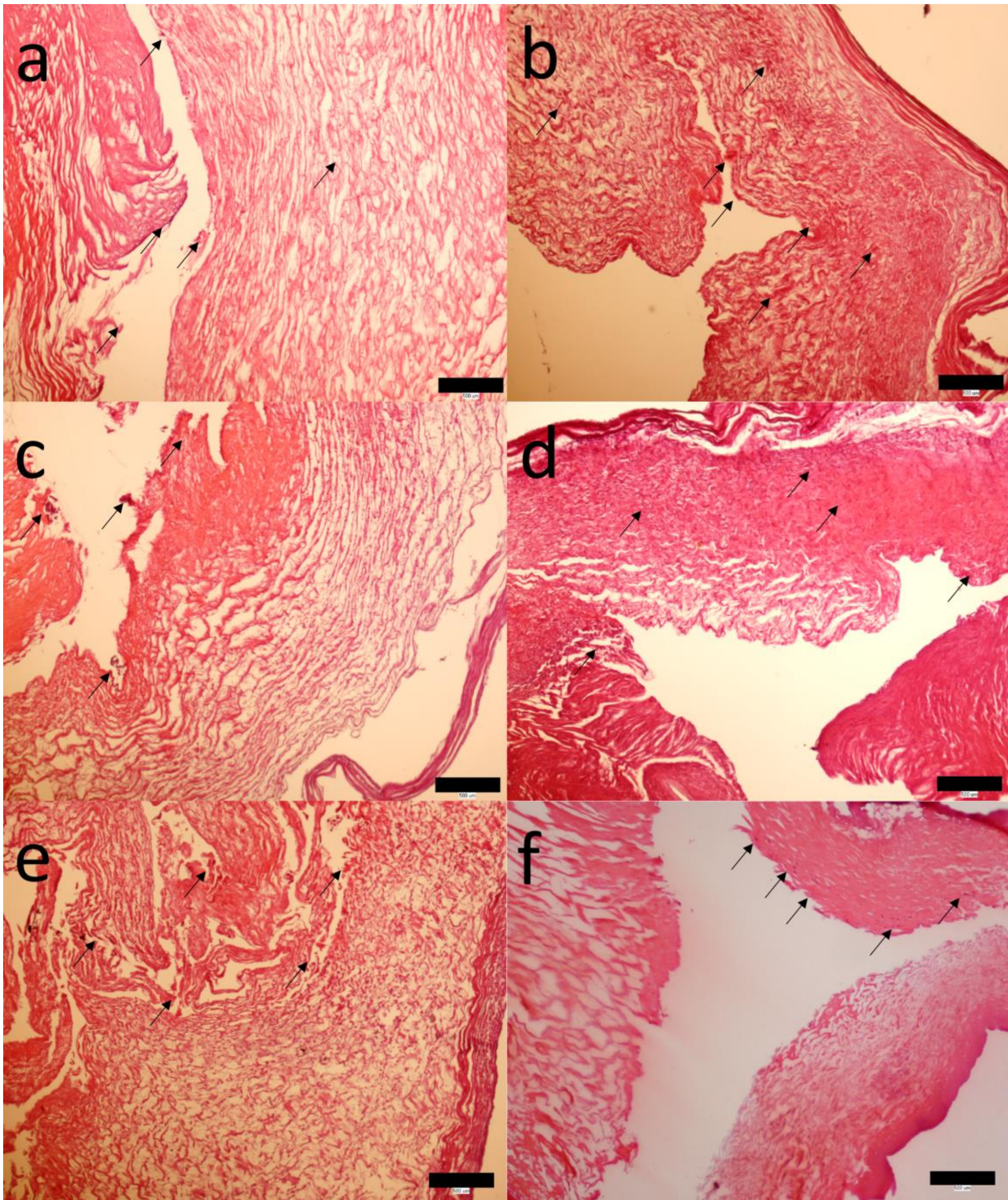


Figure 5.3: Cross-sectional views of the tissue samples at 4x magnification for static constructs. Scale bar is 500 μm. a – 7 days non-supplemented controls, b - 14 days non-supplemented controls, c - 7 days extract supplemented controls, d - 14 days extract supplemented controls, e – 7 day tenocyte culture, f – 14 day tenocyte culture. Arrows indicate cells within the scaffold.

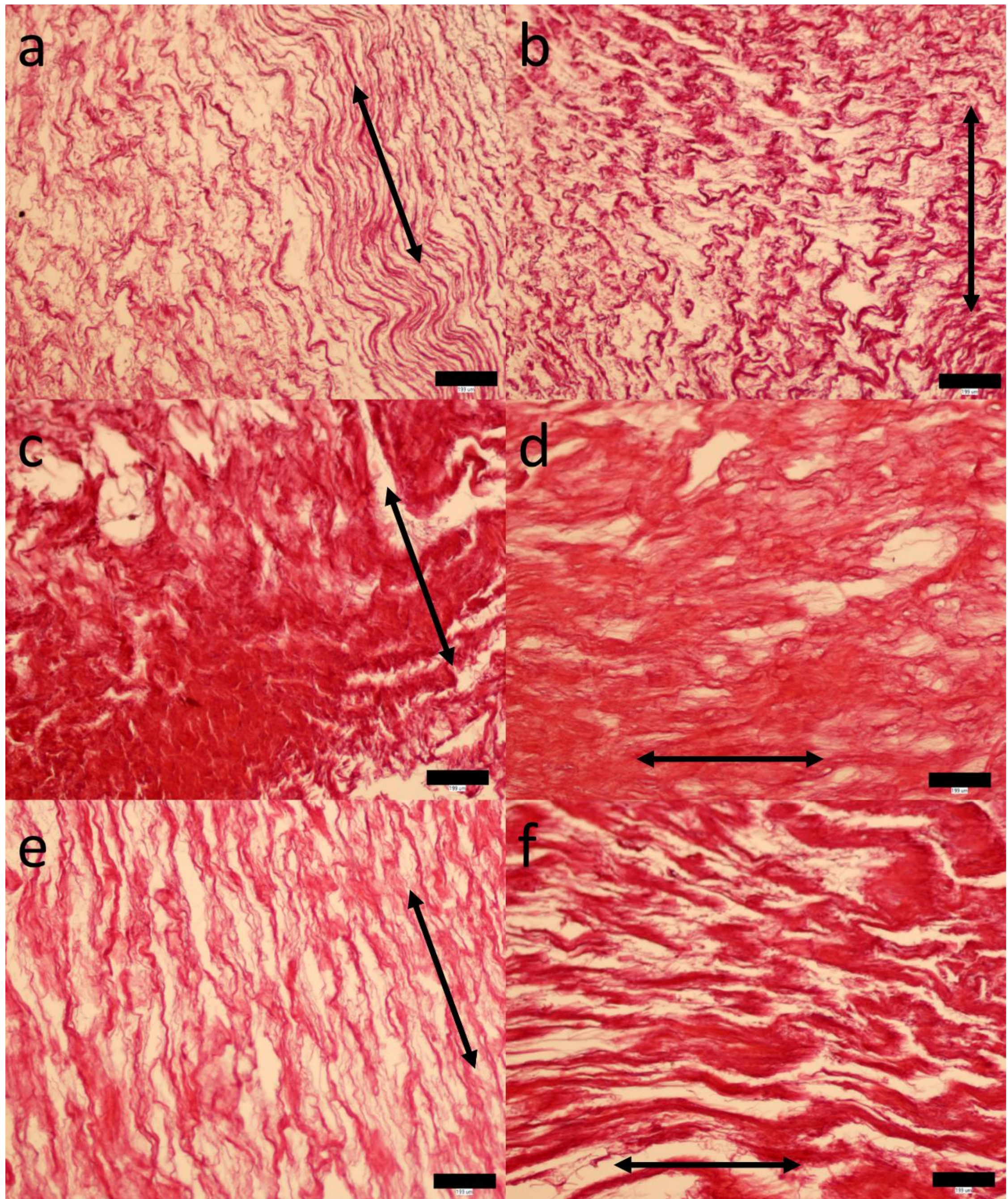


Figure 5.4: Lateral section views of the tissue samples at 10x magnification for static constructs. Scale bar is 200 μm. a – 7 days non-supplemented controls, b - 14 days non-supplemented controls, c - 7 days extract supplemented controls, d - 14 days extract supplemented controls, e – 7 day tenocyte culture, f – 14 day tenocyte culture

	Static		Dynamic	
	Mean	Std Dev	Mean	Std Dev
7 Day Non-Supplemented MSC	0.15	0.03	0.21	0.01
14 Day Non-Supplemented MSC	0.11	0.05	0.49	0.05
7 Day Supplemented MSC	0.11	0.01	0.38	0.02
14 Day Supplemented MSC	0.31	0.01	0.44	0.02
7 Day Tenocytes	0.33	0.02	0.27	0.03
14 Day Tenocytes	0.40	0.04	0.43	0.03

Table 5-1: Anisotropy data for the lateral sections of the extract experimental groups.

	Static		Dynamic	
	Mean	Std Dev	Mean	Std Dev
7 Day Non-Supplemented MSC	10%	1%	12%	5%
14 Day Non-Supplemented MSC	45%	21%	54%	18%
7 Day Supplemented MSC	8%	1%	12%	3%
14 Day Supplemented MSC	69%	11%	73%	8%
7 Day Tenocytes	14%	4%	21%	12%
14 Day Tenocytes	51%	10%	60%	7%

Table 5-2: Maximum cell penetration for the cross sections of the extract experimental groups.

Figure 5.5 shows cross sections of the dynamically cultured constructs. In all three cases after 7 days (figure 5.5a, 5.5c, 5.5e), the majority of the cells still resided on the surface of the interior of the tissue where they were seeded. However, after 7 days, they began to penetrate throughout the scaffold. The tenocyte culture (figure 5.5f) after 14 days still had most of its cells at the surface, however, there was a portion of cells that penetrated nearly 60% of the way through the scaffold. On the other hand, both MSC groups has a large portion of their cells penetrate the scaffold. The non-supplemented controls penetrated nearly 54% through the scaffold, similar to the tenocyte control, but had a much larger amount of cells penetrate. The extract supplemented construct had nearly 73% of the scaffold depth penetrated with cells.

The lateral sections in figure 5.6 indicate fiber alignment of extracellular matrix along with the direction of stretching as indicated by the arrows. After 7 days, all the constructs demonstrate some alignment with the direction of stretching, but the ECM is still somewhat disorganized and/or wavy in appearance (figures 5.6a, 5.6c, 5.6e). The non-supplemented culture in figure 5.6a especially appears somewhat more random in appearance compared to the tenocyte and extract supplemented constructs. After 14 days however, a much more aligned extracellular matrix (ECM) is seen along with a greater abundance of ECM. This is especially pronounced in figure 5.6d, the extract supplemented MSC construct, with a dense connective tissue-like appearance. In addition, some purple stained nuclei appear elongated, indicating a tendon like phenotype. For comparison, figure 5.7 shows a rat tendon, in which fibers are aligned in the longitudinal direction of the tendon along with a sparse population of cells.

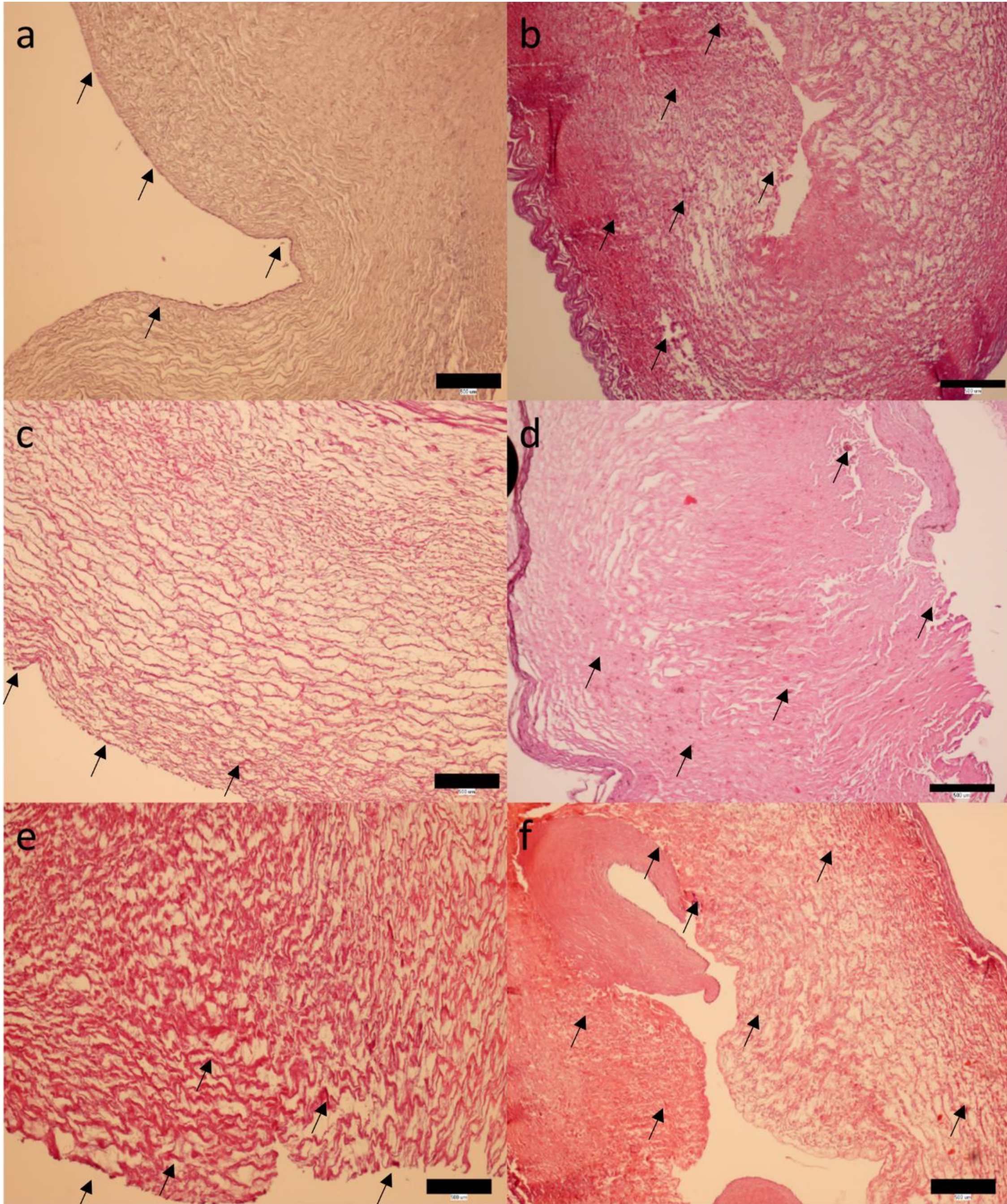


Figure 5.5: Cross-sectional views of the tissue samples at 4x magnification for dynamic constructs. Scale bar is 500 µm. a – 7 days non-supplemented controls, b - 14 days non-supplemented controls, c - 7 days extract supplemented controls, d - 14 days extract supplemented controls, e – 7 day tenocyte culture, f – 14 day tenocyte culture. Arrows indicate cells within the scaffold.

