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Proof of Concept Studies for Point of Care Rotator Cuff Tissue Engineering

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PROOF OF CONCEPT STUDIES FOR POINT OF CARE ROTATOR CUFF TISSUE ENGINEERING

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Bioengineering

by
Alison Welch
May 2016

Accepted by:
Jeremy Mercuri, PhD, Committee Chair
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ABSTRACT

Injury and degeneration of rotator cuff tendons are frequent causes of shoulder disability globally, commonly affecting athletes and the elderly. Annually, there are approximately 250,000 surgeries related to the rotator cuff performed in the United States.\(^1\) With rotator cuff tears being the largest cause of rotator cuff surgery, between 10% and 40% of all rotator cuff tears are classified as massive, extending over 5 cm in length.\(^2,3,4,5,6\) The success rate of massive rotator cuff tear repair is variable, with re-tear rates ranging from 38% to 90% depending on factors such as patient age, tear chronicity, and overall tissue quality.\(^7,8,9,10\) A primary factor causing failure is poor tissue quality due to poor tissue regeneration by sparse and inactive resident tenocytes.\(^11\) Current repair approaches (biceps tenotomy/tenodesis, muscle-tendon transfer, and biologic tissue grafts) suffer from shortcomings because they do not address tissue quality nor do they attempt to regenerate damaged tendon.\(^12,13,14,15,16,17,18,19\) Therefore, it would be advantageous to develop a therapy that can cause tissue regeneration by addressing the issues of tenocyte inactivity and poor tissue quality.

Our proposed approach for massive RC repair utilizes autologous adipose derived stem cells (hADSC) and an autologous tendon graft scaffold to biologically augment suture repair by delivering stem cells and promoting their differentiation. This approach aims to improve the quality of naturally formed matrix at the repair site over time through integration of the scaffold with the damaged tendon. This method can be performed entirely at the point of care, reducing additional processing time, external expansion of cells, and tissue contamination. Herein, we propose to address proof of concept studies...
illustrating the following: 1) the ability to reliably form macro-porous (meshed) scaffolds from a tendon source. 2) The viability of tenocytes within scaffolds. 3) The mechanical properties of the scaffold. 4) The ideal autologous tenocyte source for differentiation of hADSCs. 5) The ability of the scaffold-stem cell construct to integrate with underlying tissue.

Results presented illustrate our ability to fabricate a macro-porous tendon scaffold of 187% expansion and the properties of said scaffolds. Once scaffold fabrication was established, the properties were assessed. When tendons were meshed to fabricate a tendon graft scaffold cell viability was decreased by 30%, elastic modulus was decreased by 32.45 MPa, and ultimate tensile stress was decreased by 1.82 MPa. The ideal tendon source for fabrication of tendon graft scaffolds was determined to be semitendinosus tendon over long head biceps tendon via hADSC differentiation profile. Lastly, in order to determine if the scaffolds would improve the underlying tendon quality, the integration of the proposed construct was illustrated in vitro.
DEDICATION

I would like to dedicate this thesis to my family. My overachieving mom, understanding dad, highly underestimated sister, resilient grandmother, brilliant grandfather, “cool” aunt (good luck figuring out which one of you this is), hilarious uncles, not-so-little-anymore cousins, and friends who save me when they get to be all too much. Thank you for always supporting me in my personal and academic ventures. You are the best.
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CHAPTER ONE

LITERATURE REVIEW

1.1 Shoulder Anatomy

The shoulder consists of three bones, the clavicle, the scapula, and the humerus. The moving joint of the shoulder, the glenohumeral joint, is a ball-in-socket joint connecting the head of the humerus at the glenoid fossa of the scapula. Additionally, the clavicle is attached to the scapula at the acromion via a plane-synovial joint allowing for significantly less motion than the glenohumeral joint.

Figure 1: Illustration of the bony anatomy of the right shoulder, viewed from the posterior.
The rotator cuff is a series of four muscles connecting the scapula to the proximal humerus. The tendons of the rotator cuff are present at the humeral interface of the muscles. The supraspinatus tendon is located on the superior surface of the greater tuberosity of the humerus. The infraspinatus tendon is located on the posterior-superior surface of the greater tuberosity of the humerus. The subscapularis tendon is located on the anterior surface of the lesser tuberosity of the humerus. The teres minor tendon is located on the posterior-inferior surface of the greater tuberosity of the humerus. The muscles of the rotator cuff provide stability to the glenohumeral joint in normal movement such as rotation, elevation, and abduction. Additionally, the location of the subscapularis prevents anterior translation of the humeral head.