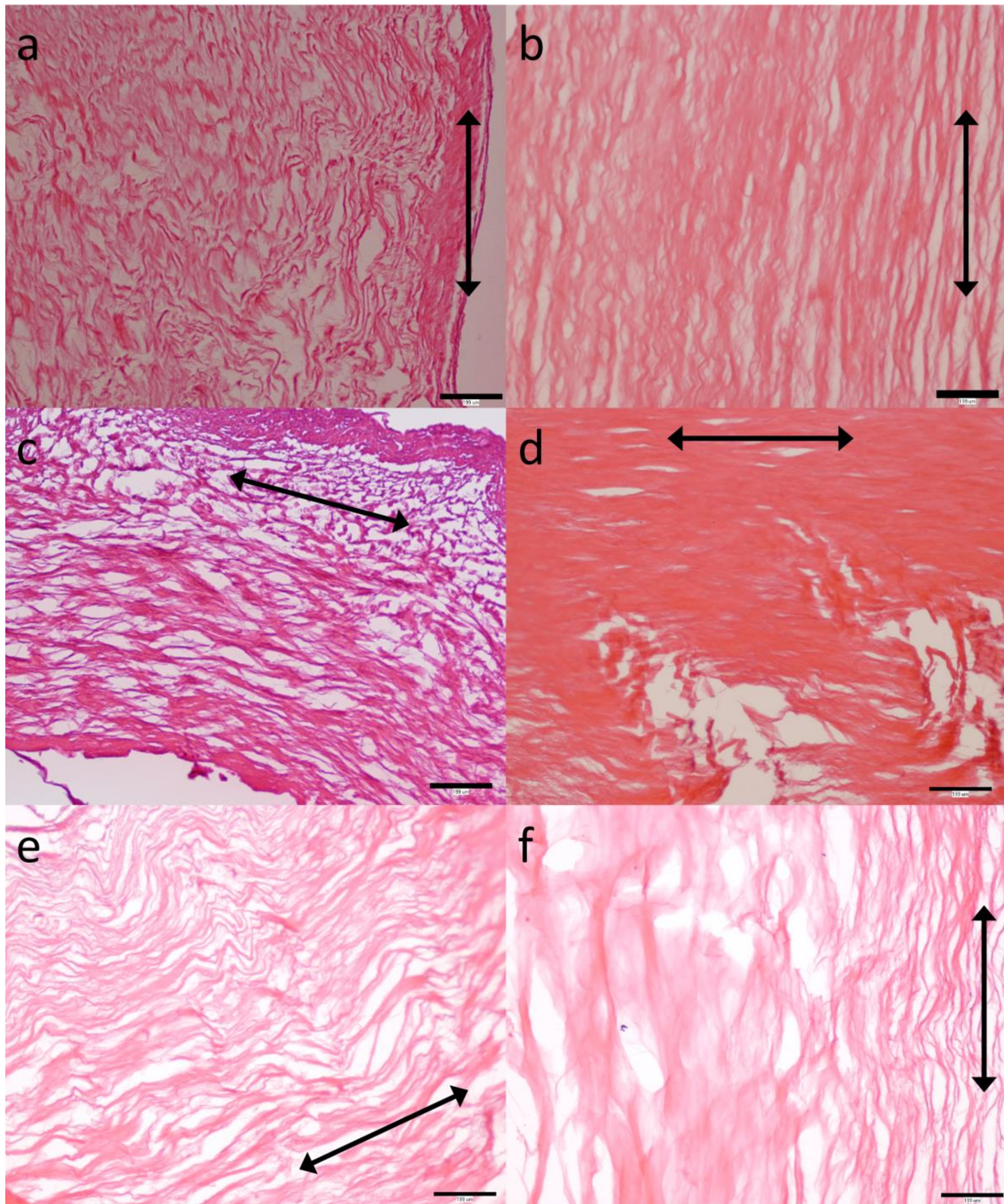


Figure 5.6: Lateral section views of the tissue samples at 10x magnification for dynamic constructs. Scale bar is 200 µm. a – 7 days non-supplemented controls, b - 14 days non-supplemented controls, c - 7 days extract supplemented controls, d - 14 days extract supplemented controls, e – 7 day tenocyte culture, f – 14 day tenocyte culture.

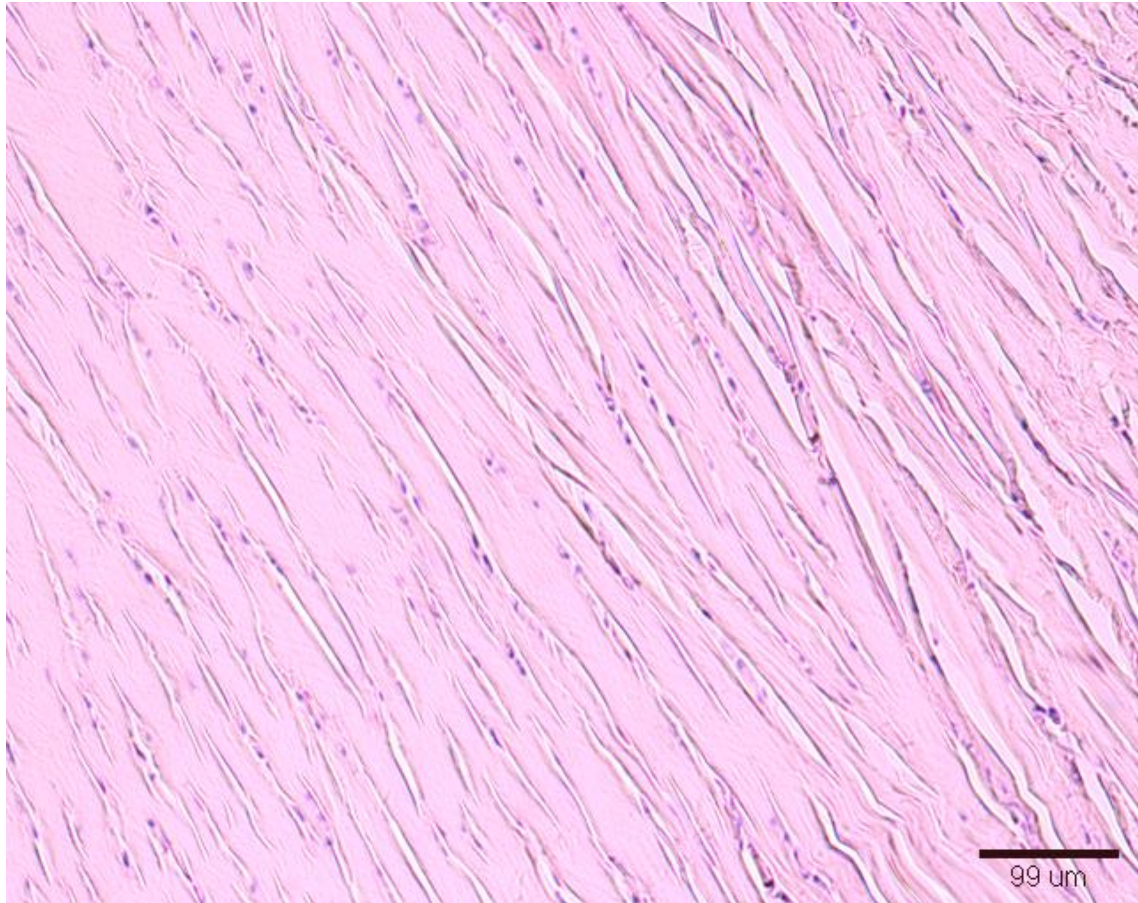


Figure 5.7: Histological staining of rat Achilles tendon. Scale bar is 100 μm.

5.3.3 Mechanical Analysis

Ultimate tensile strength of the constructs is shown in figure 5.8. All groups had significant increases in mechanical strength from the decellularized HUV which had an ultimate tensile strength of 0.47 ± 0.19 MPa. None of the static culture groups showed significant increases from 7 to 14 days, but did show increasing trends. Fourteen days static cultures resulted in 1.73 ± 0.56 MPa, 2.1 ± 0.33 MPa, and 1.84 ± 0.67 MPa for MSCs without extract, MSCs cultured with extract, and tenocyte cultures respectively. The same groups possessed ultimate tensile strengths of 2.6 ± 0.6 MPa, 3.45 ± 0.70 MPa, and 2.38 ± 0.91 MPa respectively after 14 days of dynamic culture. Each of these

increases were significant in respect to the 7 day dynamic culture period. In addition, the extract supplemented group possessed higher ultimate tensile strengths than the non-supplemented (33% larger) or tenocyte group (45% larger).

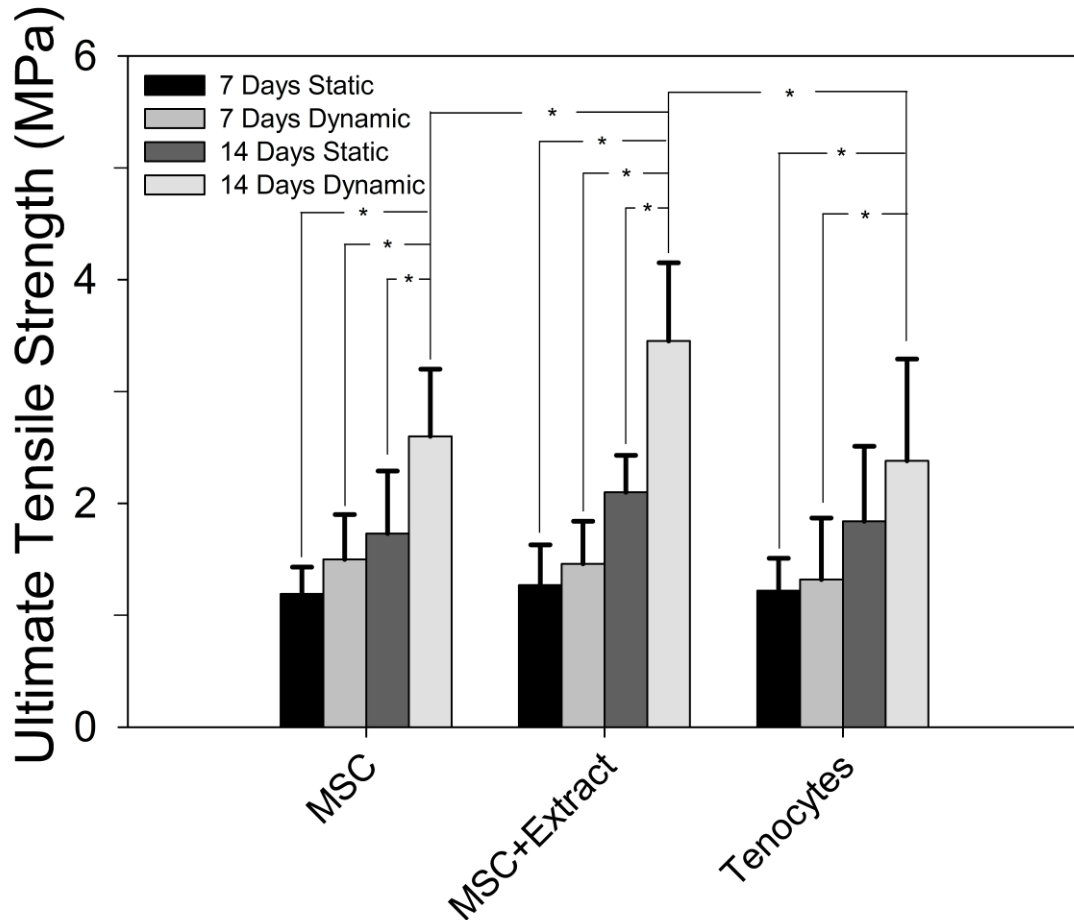


Figure 5.8: Ultimate tensile strength of the various construct groups. * indicates $p < 0.05$ between groups. Data represented as mean ± standard deviation. A sample size of $n \geq 3$ was used.

5.3.4 Gene Expression

Figure 5.9 shows the gene expression analysis for the extract supplemented controls. The static supplemented experimental group compared to the non-supplemented MSC group is given in figure 5.9a. Although most of the tendon-related genes are upregulated after 7 and 14 days (only tenomodulin after 7 days is

downregulated), none are significantly changed from 7 to 14 days. Figure 5.9b compares the static extract supplemented group to tenocytes. In this comparison, expression levels are similar to tenocytes; however scleraxis expression is upregulated after 7 days compared to tenocytes but is downregulated after 14 days, a 22 fold change. Alternatively, COMP is downregulated after 7 days but has a significant a 5.6 fold increase compared to the 7 day time point and is upregulated after 14 days.

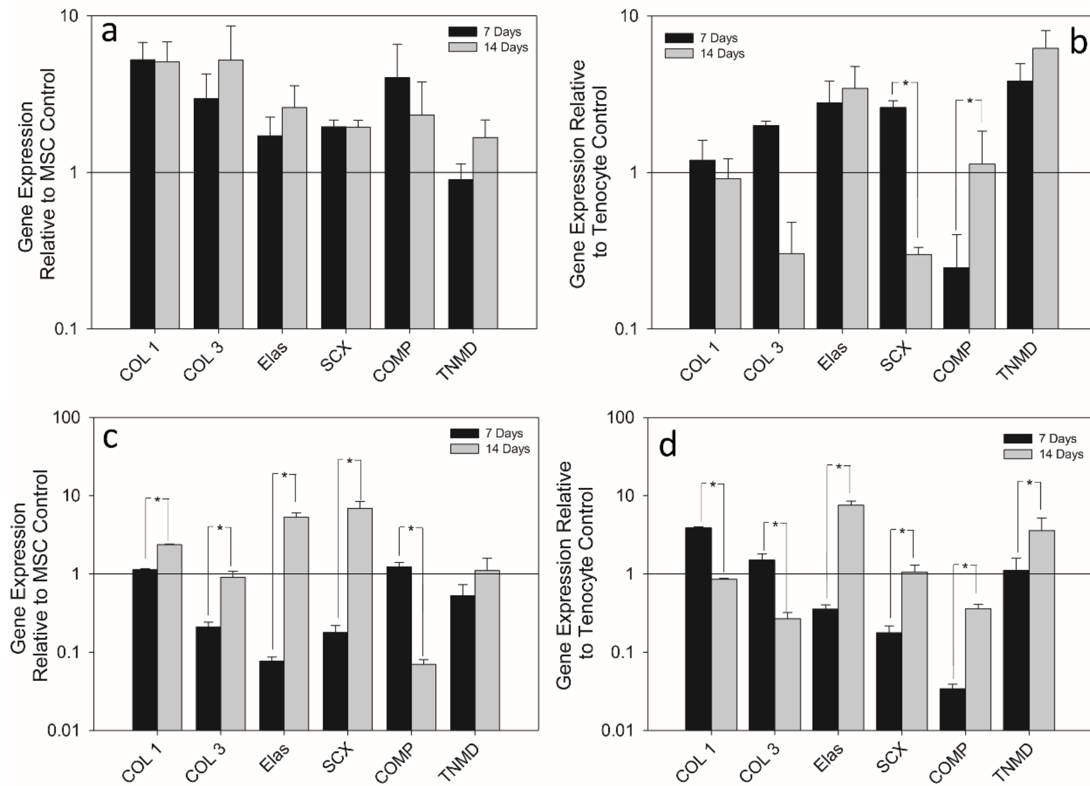


Figure 5.9: Gene expression of cells for selected tendon related genes. * indicates $p < 0.05$ between groups. Data represented as mean ± standard deviation. A sample size of $n \geq 3$ was used. a) Statically cultured and extract-supplemented constructs compared to non-supplemented MSCs, b) Statically cultured and extract-supplemented constructs compared tenocytes, c) dynamically cultured and extract-supplemented constructs compared to non-supplemented MSCs, d) dynamically cultured and extract-supplemented constructs compared to tenocytes.

Figure 5.9c provides gene expression of the mechanically stimulated extract supplemented constructs compared to the non-supplemented MSC constructs. After 7

days, only collagen type 1 and COMP were upregulated compared to the non-supplemented controls (a 1.13 fold and 1.22 fold increase respectively). Alternatively, collagen type III (4.73 fold), elastin (13.07 fold), scleraxis (5.61 fold), and tenomodulin (1.89 fold) had decreased expression. With 14 days of culture, only COMP expression decreased from 7 days. However, collagen type III was still slightly downregulated. Each of the changes between 7 and 14 days was significant with the exception of tenomodulin.

When comparing the mechanically stimulated supplemented constructs to the tenocyte control in figure 5.9d, after 7 days, only collagen type I, collagen type III, and tenomodulin were upregulated. This was a 3.87, 1.50, and 1.11 fold increase respectively. All other gene expressions were downregulated. However, after 14 days, both collagens were downregulated while elastin (7.53 fold) and scleraxis (1.05 fold) were upregulated. COMP and tenomodulin remained downregulated and upregulated respectively. Significant decreases between 7 and 14 days for collagen type I and collagen type III were present while all other genes were significantly upregulated compared to the earlier time point.

5.4 Discussion

It was desired in this study to investigate an alternative means for chemically stimulating the MSC/HUV construct to supplement mechanical stimulation in tendon tissue engineering. Growth factors have been successfully isolated from lysed mature cell lines.[325] Furthermore, it has been previously shown that extract which has been shown to contain genetic material and stored proteins (such as growth factors and other

signaling molecules) can influence stem cell fate towards the mature cell lineage.[326,329,331,332] This is done by the stem cells incorporating genetic material for reprogramming and signaling resulting from the soluble factors such as growth factors produced by the mature cell line. Tenocytes have been shown to produce many of the growth factors such as TGF-β1, BMP-12, and FGF-2 present in native tendon.[333] Although many of these molecules have half-lives of hours (FGF-2) to a day (some proteins found in the cell), the initial exposure to the growth factors and any incorporation of genetic material and transcription factors would have positive tenocytic effects on the MSCs.[334–338] By promoting a tenocytic lineage within our construct, we saw increases in cellular proliferation, tenocytic gene expression patterns, and ultimate tensile strength when MSCs were supplemented by tenocytic extract.

The tenocytic extract did have a mitogenic effect on MSC-seeded HUVs and was synergistic with mechanical stimulation as seen in figure 5.2. After 14 days, constructs that were supplemented with extract and mechanically stimulated had significantly higher amounts of cells compared to the non-supplemented group. In addition the dynamic stimulation also provided significant increases over static culturing in the supplemented group. Furthermore, the dynamically cultured and extract supplemented constructs also possessed a significantly larger amount of cells compared to tenocytes after 14 days. Tenocytic extract has been shown to stimulate DNA synthesis and contains IGF-1.[339] In addition, IGF-1 is known to increase MSC proliferation rates.[340] As for the tenocyte culture itself it has been seen that at some limiting concentration, tenocytes stop proliferating within a 3D collagenous construct.[285] This also occurred in the HUV which contains mostly collagen other

components, as the tenocytes possessed the highest amount of cells after dynamic stimulation of 7 days, but did not demonstrate any better changes. Our explanation for this behavior may be that by repopulating the construct with mature tenocytes, the cells may grow rapidly at first, but reach the concentration within the scaffold that resembles a healthy tendon, thereby halting their proliferation rates. This would result in little change from 7 to 14 days.

Similar trends were seen with the ultimate tensile strength of the constructs. Although, seeding with cells was beneficial enough to significantly increase the tensile strength of the decellularized HUV, significant increases within the experimental groups did not occur until after 14 days of culture with mechanical stimulation. In this situation, both MSC-seeded groups demonstrated significant increases compared to earlier time points and the same day static group. Once again, the extract supplemented group with mechanical stimulation possessed the best tensile strength, exhibiting a degree of synergy between the two stimulation techniques. It had a 3.45 ± 0.70 MPa ultimate tensile strength, and was significantly stronger than the static 14 day culture, either 7 day culture, and 14 day mechanically stimulated non-supplemented and tenocyte groups.

These tensile strength gains did correlate with the histological images with higher mechanical strengths correlating with more aligned ECM. When looking at figure 4 compared to figure 5.6, the lateral histological images, dynamic culturing resulted in more aligned fibers in the direction of stretching. Additional differences were seen in the mechanically stimulated lateral section images in figure 5.6. After 7 days, all three groups had some aligned ECM fibers in the direction of stretching, but

there was still random orientation, reminiscent of the decellularized HUV scaffold (figures 5.6a, 5.6c, 5.6e).[178,180] However, after 14 days, differences started appearing. Both MSC seeded groups (figures 5.6b and 5.6d) had more tissue formed in the images compared to the tenocytes (figure 5.6f) and the fibers, although fairly well aligned in the tenocyte group, were more aligned with the MSC groups. Between the supplemented and non-supplemented groups there was also some difference, with the extract supplemented group (figure 5.6d) possessing a much denser connective tissue appearance that resembles natural tendon tissue. This similarity can be seen in a magnified image of a rat Achilles tendon seen in figure 5.7.

The gene expression results for the extract supplementation when compared to non-supplemented control and tenocyte culture in figure 5.9 also relates to the previous data. In figure 5.9a, it is seen that the tendon extract does positively influence tendon gene regulation without mechanical stimulation as all genes are overexpressed except for tenomodulin at 7 days compared to non-supplemented MSCs. However, there are no significant changes from day 7 to day 14, corresponding to no significant increases in mechanical strength among static groups. Figure 5.9b shows that compared to tenocytes, static culture of extract supplemented construct results in tendon gene expression similar or less than tenocytes. In fact, scleraxis, an early marker of tendon development is actually downregulated after 14 days compared to the tenocyte control.[341]

However, gene expression experienced greater changes when mechanical stimulation was also applied. In figure 5.9c, we see that collagen type I is upregulated compared to the non-supplemented group at both 7 and 14 days, while it is only

upregulated compared to the tenocytes at 7 days. After 7 days of culture, the extract supplemented group had more ECM, which is primarily made out of collagen type I compared to the other two groups according to the histological images. After 14 days, both MSC groups had a more connective-tissue like appearance, but the extract supplemented group had more tightly packed fibrils. On the other hand, as less ECM is present in the tenocyte culture, the cells may be increasing the expression of collagen type I genes to produce more matrix to fill the void space at 14 days. Also, tendon development progression is marked by increased collagen production, another reason why the extract supplemented group may have seen a higher expression of collagen type I compared to the non-stimulated group, as it has increased tendon-like characteristics.[342] Alternatively, collagen type III is downregulated at both 7 and 14 days compared to the MSC control, while it is upregulated compared to tenocytes after 7 days and then downregulated at 14 days. Collagen type III is often found in higher concentrations at the beginning of tendon development and healing and decreases as it matures.[343] An increase of collagen type III when compared to normal MSCs between 7 and 14 days indicate a tendency towards more of a tenocytic lineage. A decrease when compared to tenocytes could indicate more developed ECM for the supplemented MSC culture, which was also seen in the histological images.

Other gene expression targets investigated indicate an increase in tendon-like development for the extract supplemented group, especially after 14 days. Elastin is often present in well-developed fibrils early on in the tendon development cycle.[342] The supplemented MSCs demonstrate a significant increase in the expression of elastin from 7 to 14 days. However, the gene is only upregulated compared to tenocytes or MSCs

after 14 days. This may be due to an increased differentiation level compared to the non-supplemented group. In addition, the tenocytes may express less elastin genes due to their mature state compared to the differentiating MSCs.

COMP helps catalyze fibrillogenesis of collagen fibers in tendons.[344] However, when compared to the tendon culture, it is downregulated in extract supplemented constructs after both 7 and 14 days, albeit with a significant increase in expression between 7 and 14 days. It has been shown that tenocytes when exposed to mechanical stimulation or mechanical stimulation and TGF-β1 and TGF-β3 increased production of COMP.[345] Therefore, the mature tenocytes could be expressing much more COMP compared to stem cells in response to the mechanical stimulation provided since the stem cells were still immature and undifferentiated, especially at 7 days. The fact that the expression in the supplemented group increases compared to the tenocytes could indicate differentiation and progress towards a tenocytic lineage. When compared to the non-supplemented control, the supplemented MSCs actually demonstrated a significant decrease in COMP expression. As seen in figure 5.6, after 7 days, the fibrils in the supplemented group (figure 5.6c) are thicker and more aligned, while in the non-supplemented group (figure 5.6a) are much more random, indicating more collagen fibril production for the supplemented group. However, after 14 days, the extract supplemented group (figure 5.6d) is denser and has less individual fibrils surrounded by void spaces as seen in figure 5.6b for the non-supplemented group. The presence of more individual fibrils and available void space indicates a greater need for collagen production and, therefore, COMP production.

Scleraxis is an early marker of tendon development.[341] It has also been shown that its production is increased in injured tendons after exposure to mechanical stimulation.[346] Taken together, mechanical stimulation and differentiating MSCs should express high levels of scleraxis. When compared to non-stimulated MSCs, the extract groups show similar trends as was seen in elastin, with a downregulation of the gene after 7 days and a significant increase in the expression after 14 days, when it is upregulated compared to the control, indicating that the extract may slightly delay differentiation in the construct. When comparing with the tenocyte group, the same effect is seen, with a significant increase in scleraxis expression between 7 and 14 days with extract supplementation, to the point it reaches a nearly identical level as the tenocytes. As the previously mentioned research has shown that tenocytes themselves will increase scleraxis expression with mechanical stimulation, so a similar level of expression between the two groups indicate a high level of scleraxis production for the extract group. Furthermore, when compared to the static groups in which the scleraxis expression decreased from 7 to 14 days, it appears that mechanical stimulation is required to maintain scleraxis levels.

In addition to being an early tendon marker, scleraxis has also been shown to positively regulate tenomodulin expression.[347] Tenomodulin, is a transmembrane glycoprotein found in tenocytes and its expression promotes tenocyte proliferation and tendon maturation.[348] When compared to the tenocyte control, a similar level of expression is seen with the extract supplementation, while it increases significantly after 14 days. This correlates with the cellularity data which showed a significantly greater number of cells present in the construct with supplemented MSCs compared to the

tenocyte culture. In addition, the histological images showed a much more tendon-like appearance with the supplemented group compared to the tenocyte group.

5.5 Conclusions

It has been shown that tenocytic extract can positively affect tenocytic-like development of a MSC-seeded HUV construct. By supplementing the seeding of the HUV with tenocytic extract, cellular proliferation increased, mechanical properties improved, more ECM was produced, and the gene expression of the MSCs followed a tenocytic differentiation progression after 14 days. While other studies have utilized growth factors to chemically stimulate MSC differentiation, this is the first study that has utilized tenocytic extract along with mechanical stimulation to positively effect tenocytic development within a tissue-engineered tendon construct.

Chapter 6: Increasing the Quality of a Modified Tissue-Engineered Tendon Construct by Long Term Culture

6.1 Introduction

One of the issues experienced within tissue engineering is mass transport within the artificial tissue. In the body, vasculature is responsible for delivering nutrient and oxygen to cells living within about 100 μm of the vasculature.[291] Unless vasculature is incorporated in the initial scaffold, the cells must rely on mass transport through the exterior of the scaffold to where the cells are located. Some bioreactors can introduce flow through the scaffold increasing transport rates utilizing convection, such as the flow perfusion bioreactor.[223] In contrast, the tendon tissue bioreactor utilized in these studies incorporates the flow outside of the tissue construct.

Previous studies with the HUV/MSC construct have shown promise with the HUV scaffold for tendon tissue engineering.[178–180] After 2 weeks, the tensile strength increased to 4 MPa, a 300% increase compared to static controls, a 20 fold increase in cell number and increased tissue formation and remodeling.[180] However, since the HUV is cylindrical and the cells were seeded in the interior of the scaffold, the major method of nutrient transport to the cells and waste transport away from the cells was diffusion to and from the circulation media external to the HUV. It was thought that as new tissue was deposited and integrated into the scaffold by the cells, diffusion rates dropped due to a reduction in effective porosity, deteriorating the construct. This limited the amount of cells supported by the scaffold and the amount of time the construct would be cultured.[180] It was found that after 2 weeks of culture within the

HUV, although construct properties were still improved, cells began to undergo apoptosis, lysed cell bodies were found, and ECM was degraded, indicating that 2 weeks was the longest viable culture time for a closed HUV construct with cells seeded in the interior.[180]

This study aimed to solve these issues. During bioreactor culture, the HUV is altered by cutting the scaffold lengthwise and opening it up into a flat sheet, where the seeded cells can be exposed to circulating media. It is hypothesized that by seeding the cells on the Wharton's jelly side of the scaffold, the efficiency of the cell seeding will be greater than on the luminal side of the HUV. It is also hypothesized that the diffusion length is decreased and eliminated at the scaffold surface, allowing for longer culture times (up to 4 weeks) and therefore superior construct properties compared to the previous methods. Finally, by culturing the construct as a closed cylinder for up to 14 days and then opened into a flat sheet, the cells seeded in the interior will be protected from the undesirable fluid shear stress stimulation which can influence MSCs towards osteogenesis.[349]

6.2 Methods

6.2.1 Scaffold Preparation

Human umbilical cords were obtained from Norman Regional Hospital (Norman, OK). The umbilical cords were then cleaned and prepared according to previous methods to extract the HUV.[179] Briefly, the umbilical cord was mounted onto a stainless steel mandrel and frozen at -80°C for at least one day. The umbilical cord was then removed from the vein using a computerized lathe. The resulting HUV had a

wall thickness of 0.75 mm. The cord was then either cut length wise if were to be cultured in an open state immediately or it was inverted and remained cylindrical if a closed bioreactor culture was to be used initially. The cords were then decellularized and washed in a 1% sodium dodecyl sulfate (JT Baker, Center Valley, PA), ethanol, and 0.2% peracetic acid washes (Sigma Aldrich, St. Louis, MO). The resulting scaffold was then pH adjusted in phosphate buffered saline to 7.2-7.4. Scaffolds were kept for a maximum of 5 days at 4°C prior to use.

6.2.2 MSC and Tenocyte Isolation

Both MSCs and tenocytes were extract utilizing similar techniques as in chapters 4 and 5.