Figure 2: Image of the rotator cuff tendons of the right shoulder shown from the medial view at their insertions onto the humerus.\textsuperscript{21}
In vertebrates, tendon is a tissue that connects muscle to bone. During muscle contraction, the tendon allows for force transfer to the bone resulting in movement of the musculoskeletal system. Due to this role, tendons are often under high levels of tensile stress. As their form follows their function, tendons are a highly aligned dense connective tissue made up of strong aligned collagen in the direction parallel to the applied forces between the muscle and bone. As a dense connective tissue, tendon has a high matrix to cell ratio.

Figure 3: Hierarchical structure of the tendon microstructure. Sub-fibrils in this image are known as fibrils and fibrils are then known as fibers.22

Tendon extracellular matrix, ECM, has a hierarchical structure similar to that seen in muscle structure (Figure 4). At the smallest level, individual molecules of Collagen I wind together to form a tropocollagen molecule. Bundles of aligned tropocollagen
molecules form fibrils, which can be full millimeters in continuous length and anywhere from 50 to 300 nm in diameter. Fibrils of all sizes are collected together into a larger fiber and a surrounded by the endotendon sheath. The endotendon is comprised of uniform and thin (50 nm) collagen microfibrils arranged helically along the length of the tendon. Fibers, along with tenocytes that align themselves with the fibers are bundled into fascicles that are then bundled again into the total tendon. Fascicles are surrounded by the epitenodinous sheath, which they are then combined and surrounded by the peritendinous sheath to form the whole tendon. The peritendon sheath is made up of the same helical microfibrils as the endotendon, but the peritendon is made up of more variable thickness, straight microfibrils. Within the ECM of tendons, 60% to 85% of the dry weight is composed of collagen. Collagen I is the major collagen component, making up 95% of the collagen, with lower levels of other collagen types Collagen III, IV, and V. Collagen III is frequently seen in tendons, especially when they are repairing from damage as the thinner fibers are more quickly produced to repair an injury site. Riley et al. saw a correlation in an increase in Collagen III presence in damaged rotator cuff tissues compared to macroscopically healthy tissue in correspondence with a slight decrease in Collagen I content. The other proteins present in the ECM are various types of proteoglycans, glycoproteins, and glycol-conjugates with differing purposes for the function of the tendon. The most abundant type of proteoglycans within the tissue is a type of small leucine-rich proteoglycan (SLRP) called decorin. Decorin makes up approximately 80% of the proteoglycan content of the tendon. SLRPs found in lesser quantities include biglycan, fibromodulin, and lumican. All of the SLRPs share similar
end functions in modification of collagen fibrils during assembly and maintenance of tendon integrity. Decorin specifically has been implicated in facilitating the formation of collagen crosslinks during fibril formation.\textsuperscript{29,30} Large aggregating proteoglycans are a less commonly found type of proteoglycan within tendon, but they are present in the forms of versican and aggrecan. These proteoglycans increase the water content of the tissue resulting in a higher compressive strength, and are therefore often found regionally in areas of tendon where compression is more commonly experienced. Glycoproteins of tendon include lubricin, collagen oligomeric matrix component (COMP), tenascin-C, and tenomodulin. COMP has been shown to promote collagen-to-collagen binding, and therefore work with the SLRPs to promote collagen fibril assembly.\textsuperscript{28} Tenascin-C is present in the interfibular matrix with COMP and the SLRPs but is most prevalent within immature tendon. Tenomodulin has been shown to be a regulator of cellular proliferation and adherence to the ECM.\textsuperscript{31} Lubricin is thought to, along with elastin, be the enabling factor in sliding and recoil between fascicles as well as being present in the epitendinous sheath surrounding the tendon body.\textsuperscript{23} Both proteins are present within the endotendinous matrices, which are less dense and collagenous than the tendon body itself, allowing for mechanical distribution throughout the tendon.
Figure 4: Histological image (H&E) of tendon, illustrating fiber and cellular alignment. Nuclei are dark and extracellular matrix is stained pink.

The cells within tendon, tenocytes, are sparse compared to other tissues but remain aligned with the collagen fibers of the matrix. Tenocytes make up less than 5% of the total volume of tendon tissue.\textsuperscript{32} Tenocytes are not characterized by one specific marker, but by an array of markers and morphological qualities. Similarly to fibroblast, of which tenocytes are often considered a derivative, tenocytes are elongated and characteristically spindle shaped. Cell markers that are known to be present on tenocytes include Scleraxis B (SCX), Tenascin C (TNC), Tenomodulin (TMD), Collagen I (COL1), and Collagen III (COL3).

In 2010, Pauly et al. investigated tendon cell cultures as a way of understanding tendon biology on a fundamental level.\textsuperscript{33} They screened cells for markers of collagen I, collagen III, decorin, biglycan, aggrecan, tenascin C, scleraxis, tenomodulin, collagen II,
and osteocalcin. Comparing collagenase isolated rotator cuff tenocytes to osteoblasts and chondrocytes, they sought to determine what markers were indicative of the tenocyte phenotype. In terms of markers, no singular marker was shown to be a clear indicator of tenocytes. Scleraxis was shown in differing levels in all cell populations, as well as tenascin and tenomodulin. It is therefore important to show the combination of these markers with collagen I and collagen III in the correct ratios in order to determine if a cell population contains tenocytes.

1.2 Clinical Significance of Rotator Cuff Tears

Injury and degeneration of the rotator cuff tendons are frequent causes of disability with approximately 250,000 surgeries related to the rotator cuff performed annually in the United States. The most common surgical procedure of the shoulder is the rotator cuff tear repair. Rotator cuff tears may occur acutely or chronically. Acute injury of the tendons often occurs in younger patients due to rapid, forceful movement such as those found in athletes. When an injury persists over an extended period of time or a small injury gradually increases in severity over time due to repeated functional movements, a tear is classified as chronic. Chronic tears are often found in the elderly and result in gradual degeneration of the tendon tissue and inflammation at the injury site. Rotator cuff tears can be categorized by many methods. A tendon tear may be graded as full or partial thickness, either penetrating part or all of the way through the tendon respectively. Partial tears are simpler to repair as they retain some connection between the tendon edges and humerus and do not require tensing the edges together to repair. A full
thickness tear is then often classified again by size. Two methods of classification are the Colfield and Bateman methods.\textsuperscript{3} Both classified tears by their length torn from the humeral head in the anterior-posterior direction. Colfield classified tears at the time of surgery a small tear as less than 1cm in length, medium as 1-3cm, large as 3-5cm, and massive as greater than 5cm.\textsuperscript{2,4,5,6} Bateman, however, in a slightly less commonly used system graded the tears after surgical debridement as Grade 1 for less than 1cm in length, Grade 2 for 1-3cm, Grade 3 for 3-5cm, and Grade 4 for a global tear with no remaining cuff left after debridement. Massive tears, as classified by Colfield, account for between 10% and 40% of all full thickness rotator cuff tears.\textsuperscript{4} Unfortunately, within massive rotator cuff tears, repair failure occurs at a rate of 38% to 90% depending on factors such as patient age, tear chronicity, and overall tendon quality.\textsuperscript{7,8,9,10} The most common method seen of re-tear after rotator cuff repair surgery, regardless of patient age or suture anchor method used, is suture tear through at the suture-tendon interface.\textsuperscript{34} This exemplifies that re-tear is highly effected by tendon quality, which is reduced by cellular senescence of resident tenocytes. Cellular senescence is a state in which the cell cycle is arrested and is unable to be reactivated leading to poor proliferation and survival.\textsuperscript{11} Senescent cells have been shown to express high levels of matrix metalloproteases (MMPs), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), and pro-inflammatory cytokines. MMPs and ADAMTS are cytokines that break down collagen and proteoglycan molecules respectively, thus causing overall matrix breakdown. Pro-inflammatory cytokines upregulate inflammatory reactions in the tissue. In tenocytes, cellular senescence can occur due to aging or, in younger patients, due to
injury. Due to the high instance of re-tear in some surgical conditions, specifically in chronic and massive rotator cuff tears, a complete rotator cuff repair is not always possible. Talbot et al. discussed the theory of extrinsic tear, or impingement, where damage is caused by repetitive micro-trauma under the acromion. Intrinsic theory considers degeneration of the rotator cuff with age, with relative de-vascularization of the tendon making the cuff more prone to injury.11,12,27