6.2.3 Tenocytic Extract Preparation

Extract was prepared as in chapter 5. A Bradford Assay indicated that there was 46.1 ± 3.7 mg/mL of protein in the extract.

6.2.4 Experimental Design

MSC-seeded HUV constructs were cultured in a custom made bioreactor for periods of up to 4 weeks as pictured in figure 6.1. Two types of culture were performed: either a flat, open construct from the beginning or an initial culture with the cylindrical form the HUV as had been done previously. If the construct was cultured initially in the cylindrical from, after 2 weeks, the construct was then opened up into a flat sheet and cultured for an additional 2 weeks, for a total of 4 weeks time.

To determine the best surface to seed the MSCs onto the flat sheet, a seeding efficiency test was performed. 1.8 million cells were seeded on either the luminal or Wharton's jelly surface of the HUV in a 250 μL suspension of a-MEM with 40 mg/mL extract concentration. Both surfaces can be seen in figure 1. The suspension was placed evenly throughout the scaffold and then cultured for 4 hours, 1 day, or 7 days to determine efficiency and initial growth patterns. The surface determined to be more conducive for cell growth was used for all further experiments.

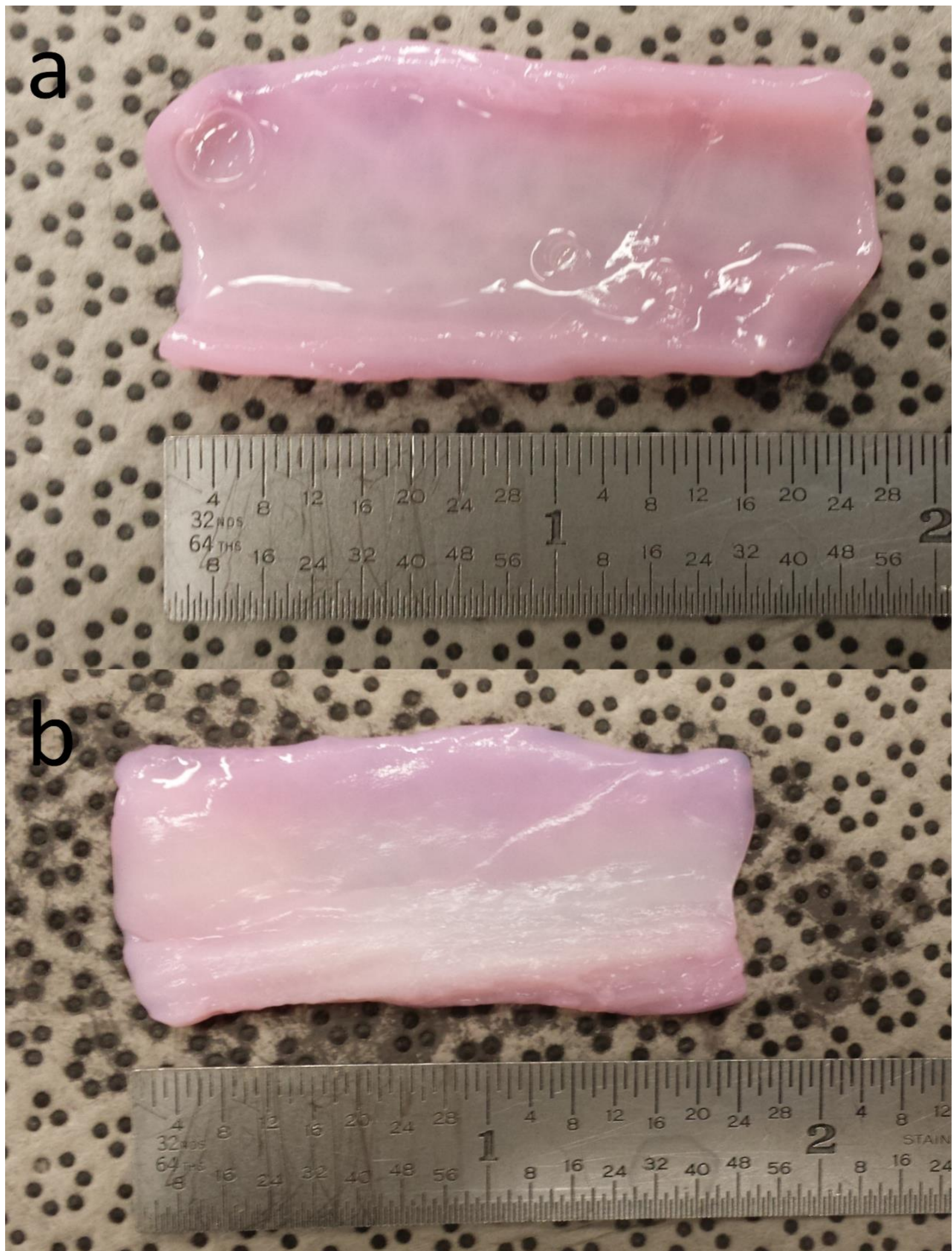


Figure 6.1: (a) Luminal surface used for seeding efficiency test. (b) Wharton's jelly surface used for seeding efficiency test.

For an initially closed bioreactor culture, established procedures were followed. MSCs were suspended in a mixture of 2 mg/mL collagen type I and tenocytic extract resulting in an extract concentration of 16.8 mg/mL and 3 million cells/mL. 0.6 mL of the solution was then injected into the middle of the HUV and sealed with stainless steel adapters. The collagen hydrogel was allowed to gelatinize for 2 hours at 37°C. Afterwards, the constructs were placed into the bioreactor. After 2 weeks, the constructs were removed and cut open lengthwise to result in a flat sheet. Closed culture was not continued after 2 weeks due to previous studies that showed ECM degradation and cell death in the construct after 2 weeks of closed culture.[180] Stainless steel clips were then used to attach the construct into the bioreactor. Figure 6.2 shows a comparison of the flat and cylindrical constructs and their placement in the bioreactor. These constructs were then cultured for 1 or 2 additional weeks for a total of up to 4 weeks. Every day during culture, the construct was cyclically stretched for 0.5 hours/day at 0.5 cycles/min at a 2% strain. After the end of the culture period, constructs were removed and prepared for further analysis.

Figure 6.2: (a) Cylindrical, closed configuration of HUV for initial bioreactor culture. (b) Open flat configuration of the HUV for extended culturing. (c) Bioreactor culture of the cylindrical constructs. (d) Bioreactor culture of the flat constructs.

To seed initially flat constructs for bioreactor culture, 1.8 million cells were suspended in 250 μL of α-MEM supplemented with 40 mg/mL of tenocytic extract. These constructs were then cultured for 1 day in a petri dish prior to bioreactor culture to allow for sufficient MSC integration onto the scaffold. This was done due to the direct exposure to circulating media and the potential to detach cells. After the static period, the constructs were placed in the bioreactor with stainless steel clips as described prior. The culture was then performed for up to 4 weeks with the same mechanical stimulation as was performed with the initially closed cultures.

As a control, static cultures were performed on the flat constructs in a petri dish. These were prepared exactly the same as the experimental cultures. However, they remained in the petri dish during the entire 4 weeks and were not subjected to stimulation.

For all bioreactor cultures, α -MEM without extract was circulated at 1 mL/min and replaced every 3 days. The cultures were performed at 37°C and 5% CO_2.

6.2.5 Cellularity

Cellularity analysis was done as described in chapter 4.

For seeding efficiency tests, the same protocol was performed, however, the entire scaffold was digested analyzed instead of strips or ringlets.

6.2.6 Mechanical Testing

Mechanical analysis was done as described in chapter 4.

6.2.7 Histology

Histology was performed in the same manner as chapter 4.

6.2.8 Gene Expression

RNA and DNA were isolated and produced utilizing the same methods as chapter 4. The tendon genes investigated for this study were: collagen type I, collagen type III, biglcyan, elastin, COMP, scleraxis, and tenomodulin. These primers were identical to chapter 4. In addition, two osteogenic markers were investigated, Sp7 (Osterix) (Forward 5' to 3': CTTTCCCCACTCATTTCCTG Backwards: 5' to 3': CTAGGCAGGCAGTCAGAAG) and osteocalcin (Forward 5' to 3': AAGCCCAGCGACTCTGAGTC Backwards: 5' to 3': GCTCCAAGTCCATTGTTGAGG).[310,350] These primers were the same sequences as chapter 4. RT-PCR and preparation was also done the same as in chapter 4. The analysis was also done utilizing the $2^{-\Delta\Delta Ct}$ method with static MSCs as a control.[311]

6.2.9 Statistics

All analysis was performed utilizing ANOVA and Bonferroni Post tests for significance between individual groups. A $p < 0.05$ was used for significance. Sample sizes of 3 or more was used for each analysis. All data was reported as as mean ± standard deviation.

6.3 Results

6.3.1 Seeding Efficiency

The seeding of the luminal side of the scaffold resulted in lower cell numbers compared to seeding on the Wharton's jelly at all time points as seen in figure 6.3. After 4 hours, the luminal seeded scaffold had 0.5 ± 0.04 million cells while the Wharton's jelly seeded scaffold has 1.2 ± 0.3 million cells, a significant difference of 167%. At 7 days, a similar difference existed, with the lumen having 0.9 ± 0.1 million cells and the Wharton's jelly scaffold had 2.1 ± 0.4 million cells, another significant difference of 133%. Both the luminal and Wharton's jelly groups showed significant increases from initial seeding to 7 days with 95% and 75% increases respectively.

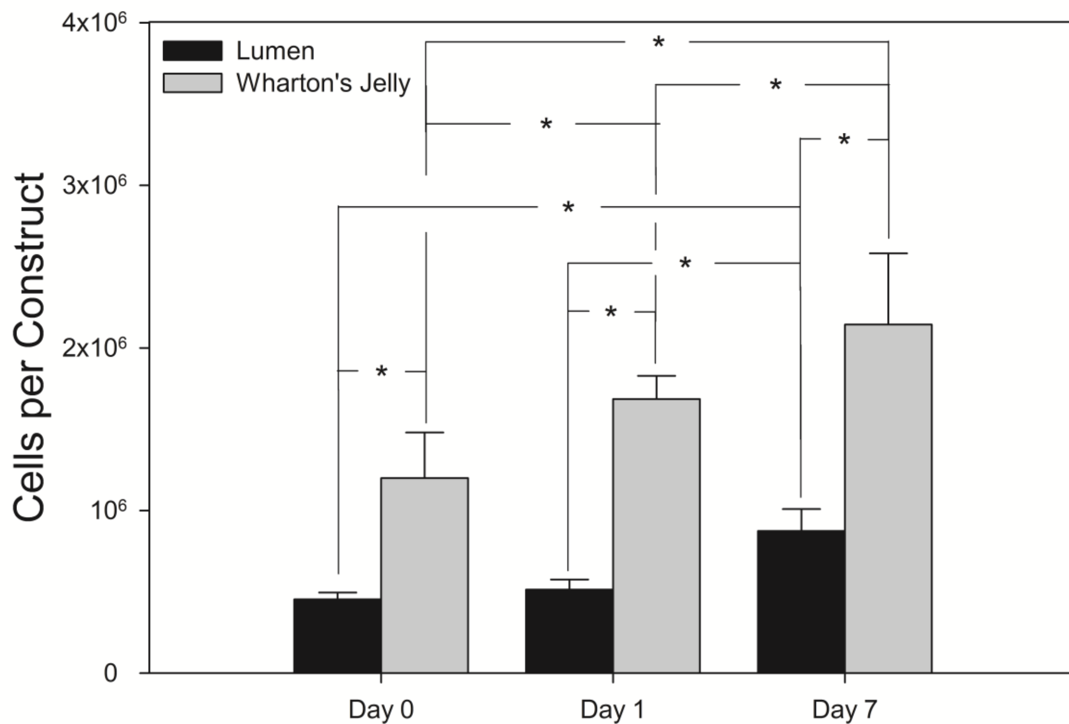


Figure 6.3: Cellularity of scaffolds used for seeding efficiency tests. * indicates p < 0.05 for specified groups. Data represented as mean ± standard deviation. A sample size of n ≥ 3 was used.

6.3.2 Cellularity

Bioreactor culture of the initially flat scaffold demonstrated significant increases of cells from week to week, shown in figure 6.4. At initial bioreactor culture of the flat construct, there was 1.7 ± 0.1 million cells as reported for the seeding efficiency. After 4 weeks of culture, there was 26.0 ± 6.4 million cells, a 15.3 fold increase in cells. Continuous open culture also possessed a significant increase in the amount of cells compared to the initially closed culture, with 63%, 71%, 92%, and 72% increase at 1, 2, 3 and 4 weeks respectively. The initially closed culture did have significant increases in cell number after 14 days, increasing to 3.87 ± 0.5 million cells. After 28 days, the initially closed then opened culture had reached a maximum cell number of 15.1 ± 3.05 million cells.

Static controls did see a significant increase in cell number from the initial seeding efficiency cellularity of 1.2 ± 0.3 to the 7 day culture, which had 2.1 ± 0.4 million cells. However, after 7 days there was no more significant increase in the number of cells. After 28 days, the cellular concentration of the scaffold had reached 3.2 ± 0.5 million cells. At each time point, the application of mechanical stimulation, either initially closed or open, increased the number of cells significantly compared to the static control. After 4 weeks of culture, the initially closed scaffold had 371% more cells compared to the static control while the initially open scaffold had 706% more.

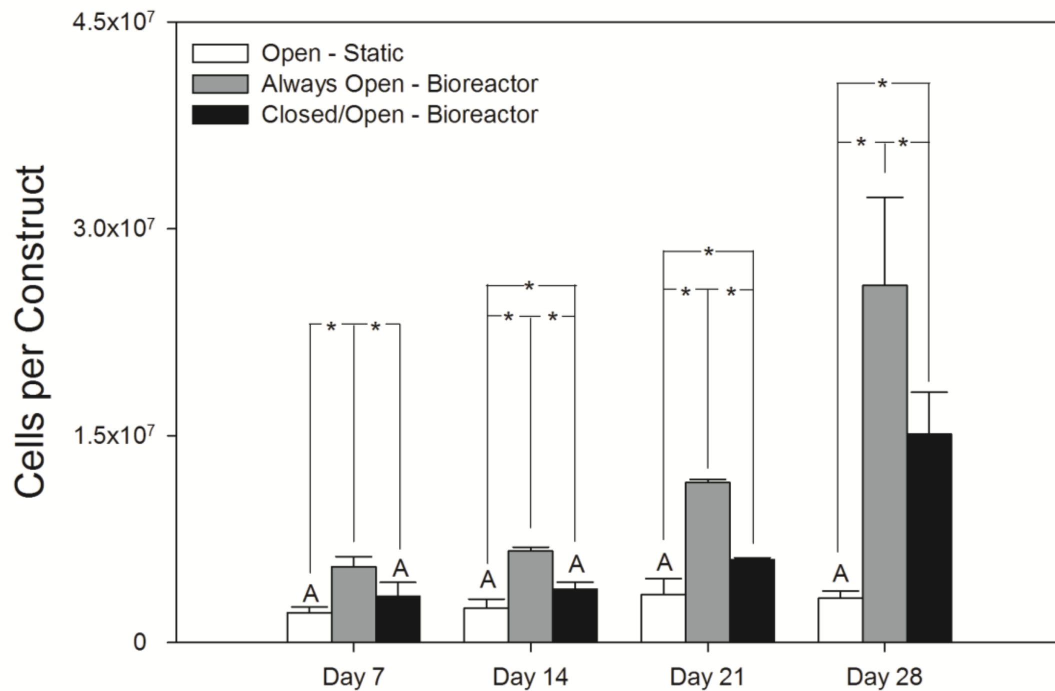


Figure 6.4: Cellularity of experimental constructs cultured in the bioreactor and the static controls. * indicates $p < 0.05$ between experimental and control groups. Letters indicate $p < 0.05$ between various time points in the same experimental group. Data represented as mean ± standard deviation. A sample size of $n \geq 3$ was used.