1.3 Current Treatment Options for Rotator Cuff Repair

The most common surgical procedure of the shoulder is the rotator cuff repair. The repair begins with debridement of the torn tissue ends, the tendon end to remove dead tissue, and the bone insertion to remove dead tissue and stimulate the cancellous bone to bleeding in order to stimulate bone marrow. Suture anchors are drilled into the bone to affix the suture to the bone side of the repair. Multiple suture anchors can be used, depending on the size of the tear. Suture is used to connect the two sides of the repair using single or multiple rows, simple or mattress style knotting methods.35
Tension is then applied across the suture to tighten and close the gap between the tendon and bone. This may be difficult in larger tears as less tissue is remaining to cover the area previously spanned by the tendon, resulting in an increased tension applied to the remaining tendon. Rotator cuff surgery may be performed either open or arthroscopically within the joint, however with an increase in size of tear surgeons may choose to perform an open surgery more often than an arthroscopic one. In 2012, Colvin reviewed national trends in rotator cuff repair and how they have changed from 1996 and 2006. In 2012, Colvin reviewed national trends in rotator cuff repair and how they have changed from 1996 and 2006. They saw an increase in outpatient procedures in 2006 especially for those patients between 45 and 64 and over 65. More patients over 65 were

Figure 5: Image of suture repair methods for rotator cuff repair. A) Suture anchors within the humeral head. B) Double row simple suture configuration. C) Double row mattress suture configuration.
also having open procedures, but less were having them in the 45-64 age group and only slightly more under 45. Generally they noticed that old age was associated with the increased prevalence of RC tears but that surgeons have become more aggressive with early repair. Massive tears have been more often seen in older patients, as they have had more time to degenerate to a larger form.

There is also an option to avoid surgery and seek physiotherapy. Physiotherapy consists of a series of exercises to correct upper body posture and to improve scapulothoracic and glenohumeral muscular control and stability. In 2014, Moosmayer et al. reported on a study in which 103 patients were subjected to surgical treatment (open or mini open) (52) or physiotherapy (51) for rotator cuff tears. The tears were full thickness but less than 5cm in length. The physiotherapy exercises were performed for 40 min 2 times weekly for 12 weeks. 12 patients chose to have surgical repair after seeing insufficient effects from physiotherapy. Results were better in the primary tendon repair group but differences between the surgical repair groups were small. Constant score, or the reported comfort and functionality, was significantly higher in primary tendon repair group compared to physiotherapy only. When compared to secondary repair, the difference was not significant. 38 physiotherapy patients saw an increase in tear size of less than 5mm and greater than 5mm in 14 cases. Full thickness re-tear was seen in 8 and partial thickness re-tear in 7 of the surgical cases. So, when comparing results between surgical and physiotherapy interventions, surgery seemed to have the better patient outcome. However, if other physiotherapy methods are found over time that surpass the
quality of the ones used by Moosmayer and his team, this study would have to be re-analyzed with the new exercise options.

In 2012, George et al. looked at the causes, complications, and approaches in healing small and medium size tears after failed rotator cuff repair. They noted that failed repairs were usually due to multiple factors including surgical complication, diagnostic errors, hidden lesions, technical errors, and poor tissue. The overall surgical complication rate was 10% from factors such as failure of the deltoid, infection, foreign body reaction, stiffness, and neurologic injury. Diagnostic errors, such incomplete diagnoses were shown to be a cause of some instances of re-tear. Additionally, supraspinatus tears were shown to have hidden lesions of the rotator interval, long head biceps tendon, or subscapularis. Technical errors in repairing specific tear shapes can cause re-tear as there are many tear shapes that may present difficult in a rotator cuff repair such as crescent, u-shaped, l-shaped, or massive contracted immobile. However, regardless of other factors, re-torn cuff tissue is usually of poor quality with increased muscle atrophy and fatty infiltration. Authors suggested augmentation of repairs when tissue quality is found to be poor or lacking.