6.3.3 Histology

Histological sections of the construct show the amount and alignment of the ECM present in the constructs. Figure 6.5 shows the static control throughout 28 days of culture along with the original decellularized construct. ECM content increases throughout the 28 days, however, up until 14 days the fibrils are irregular, thin, and disorganized (figures 6.5b and 6.5c), similar to the decellularized construct (figure 6.5a). At 21 days, the ECM becomes thicker, but still disorganized and at day 28, some fibrils are straight while other portions of the construct has irregular matrix still. Table 6-1 quantifies that the fibril alignment does increase somewhat through 28 days.

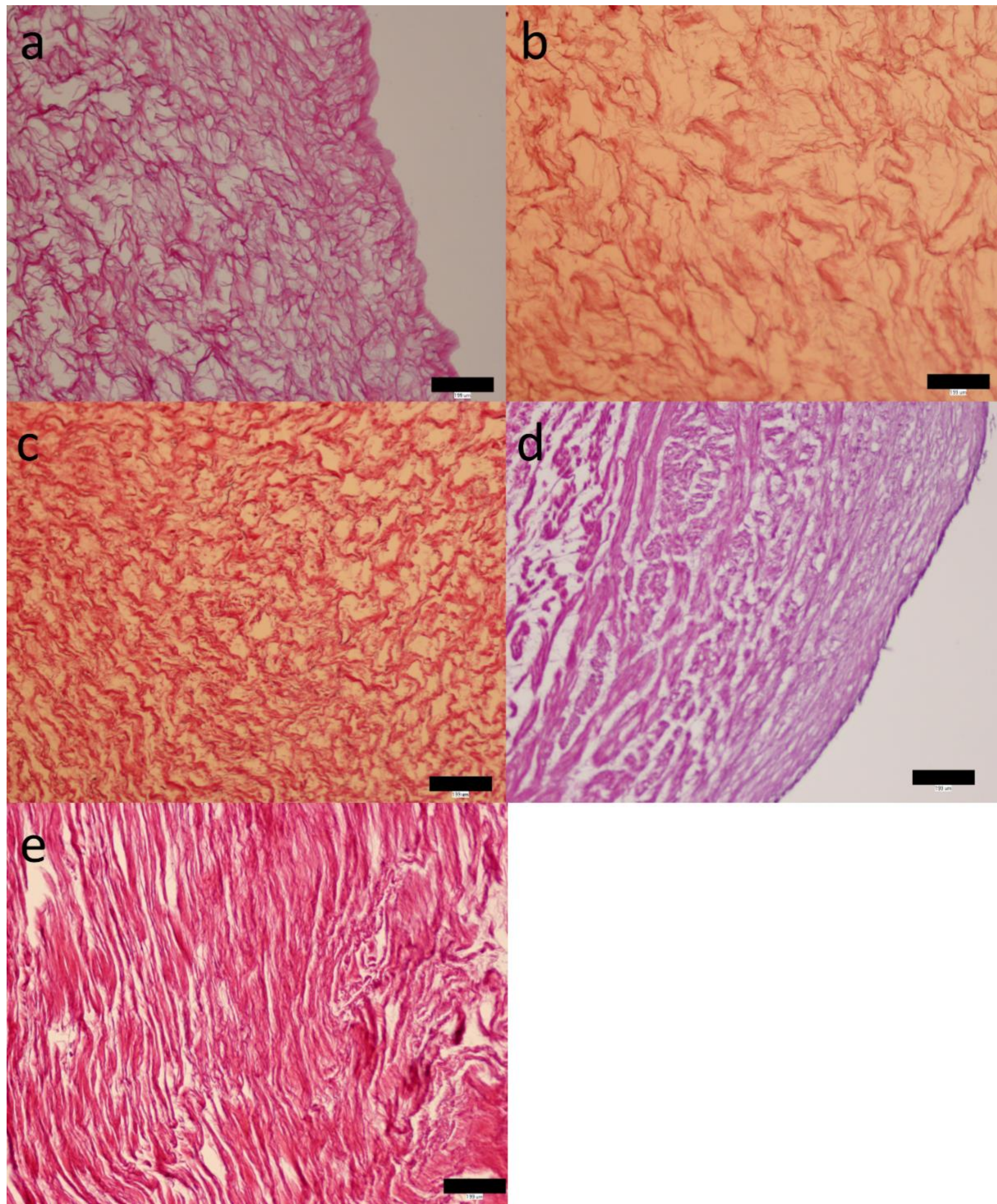


Figure 6.5: Histological sections of statically cultured flat constructs. Magnification is 10x and scale bar is 200 μm. (a) Decellularized scaffold, (b) 1 week, (c) 2 weeks, (d) 3 weeks, (e) 4 weeks.

The addition of mechanical stimulation increases the alignment of fibrils of the ECM as seen in both the initially open scaffold (figure 6.6) and the initially closed then opened scaffold (figure 6.7). With the initially open scaffold, ECM deposition increases throughout the 28 days, similar to the static controls. However, even after 7 days, ECM fibrils are fuller and aligned in parallel, which was not the case with the static controls. Similar trends were seen with the initially closed scaffold. ECM amounts appear to be similar through 21 days. At 28 days (figure 6.7d), the tissue appears to resemble dense connective tissue with parallel fibrils. This is in comparison to the 28 day construct that was initially open (figure 6.6d), it also has similar amounts of ECM, however, it is less aligned in the direction of stretching, as also indicated in tables 6-2 and 6-3.

Cross sections of the HUV shown in figure 6.8 show cellular penetration within the scaffold. The initially open scaffolds showed less penetration (figures 6.8a-e) than the initially closed then opened scaffolds (figures 6.8f-j). Cells remained at the surface or within 40% of the surface depth through 28 days on the initially open scaffolds (table 6-4). After 28 days, cells did penetrate more, reaching at least half the depth of the scaffold. In contrast, the initially closed culture demonstrated continuing penetration from the start of the culture through 14 days, while the scaffold was still closed (figures 6.8f-h). After opening the scaffold up for additional culture, cells remained throughout the scaffold (figures 6.8i and 6.8j).

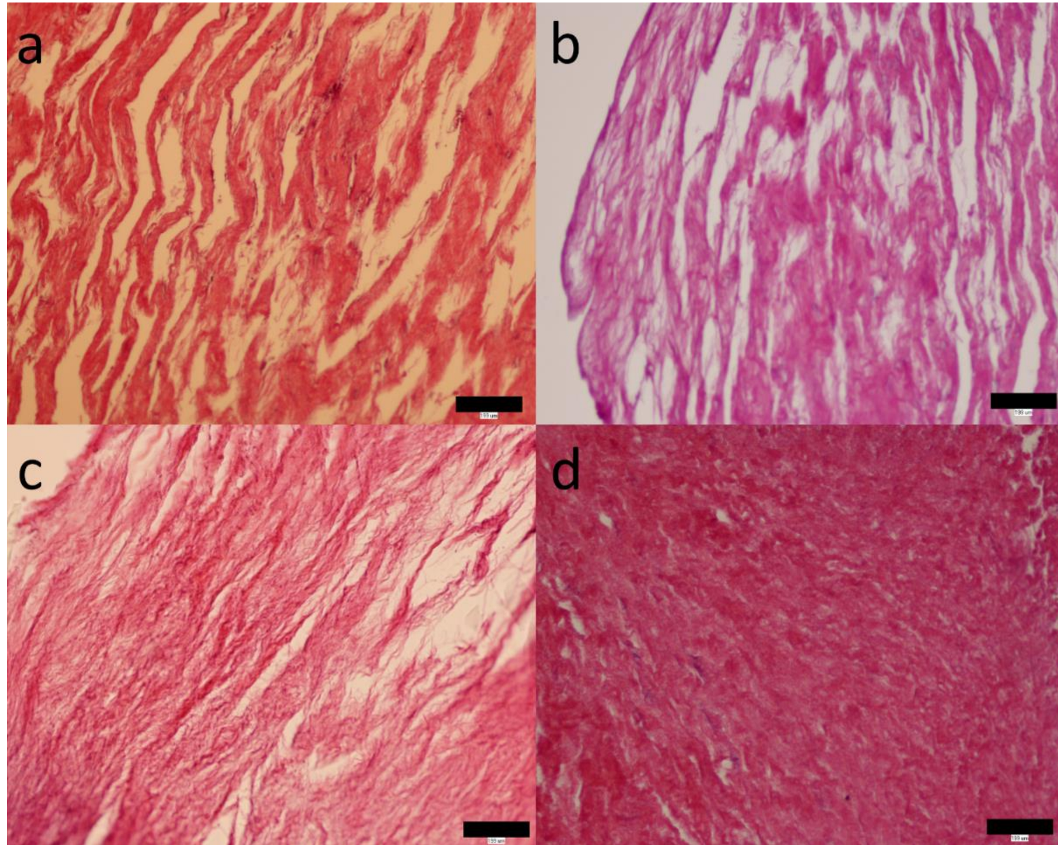


Figure 6.6: Histological sections of always open constructs cultured in the bioreactor. Magnification is 10x and scale bar is 200 μm. (a) 1 week, (b) 2 weeks, (c) 3 weeks, (d) 4 weeks.

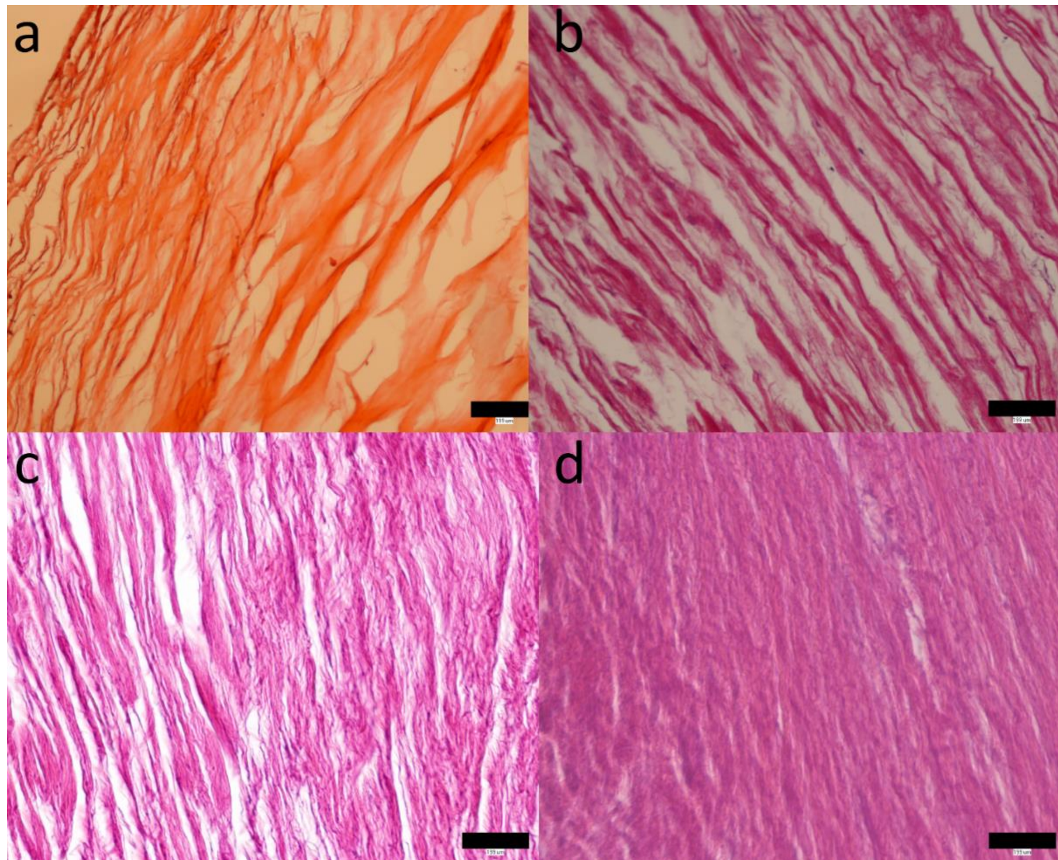


Figure 6.7: Histological sections of constructs that were closed for 2 weeks and then open for 2 weeks cultured in the bioreactor. Magnification is 10x and scale bar is 200 μm. (a) 1 week, (b) 2 weeks, (c) 3 weeks, (d) 4 weeks.

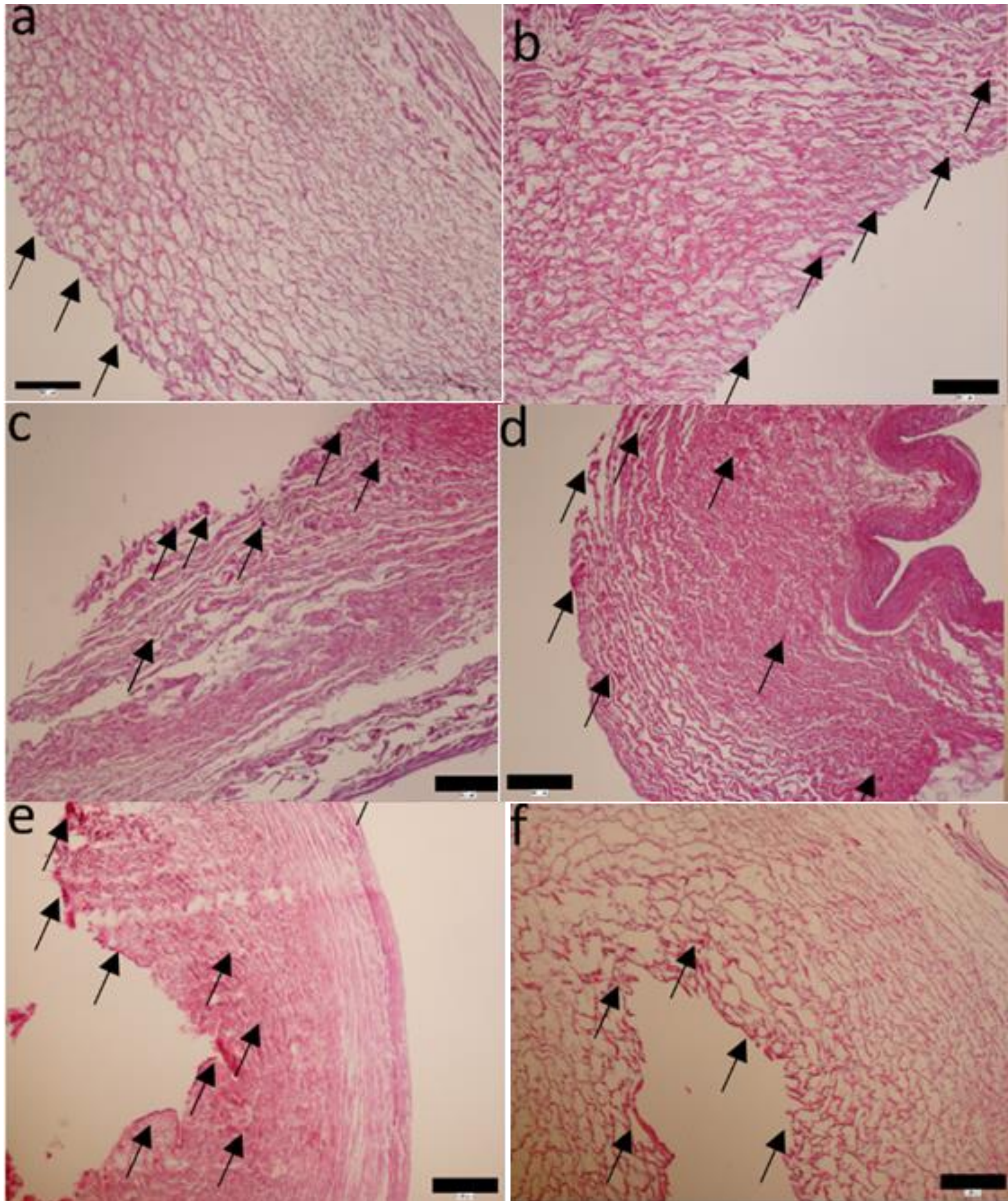


Figure 6.8: Cross sections of HUV/MSC constructs. Magnification is 4x and scale bar is 500 μm. a) Seeded initially open culture, b) 1 week initially open culture, c) 2 weeks initially open culture, d) 3 weeks initially open culture, e) 4 weeks initially open culture f) Seeded initially closed culture. Arrows indicate cells within the scaffold.

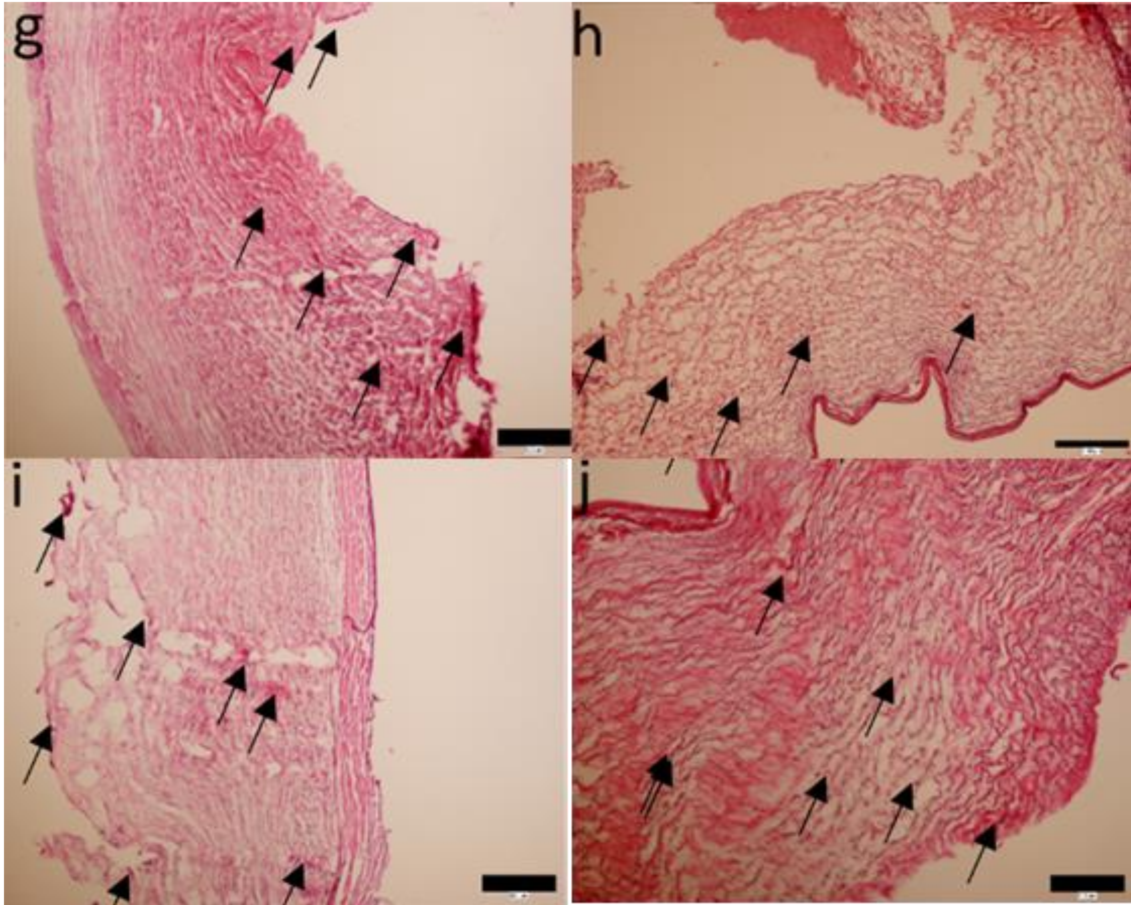


Figure 6.9: Continued cross sections of HUV/MSC constructs. Magnification is 4x and scale bar is 500 μm. g) 1 week initially closed culture, h) 2 weeks initially closed culture, i) 3 weeks initially closed culture, j) 4 weeks initially closed culture. Arrows indicate cells within the scaffold.

	Mean	Std. Dev.
0 Days	0.15	0.04
7 Days	0.14	0.05
14 Days	0.08	0.03
21 Days	0.23	0.06
28 Days	0.35	0.09

Table 6-1: Anisotropy values for the closed open group based on lateral histological sections.

	Mean	Std. Dev.
7 Days	0.29	0.06
14 Days	0.40	0.01
21 Days	0.26	0.02
28 Days	0.09	0.04

Table 6-2: Anisotropy values for the closed open group based on lateral histological sections.

	Mean	Std. Dev.
7 Days	0.47	0.03
14 Days	0.52	0.01
21 Days	0.47	0.06
28 Days	0.40	0.08

Table 6-3: Anisotropy values for the closed open group based on lateral histological sections.

	Always Open		Closed/Open	
	Mean	Std. Dev.	Mean	Std. Dev.
7 Days	18%	5%	42%	6%
14 Days	16%	7%	72%	7%
21 Days	39%	7%	71%	7%
28 Days	23%	4%	77%	10%

Table 6-4: Maximum cell penetration determined by histological cross sections of the always open and closed/open experimental groups.

6.3.4 Mechanical Properties

Figure 6.9 shows that the addition of cells significantly increased the ultimate tensile strength of the scaffold compared to the decellularized scaffold, which had an initial ultimate tensile strength of 1.0 ± 0.4 MPa. This occurred in the static cultures at day 14 and both mechanically stimulated cultures after 7 days of culture. However, the static cultures did not have significant improvements after 14 days of culture. After 14 days, the ultimate tensile strength was 2.5 ± 0.6 MPa and after 28 days it was 2.9 ± 1.1 MPa. The addition of mechanical stimulation via the bioreactor did increase the ultimate tensile strength after 7 days of both the initially open construct (3.2 ± 0.7 MPa) and the initially closed construct (1.7 ± 0.4 MPa). However, no other significant gains were seen even after 28 days for the always open construct which had an ultimate tensile strength of 3.7 ± 0.7 MPa. At 28 days there was a significant difference between initially culturing with a flat or cylindrical configuration, as the closed then opened culture had an ultimate tensile strength of 5.6 ± 0.7 MPa, a 50% increase compared to the initially open construct.

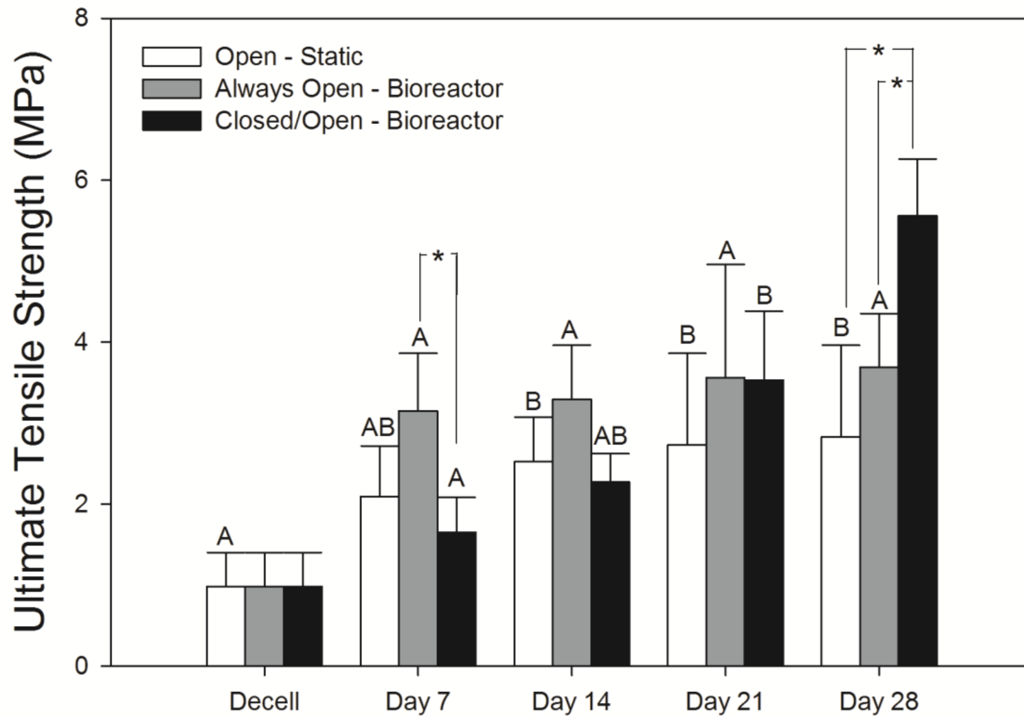


Figure 6.10: Ultimate tensile strength of experimental constructs and static controls. * indicates p < 0.05 between various experimental and control groups. Letters indicate p < 0.05 between various time points in the same experimental group. Data represented as mean ± standard deviation. A sample size of n ≥ 3 was used.

Elastic modulus results are shown in figure 6.10. Static culture of the constructs had a significant differences in the elastic modulus after 14 days compared to the decellularized scaffold: 4.1 ± 0.1 MPa vs 0.7 ± 0.3 MPa, a 5.9 fold increase. However there were no other significant differences for the static cultures. For the initially open construct, mechanical stimulation increased the elastic modulus after only 7 days of culture compared to the decellularized construct with an elastic modulus of 6.4 ± 3.6 MPa, an 814% increase. The elastic modulus thereafter did not show any significant increases. The initially closed construct only significantly increased its elastic modulus

after 14 days to 3.2 ± 2.2 MPa. However, it had the highest elastic modulus after 28 days, 9.55 ± 3.92 MPa, a 338% increase from the beginning of the bioreactor culture.

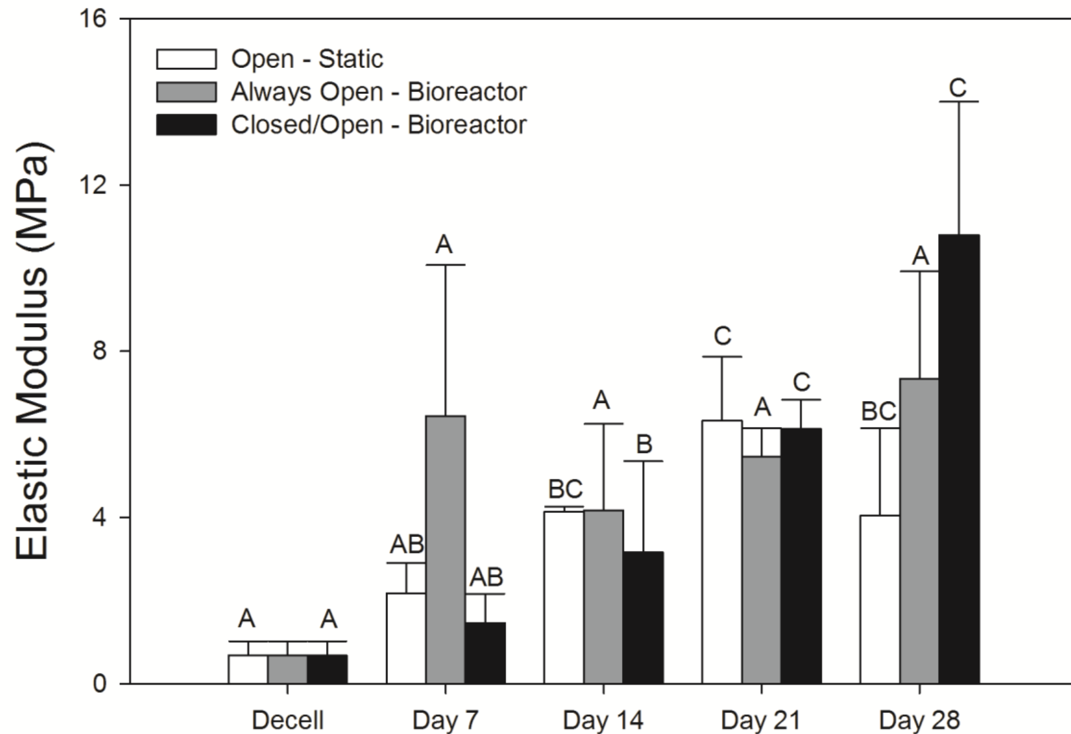


Figure 6.11: Elastic modulus of experimental constructs and static controls. * indicates $p < 0.05$ between various experimental and control groups. Letters indicate $p < 0.05$ between various time points in the same experimental group. Data represented as mean ± standard deviation. A sample size of $n \geq 3$ was used.