Due to high re-tear rates and a relatively low amount of reparable tissue, chronic massive rotator cuff tears are often deemed irreparable and not operated on. In these cases, physical therapy for strengthening of the surrounding muscles is often used to retain some shoulder function. Talbot et al. looks at the other options for dealing with massive rotator cuff tears that are deemed irreparable. Patients with massive irreparable rotator cuff tears present with variable function and pain. Tendons that cannot be mobilized to repair are
considered irreparable. Non-operative management of these tears includes analgesics, steroid injections, and activity adaptations. These management methods, however, have not been shown to alter the eventual degenerative course of a chronic tear. Deltoid “re-education programs”, where patients have used physical therapy to train the deltoid to function in place of the torn muscle, have shown marked increases in Constant scores, especially within the elderly. Operative options that are not complete rotator cuff repairs include simple debridement of dead tissue, subacromial decompression, and biceps tenotomy. These options reduce the amount of extra degenerating tissue in the injury area and often show reduction of pain, though do not cause a change in function. In subacromial decompression, the tissues in the capsule under the acromion are released to increase the range of motion of the join and reduce the likelihood of impingement. Biceps tenotomy also reduces pain by removing the long head of the biceps tendon and freeing that area from additional wear and inflammation. The short term pain reduction benefits of any of these methods may not be retained over time. Supra-scapular nerve ablation showed improvements in pain scores that were not maintained over 3 months by desensitizing the nerve. Partial rotator cuff repairs focus on reinforcing mechanical properties rather than repairing anatomical structure. Tendon transfer surgeries (latissimus dorsi, deltoid) aim to restore function in younger and higher demand patients. These surgeries remove the tendon and muscle from their original insertion and replace them at the torn rotator cuff insertion, where their contraction then functions to move the shoulder rather than their original purpose. Functional scores increase about 25% after surgery but loss of strength is not corrected and loss of functionality is present at the site of the
removed tendon-muscle pair. Augmented repairs are often used, but there are many options that require more study. Arthroplasty, or shoulder joint replacement, is a last ditch solution and does have high benefits when successful. However it can wear over time in young patients and has a variable success rate especially in those with previous shoulder surgery.

1.4 Tendon Tissue Engineering

Tissue engineering in application for rotator cuff repair is the manipulation of cells, scaffolds, and tissues to repair and hopefully regenerate the tendon tissue in the rotator cuff back to a pre-torn state.

1.4.1 Scaffolds

In the repair of tendons, including rotator cuffs, there is often a need for augmentation of the original tissue if that tissue is damaged or removed due to injury or disease. Scaffolds are a material used to augment tissue, provide a delivery environment for cells, and provide signaling for healing. Scaffolds can act as a replacement for that tissue either in temporary or a long-term state. Some scaffolds are used to off-load the tissue at the repair site from experiencing excess tension by acting as a mechanical augmentation while others may act to help guide the healing of the repair site through biological augmentation. In biological augmentation, scaffolds can act as a delivery vehicle for chemicals, growth factors, or cells to the repair site.
Scaffolds can be biologically or synthetically based, being made from protein extracellular matrices or synthetic polymers respectively. Advantages attributed to biological scaffolds include having a well-defined 3D surface protein microstructure and a natural porosity. They can quickly interact with host tissue and induce new tissue formation faster than synthetic grafts. Limitations of these grafts include relatively low mechanical properties, non-specific induction, undefined degradation, and variable biocompatibility compared with synthetic materials. Synthetic scaffolds are manufactured from chemical compounds, allowing control of chemical and physical properties, higher mechanical strength, and consistent quality. However, synthetic materials often come with poor biocompatibility and a lack of integration potential.

In 2010, Longo et al. performed a systematic review of the most commonly used biological and synthetic grafts for tendon on the market at the time. The review gave a brief overview of the products’ benefits and drawbacks found for the rotator cuff area. Biological scaffolds are usually small intestine submucosa (SIS), dermis, or pericardium and are de-cellularized. Figure 5 shows the table of grafts analyzed in the article.
Table 1: The most popular commercially available grafts as analyzed by Longo et al. (2010).\textsuperscript{41}

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In the lab, SIS has been shown to have poorer results than autologous implants. However, some success was seen with cell seeding on said grafts. Collagen patches tended to cause high inflammation. PLA patches also did not see significant load increases. Additionally, in a clinical study patients with xenografts had less strength than control repairs, more impingement of rotation, and slower healing rates, specifically with SIS that was shown to not be fully de-cellularized as claimed by the manufacturers. GraftJacket, human dermal matrix, showed increased strength, and statistically significant
improvements in pain, forward flexing, external rotation, and full graft incorporation in 13/16 patients in clinical trial. In terms of synthetic grafts, polymer filamentous carbon composites and BMSC cell seeded knitted PLGA fiber scaffolds showed good histological and mechanical outcomes and a higher volume of regenerated tissue, higher tensile stiffness, and higher modulus compared to non-seeded controls respectively. The authors noted after these reviews that both fiber alignment and cellular seeding showed benefit in grafting, in addition to full de-cellularization of xenogenic materials.

Other types of scaffolds are still under investigation, such as those reported on in 2015 by Alberti et al. The researchers use a technique they call “bioskiving” to create scaffolds from de-cellularized tendon. They cryosection de-cellularized tendon into 50um thick slices, stack them while frozen, and then air dry to fuse the stacks. The dried tendon stacks are then washed in 5% glutaraldehyde and PBS for 30 minutes and air dried again before use. Alberti and his team investigated the effects of section orientation and glutaraldehyde crosslinking concentration on tensile strength of the scaffolds produced. Non-crosslinked samples had the lowest UTS and modulus values. They also showed sliding of fibers in the midsection. 2.5% glutaraldehyde for 20 min resulted in the highest UTS value and a modulus. According to scanning electron microscopy (SEM) imaging, non-crosslinked samples fail with smooth edges as if the failure occurred by adjacent fiber pull apart, whereas crosslinked samples fail at a rough edge where fracture may have propagated and ended fibers on a micro scale. When force was applied along the direction of the fibers (0 degrees), the UTS was the highest. When force was applied at 45 degrees and 90 degrees (perpendicular) the UTS values fell with an increase in
angle. The moduli followed the same trend. When fiber angle was alternated, the strongest samples other than samples that were all aligned had were aligned at 45 degrees.

The methods of using grafts are still under research to determine the most beneficial application of grafts for rotator cuff augmentation. In 2012, Mihata et al. looked to cadaveric models to determine the best of 3 tested patch-grafting methods. The three methods were tendon reconstruction, capsule reconstruction, and a method that includes both (hybrid). The grafts were made from fascia lata of allografts that was about 5mm thick when folded 2 to 3 times and sutured at the open edge. Lengths and widths of the grafts were best matched to lengths and widths required for repairs. Balanced load and superior force tests were performed with a total of 110 N distributed. Under superior force, the tendon repair resulted in a significant superior translation. Under capsule and hybrid repairs, the translation was not significant, nor was the difference between the movement of capsule and hybrid repairs. Capsule reconstruction reduced the range of motion by 16 degrees, and there was not a significant difference between that and hybrid. Glenohumeral force was not restored by any of the methods tested.

In 2008, Moffat et al. developed an electrospun poly(D,L-lactide-co-glycolide) (PLGA) polymer scaffold for rotator cuff repair. They used electrospinning to create aligned nanofibers on which they then cultured rotator cuff fibroblast-like cells for 3 and 14 days. Gene expression of collagen type I appeared after 3 days of culture whereas collagen type III expression remained the same in aligned matrices, non-aligned matrices,
and monolayer controls across all timepoints. Mechanically, *in vitro* culture decreased the ultimate tensile stress (UTS) and yield strength of the scaffolds over time when they were not seeded with cells. However, the application of cells caused the mechanical changes to halt, resulting in no significant changes to mechanical properties over time. Additionally, at all time points, the aligned scaffolds had greater tensile properties than those that were not aligned in both cellular and acellular groups.