6.3.5 Gene Expression

Figure 6.11 demonstrates that the initially closed then opened construct provided many significant responses in gene expression. This configuration had a significant increase in collagen type I gene expression compared to the static control from 7 to 14 days:

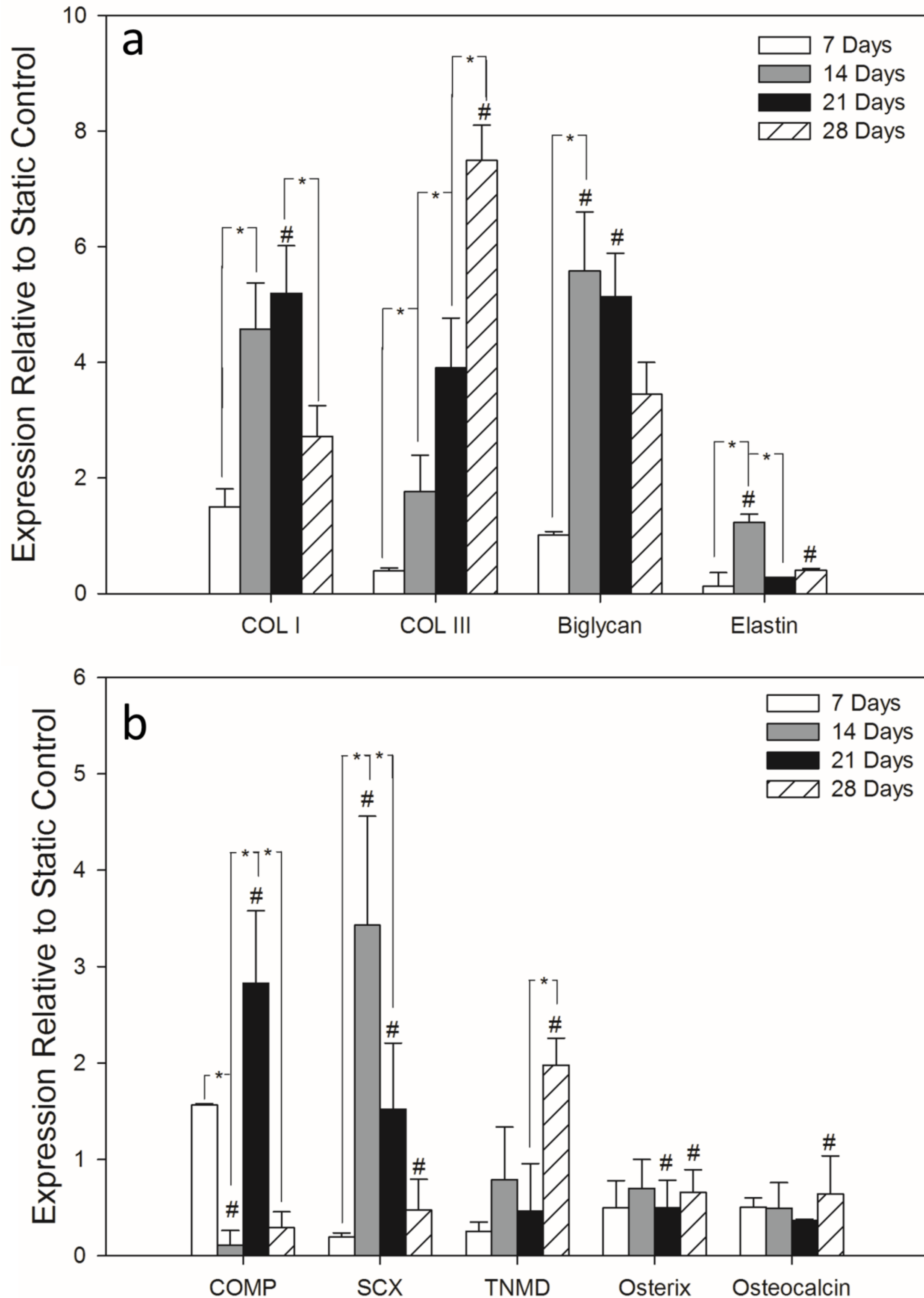
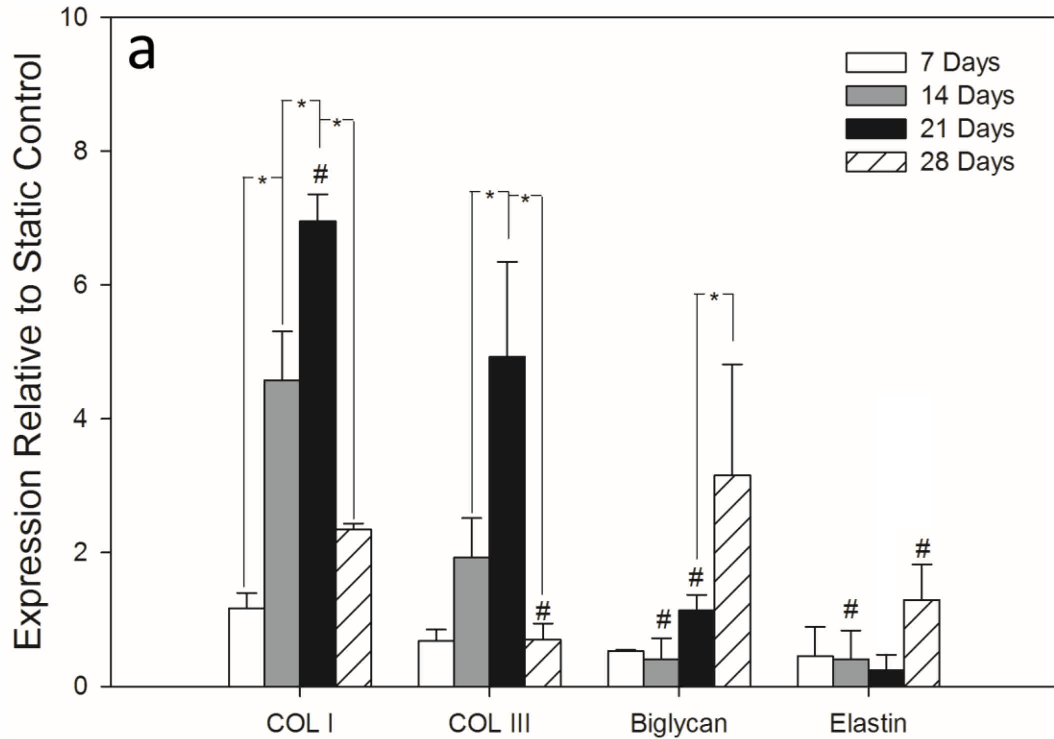


Figure 6.12: Gene expression for initially closed constructs. (a) Tendon-related ECM molecules and (b) Tendon markers and osterix, an osteogenic marker. * indicates $p < 0.05$ between time points, # indicates $p < 0.05$ between the initially open and initially closed constructs. Data represented as mean ± standard deviation. A sample size of $n \geq 3$ was used.

from 1.5 ± 0.3 to a 4.6 ± 0.8 fold increase in gene expression compared to the static control. The expression then significantly decreased from 21 days (5.2 ± 0.8 fold) to 28 days (2.7 ± 0.5 fold). Collagen type III significantly increased from the first to fourth culture week, increasing from 0.4 ± 0.04 fold to 7.5 ± 0.6 fold. Biglycan expression only increased between the first and second weeks of culture from 1.0 ± 0.1 fold to 5.6 ± 1.0 fold, an increase of 450%. Elastin was mostly downregulated, with the exception of an increase after 14 days to a 1.2 ± 0.1 fold increase in expression.

Figure 6.11b presents the expression levels for several tendon markers and one osteogenic marker for the closed then opened. COMP alternated upregulation and downregulation from 1 to 4 weeks, with a maximum expression after 21 days of 2.8 ± 0.8 fold increase compared to the control. Scleraxis expression was significantly increased after 14 days with a 3.4 ± 1.1 fold increase in expression levels. It then decreased after 21 days to a 1.5 ± 0.7 fold increase compared to controls. Tenomodulin only significantly increased after 28 days, with a 2.0 ± 0.3 fold increase. Osterix and osteocalcin, showed no appreciable changes and was under expressed throughout.

The bioreactor cultures that were always open from the beginning also had significant changes in gene expression, however it was different time and amounts compared to the initially closed scaffold configuration. These are presented in figures 6.12a and 6.12b. Collagen type I expression compared to the control increased until day 21, where it had a 7.0 ± 0.4 fold increase in expression compared to the static control. However, it then decreased in levels to 2.3 ± 0.1 after 28 days. Collagen type III demonstrated similar trends, however, it was not significantly increased from 7 to 14 days. However, it also



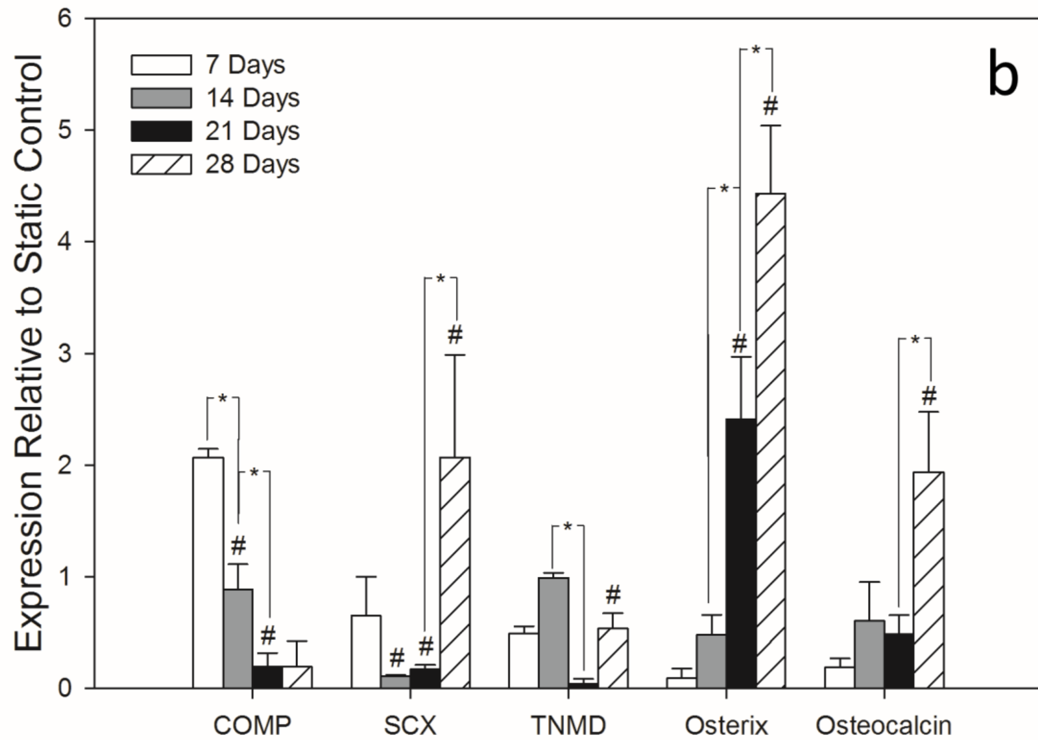


Figure 6.13: Gene expression for initially open constructs. (a) Tendon-related ECM molecules and (b) Tendon markers and osterix, an osteogenic marker. * indicates $p < 0.05$ between time points, # indicates $p < 0.05$ between the initially open and initially closed constructs. Data represented as mean ± standard deviation. A sample size of $n \geq 3$ was used.

reached a maximum after 21 days (4.9 ± 1.4 fold) and then decreased in levels after 28 days (0.7 ± 0.3 fold). This expression also indicated a downregulation compared to the static controls after 28 days. Biglcyan possessed a significant increase in expression after 28 days, with a 3.2 ± 1.3 fold increase compared to the static control. Elastin was also relatively downregulated as was the case in the initially closed configurations, but did was upregulated after 28 days to a 1.3 ± 0.5 fold increase compared to the static control. COMP continually decreased in expression levels through 21 days, initially being overexpressed at 7 days (2.1 ± 0.1) to becoming downregulated at 21 days (0.2 ± 0.1). Scleraxis behaved similarly to biglycan in the open configuration, as it only significantly increased after 28 days to a 2.1 ± 0.9 fold increase compared to the static controls. Tenomodulin remained downregulated throughout the culture times, even decreasing from 14 to 21 days: 1.0 ± 0.04 fold to 0.04 ± 0.04 fold respectively. The largest difference in gene expression between the initially open and closed configurations was seen with the osterix gene. As an always open flat sheet, expression was initially downregulated after 14 days (0.5 ± 0.2 fold), but then significantly increased in expression at 21 days and once again at 28 days, reaching its largest expression level of a 4.4 ± 0.6 fold increase. Osteocalcin also demonstrated a significant increase in expression resulting in upregulation of the gene, but only after 28 days of open culture, as it increased 296% from day 21 to a 1.9 ± 0.5 fold increase compared to the static control.

There were also some significant differences between the gene expressions for not only the culture time points, but also between the initially open and closed configurations. After 1 week, there were no differences. However, after 2 weeks,

biglycan (1268%), elastin (203%), and scleraxis (2990%) expressions were all higher for the closed configuration and COMP (707%) was lower. After 3 weeks, collagen type I (33%) and osterix (382%) were lower for the closed configuration while biglycan (353%), COMP (1343%), and scleraxis (794%) were higher. Finally, after 4 weeks of culture, collagen type III (975) and tenomodulin (74%) were higher while elastin (216%), scleraxis (336%), osterix (571%), and osteocalcin (202%) were lower.

6.4 Discussion

Previous culturing of the HUV/MSC construct for tendon tissue engineering showed much promise. However, the closed nature of the construct limited the length of time the construct could be cultured to two weeks due to nutrient transport limitations that caused cell death and ECM degradation.[180] By opening up the construct, either initially or after two weeks of closed culture, longer culture times could be accomplished. With the longer culture times, cellularity, mechanical properties, construct quality, and gene expression improved or changed compared to the shorter time points. However, there were differences depending on when the cylindrical construct was opened up into a flat sheet. The always open construct did show greater cell content, however, its gene expression and mechanical properties were poorer compared to the construct that was closed for 2 weeks and then opened into a flat sheet for 2 weeks in the bioreactor.

An initial seeding efficiency test was first performed to determine the best surface of the newly flat tendon for cell attachment. With previous HUV/MSC constructs, the HUV was always inverted so that any residual Wharton's jelly tissue

would be on the inside of the scaffold where the cells were seeded.[179,180] This was done to enhance initial cell attachment since the Wharton's Jelly is a rougher surface than the luminal side as seen in figure 6.1. It has been shown that increased roughness of the scaffold surface can positively influence MSC attachment and differentiation.[351–353] In addition, Wharton's jelly has large amounts of glycosaminoglycans and proteoglycans such as hyaluronic acid and heparin and chondroitin derivatives that can assist with cell attachment and growth.[318,354–357] In addition, a study coated the human umbilical artery with fibronectin to facilitate cell seeding.[186] However, it was never initially tested with this specific construct that this was actually more beneficial, and with the addition of fluid shear, the seeding density experiment was performed to ensure the best cell attachment. It was found that it was true that the Wharton's jelly surface was more beneficial, as after both four hours and 7 days, there was significantly more cells on the Wharton's jelly seeded surface than the luminal surface: 167% and 230% respectively. Therefore, for any further experiments, cells were always seeded on the Wharton's jelly surface of the scaffold, whether it be initially a flat sheet or a closed cylinder.

When cultured in the bioreactor, cellularity continued to increase. When initially cultured as a flat sheet, the cellularity significantly increased week over week, reaching a peak cellularity after 28 days of 26.0 ± 6.4 million cells. The same was true for the construct that was initially cultured in cylindrical form and then opened, and after 4 weeks it had 15.1 ± 3.0 million cells. In each case, the always open construct contained more cells than its closed then open counterpart. This could be attributed to the extra initial exposure to fluid shear on the surface of the scaffold where the majority

of the cells resided in early time points. It has been shown that fluid shear does positively influence cellular proliferation, and the cylindrical configuration shields the cells from this influence.[358,359] In both experimental groups, the cells also experienced stimulation from the cyclical stretching of the bioreactor, which also has been shown to increased proliferation rates.[179,180,360] This can be seen by comparing either bioreactor culture group to the appropriate static control. Overall the static group saw no significant gains after 1 week in the petri dish.

Previously, cylindrical constructs seeded with 1.8 million cells reached an ultimate tensile strength of 2.7 ± 0.8 MPa after 2 weeks of culture, the maximum culture time due to diffusional limitations for a closed construct.[180] It was hypothesized that longer culture times should increase the ultimate tensile strength of the construct due to increased cellularity and ECM production and remodeling. This turned out to be true, as both the always open or closed then open cultures both had improvements in the ultimate tensile strengths over the previously reported value after 4 weeks – 3.7 ± 0.7 MPa for the initially and 5.6 ± 0.7 MPa for the initially closed group. As seen in table 1, this is similar to ultimate tensile strengths of certain rotator cuff tendons. This difference between the two groups was also statistically significant. In addition, the initially closed scaffold also saw significant increases between 3 weeks and 4 weeks culture time, where the initially flat scaffold did not. When looking at the elastic modulus, there is a significant increase when cyclical stretching is applied to the construct in the bioreactor when compared to the decellularized scaffold. Otherwise there is no strong correlation between being initially open or closed or increasing culture times.

The histological sections correlated to tensile strength data. The static constructs throughout the time points had a disorganized extracellular matrix, which coincided with no significant increases in tensile strength throughout the culture until day 14, where there was more ECM present than the day 0 or day 7 cultures to provide more structural integrity. Comparatively, dynamically cultured constructs, both the initially open and closed, showed ECM alignment in the direction of stretching after 7 days and also had significant increases in their ultimate tensile strength compared to the decellularized construct. However, the always open construct had shorter, thicker fibrils up until day 28, while the initially closed then open construct had longer, more aligned fibers. At day 28, both bioreactor cultures possessed an almost continuous matrix. However, a closer look shows that there appears to be a more fibrillar appearance with the initially closed construct compared to the initially open construct, which appears more discontinuous and wavy. The fibrillar alignment in the longitudinal direction provided more tensile strength in the initially closed construct than the initially open construct, which was not as organized, resulting in significant increases in the ultimate tensile strength at day 28.

In terms of cellular penetration, the initially open cultures maintained most of the cell population near or at the surface until day 28 where penetration occurred further into the scaffold. This is in contrast to the initially closed scaffold, which promoted cellular penetration at the beginning of culture, resulting in cellular penetration through nearly the entire scaffold at day 14. These cells remained distributed throughout the scaffold even after opening the scaffold up into a flat sheet. This penetration difference could be due to chemotactic migration of the MSCs which can be initiated by FBS

supplemented media.[361] In the initially open scaffold, surface cells are exposed to circulating media directly and an immediate source of nutrients and oxygen. While in the initially closed scaffold, the cells are in the interior of the scaffold isolated from circulating media on the outside of the scaffold. Therefore, the cell would migrate towards the exterior of the scaffold where nutrients are in higher concentration.

The gene expression profiles of the groups indicate that always open scaffold may delay and even hinder tenocytic development in favor for some osteogenic development. This could be due to the immediate exposure of the MSCs which are mainly at the surface of the scaffold to fluid shear stresses, which have been shown to induce osteogenic differentiation even without soluble cues such as dexamethasone or BMP-2.[349] This pathway could provide competition with the tenocytic pathway which is favored by the cyclical stretching provided by the bioreactor.[235] For example, scleraxis, which is an early marker of tendon development is significantly more expressed in the 14 and 21 day culture time points in the initially cylindrical construct compared to the flat construct.[362] Furthermore, scleraxis is downregulated during those two time points for the open construct, and isn't significantly upregulated until day 28, where it is actually significantly greater than the closed construct. However, scleraxis is an early tendon marker and regulates tenomodulin which is a late term tendon marker.[62] Compared to the initially closed construct, in which tenomodulin is significantly upregulated after 4 weeks when compared to earlier time points or the open construct, indicating a more mature tendon phenotype of the cells after 4 weeks.

Another possible explanation for the initially closed construct having more positive tendon-related gene expression could be due to the tenocytic extract and how it

was delivered. As a closed cylinder, the extract is placed within the sealed construct along with the cells, protecting and containing it. When it supplements the seeding of the always open scaffold, there is a potential for some loss of the extract as it is on the surface of the scaffold along with the cells. This would provide the initially closed scaffold with a potentially stronger exposure initially to the tenocytic extract that was shown in chapter 5 to have beneficial tenocytic differentiation capacities.

In addition to tendon markers, osterix and osteocalcin were investigated as osteoblastic differentiation markers. Osterix is a transcription factor required for osteogenic differentiation and osteocalcin is a protein secreted mainly by osteoblasts.[363,364] When looking at this marker, there is no increasing trend or signal from the initially closed then opened construct. For the always open group, the cells had been experiencing fluid shear from the start, and after enough time such as 3 and 4 weeks, there is significant upregulation of osterix compared to earlier time points and the initially cylindrical construct. Osteocalcin behaves similarly after 4 weeks of culture in the always open construct. Whereas the initially closed then open scaffold possesses cells that are penetrated into the scaffold by the time the scaffold is opened up, protecting them from the fluid shear stimulus (figure 6.8). This indicates that the additional exposure to fluid shear by the initially open construct may actually promote osteogenic differentiation of some cells, even with the presence of the cyclical stimulation of the bioreactor.

In terms of collagen content, both groups did show increasing amounts of collagen up to 21 days. However, the always open configuration actually had a larger expression of collagen type I after 3 weeks. The same trends hold true for collagen type

III, however, at 28 days, the closed then open configuration significantly increases its expression, while the open configuration actually decreases its expression. This could be due to the initially flat construct possibly preferring an osteoblastic lineage. Collagen type III is often found in developing tendons and also comprises about 5% of the total collagen content in mature tendons along with collagen type V.[43,44] Comparatively, bone development begins with mostly collagen type I and small amounts of collagen type III and which provides the base for mineralization.[365,366] This could be the reason they are upregulated significantly at day 21 and then decreased in expression at day 28, as the osteogenic marker, osterix was also upregulated starting at day 21, indicating osteoblastic tendencies. Meanwhile, since mature tendons still are comprised of collagen type III in small amounts, its expression remains upregulated.

Other genes such as COMP, biglycan, and elastin were also investigated. Biglycan contributes to the musculoskeletal system development and is also present in the HUV, although in smaller amounts compared to other proteoglycans.[318,367] It is expressed in high amounts in the developing tendon and responsible for ECM organization.[368,369] By differentiating into musculoskeletal lineages, whether it is osteogenic or tenocytic, the cells appear to be producing more biglcyan by increasing the expression the biglcyan gene to make up whatever deficit may be present. This expression occurred earlier with the cylindrical construct (2 and 3 weeks) compared to the open construct (4 weeks) further indicating construct development may be delayed with the always open configuration. This is also true with the elastin expression, which is another small, but critical component for tendon function.[370] The upregulation of this gene occurs at 2 weeks for the closed system while it is expressed in high amounts after

4 weeks for the always open construct. COMP, a glycoprotein serves many purposes and is present in abundance in tendons.[371] It can help in ECM production by catalyzing fibrillogenesis of collagen fibers and it is also thought to bind and present BMP-2 in bone tissue.[344,372] However, for the always open construct, the highest expression of COMP is present at 7 days. The cells may be producing COMP for fibrillogenesis at this point, and as they are exposed to fluid shear and tending towards osteogenesis, the existing COMP may also possibly be used as a presenter molecule.

6.5 Conclusions

This work has shown that by opening the HUV/MSC construct into a flat sheet, long term bioreactor culturing for tendon tissue engineering can be accomplished. Along with this increased culture time, construct properties such as cellularity, tensile strength, and ECM quality and quantity are shown to be increased. However, by opening up the construct initially, the immediate influence from fluid shear from circulating media caused a delay in tenogenic development and possible osteogenic differentiation of the MSCs present on the scaffold, even with cyclical stretching. This was prevented by allowing the construct to be cultured in a closed cylindrical shape initially for 2 weeks to protect the cells from the osteoblastic influence of fluid shear. Another two weeks of open culture removed any diffusional limitations that had previously occurred within the closed scaffold for long term culture. This 4 weeks of increased culture time resulted in a superior construct compared to previous work that had only cultured the construct for 2 weeks. The increased mechanical and ECM

properties cultured *in vitro* could allow for faster and better integration if the construct were used as a graft for tendon supplementation in the injury recovery process.

Chapter 7: Conclusions and Future Directions

7.1 Conclusions

The studies discussed previously built upon the previous studies of the HUV/MSC construct as a viable option for tendon tissue engineering. Ideally, these studies have pushed the HUV/MSC construct towards a more viable path as a graft alternative in surgical treatment of tendon injuries.

The first study investigated how altering the duration and frequency of mechanical stimulation (previously done at 1 cycle/min and 1 hour/day) at 2% strain. It was found that long duration and faster frequencies did not increase cellular proliferation at 7 days, while the slower frequencies and shorter durations did compared to the static control. In addition, ECM amounts and alignment directly correlated the groups that had significant increases in cell number. When investigating the levels of tendon related genes, it was found that the MSCs expressed high levels of biglcyan and low levels of elastin, which are found in low and high amounts respectively in the HUV, indicating a remodeling by the MSCs into a more tendon-like construct. Furthermore, differentiation appeared to begin after 7 days, with most stimulations upregulating some of the tendon markers, while downregulating the non-tendon markers, with the exception of the 1 hour/day and 1 cycle/min stimulation, which also increased non-tendon markers. Overall, shorter durations and slower frequencies such as 0.5 hours/day and 0.5 cycles/min were most beneficial at least in early culture times of the HUV/MSC construct.

The second study was concerned about supplementing the altered mechanical stimulation with chemical stimulation through the use of tenocytic extract, the

biochemical factors and genetic material released through lysing of mature tenocytes. When this was supplemented during seeding of the HUV with MSCs, the properties of the construct improved, even more so when combined with mechanical stimulation, providing a synergistic effect. Tensile strengths increased by 33% compared to non-supplemented groups, along with an increase in cell proliferation and ECM production. Gene expression indicated tenocytic differentiation was delayed slightly until after two weeks, however, the overall construct was greatly improved compared to not supplementing with extract

Finally the third study investigated how long term culture would affect the construct. Prior to this study, the original cylindrical HUV was limited to 2 weeks culture time in the bioreactor due to mass transport limitations. By opening up the HUV into a flat sheet and exposing it to circulating media, culture times were increased to four weeks. Opening of the construct at the beginning of the culture hindered tendon-like development of the construct, even upregulating osteogenic genes in the MSCs. However, by culturing the construct as a cylinder initially and then opening it after 2 weeks of culture, development improved. After 4 weeks of culture, the ultimate tensile strength had reached 5.6 ± 0.7 MPa and gene expression results indicated the MSCs committed towards a tenocytic lineage after 2 weeks and maintained levels through the 4 weeks of culture.

Overall, these studies built upon and improved the HUV construct. The now 4 week culture time showed increases in ECM production and gene expression compared to the previous studies. In addition, the ultimate tensile strength of the construct increased by 36.6% from 4.1 ± 0.5 in previous studies to MPa 5.6 ± 0.7 MPa.[180] This

tensile strength is approaching some of the physiological levels of some of the weaker tendons in the body such as the flexor tendon and rotator cuff. Therefore, the HUV/MSC construct has a promising future with further improvements as a potential tissue-engineered tendon graft.

7.2 Future Directions

7.2.1 Long-term Mechanical Stimulation Variables

In this study, chapter 4 investigated the frequency and duration of mechanical stimulation. However, this was only for a 7 day culture period. It found that a slower frequency and shorter duration was beneficial at earlier time points. However, other studies have found that different that higher frequencies and durations on other types of tendon tissue engineering constructs have also been beneficial, along with higher strains.

As the HUV/MSC construct matures, it is possible that different stimulation regimes may prove more beneficial than what was most beneficial at early time points. Since the construct requires more than 1 week of culture to develop sufficient tissue properties for in vivo use, it is pertinent to investigate whether different mechanical stimulation parameters may improve upon existing long term results or hasten the achievement of these results. This could include investigating a wider range of durations and frequencies investigated in chapter 4 or also changing the amount of strain provided by the bioreactor, which was not changed in chapter 4.

Furthermore, strain rates were not varied in these studies. It would be beneficial to see if changing the strain rates at early time points and also later time points (when the ECM has matured and improved) may impact construct development.

7.2.2 Supplementation with Growth Factors and Extract Improvement

The tenocytic extract has shown to significantly increase the properties of the HUV/MSC construct. However, there are three potential issues with this technique.

First, the mature tenocyte most likely does not provide all of the signaling to developing tendons in terms of ECM deposition, stem cell differentiation, and vascularization. Vascularization is absolutely necessary at some point during tissue maturation, without it, when in the body, transport in the interior of the tissue will eventually be limited without adequate blood flow. VEGF supplementation either at the end of *in vitro* culture or prior to implantation could potentially initiate neovascularization when the construct is placed into the body. This is only one example that specific growth factor supplementation may provide that the extract cannot.

Second, the exact composition of the extract is not known. A better understanding of the composition of the extract could be beneficial. The extract can then be supplemented further with missing growth factors to further enhance construct development.

Finally, the life of this extract during initial supplementation is short lived. To circumvent this, two options present themselves. The extract could be encapsulated prior to placing in the HUV to control release rates and protect the extract. Second,

when the construct is opened up in bioreactor culture, more extract or growth factors could be placed in the circulating media to maintain soluble factor levels. This may further improve construct properties. As it is now implemented, only the early culture is exposed to the biochemical cues, although as it is seen it does positively affect future development.

7.2.3 Engineering of Bone-Tendon Enthesis

As discussed in the introductory sections, the tendon consists of three major zones: the main tendon midsubstance, the bone-tendon junction, and the muscle-tendon junction. To facilitate greater acceptance of the graft and more complete healing, the ideal tendon construct would mimic this architecture.

Of these, the tendon-bone enthesis is the most complex structure. It consists of transition zones from tendon to fibrocartilage to bone. It is also the location of many tendon repair failures after surgery due to a lack of quality in the enthesis development.[373] To facilitate stronger attachment, a fibrocartilage end of the construct should be developed. If the construct was attached directly to the bone where a zonal enthesis is normally present, mechanical stability could be hampered initially or development of the enthesis could be poor compared to a natural enthesis. This could be done utilizing gene transfection of the cells on one end of the construct or a hydrogel with cartilage related growth factors or chondrocytes attached to the end of the scaffold. Gene transfection has been done in MSCs in prior studies to influence chondrocyte development.[374,375] Furthermore, it has been shown that uniaxial stretching is beneficial in fibrocartilage formation from MSCs differentiated into chondrocytes, creating a

symbiotic relationship with the cyclical stretching already present within the bioreactor. This fibrocartilage zone could then be further developed by adding osteogenic stimuli to promote osteogenesis in at the surface to further mimic the enthesis of the tendon.

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