In 1998, Young et al. studied the use of collagen gel as a scaffold for delivery of stem cells to an Achilles tendon defect. They created a construct from bone marrow derived stem cells and type I collagen derived from bovine skin by suspending the cells in the collagen solution and then gelating it between two sutures. The sutures were then used to implant the constructs into the Achilles tendons of rabbits after 40 hours of culture. After 4, 8, or 12 weeks, the tendons were mechanically tested. Samples with the constructs measured higher on average for stiffness, maximum force, modulus, maximum stress, reaction force energy, and failure energy at every time point than suture repaired controls. The constructs also exhibited aligned and elongated cell morphology within the repair as compared to variable morphology within the controls. These results supported their initial hypothesis that cells and an organized collagen matrix would improve repair biomechanics.

In 2014, Pawelec et al. looked at the influence of fibrin gel on the morphology change of tenocytes. Fibrin is a gel that does not inhibit ECM formation. In Pawelec’s study, gel was made using 1% fibrin and aprotinin (.15 U/ml) in PBS. Gelation occurred by immersion in 300ul of 0.4 U/m thrombin for 10 min. Samples of tenocytes were either
plated alone, with fibrin gel, or with fibrin gel on type 1 collagen scaffolds. Metabolic activity of cells was seen to increase with the addition of fibrin gel. Fibrin gel on scaffolds yielded the highest number of cells, scleraxis expression, and tenomodulin expression. The addition of fibrin gel did not cause an increase in COL1 production when normalized to cell number. This study showed the benefits of applying physiological proteins for the expression of tendon properties but that mechanical support via a scaffolding or graft system proves more beneficial than unsupportive proteins.

In 2014, Beitzel et al. compared highly crosslinked collagen, non-crosslinked collagen, and fibrin matrices to human rotator cuff tendon. They loaded them with bone marrow derived stem cells and performed cell adhesion, proliferation, histology, and scanning electron microscopy (SEM). They determined that greater numbers of cells adhered to the non-crosslinked and fibrin scaffolds compared to the samples. Non-crosslinked collagen samples saw higher proliferation rates compared to fibrin but no significant differences with other scaffolds. In histological analysis, cells adhered to the edges of all scaffolds. Both collagen membranes allowed cellular migration into the center of the scaffold. However, the rotator cuff tendon showed the highest number of cells compared to the highly crosslinked collagen and the fibrin. Using SEM, porosity was measured, showing that rotator cuff tendon has 20-30% porosity, while non-crosslinked collagen was 60-70% porous. These differences were attributed to the varying matrix components and constructions, indicating the necessity of tailored matrix properties to match those found in rotator cuff tendon.
In 2010, Sano et al. reported on a clinical study in which 20 patients underwent repair of massive rotator cuff tears with long head biceps tendon grafting in order to study the repair integrity. The protocol used in this paper included the removal of the long head biceps tendon (LHBT) by tenodesis and splitting the tendon graft into two layers width-wise if it was smaller than the residual defect. The graft was attached to the original cuff tendon with suture that then snaked through the patch tendon and ended at a bony insertion. 14 of the 20 patients followed up for analysis after 12 to 48 months. They were analyzed via Japanese Orthopaedic Association (JOA) shoulder scores which monitored levels of shoulder pain, range of motion, muscle strength, muscle durability, and ability to perform activities of daily living. All JOA parameters increased significantly on average after follow up. Repair types shown Radiologically, 13 patients showed no re-tear and 1 showed a minor discontinuity. Of the 13 without re-tear, 3 showed sufficient thickness of repair and 10 showed no discontinuity but insufficient thickness compared to a healthy tendon. Though the study size was small, the results did show results of a decrease from normal re-tear rates with the application of the graft. The insufficient thickness, however, indicates that additional help would be required to bring the cuff back to a healthy state on a reliable basis.

1.42 Cell Sources

As tendons have a low cell density and damaged tendons have less active resident tenocytes, the application of an alternative cell source is often required for tissue engineering. When a healthy alternative cell source is applied, active cells are then
present to re-build the damaged matrix to a one more fitting of the healthier cells. This can result in a final tissue with mechanical and chemical properties more similar to an undamaged tissue.

In 2015, Gungormus et al. performed a study to determine if cells would improve the effects of a graft in a tendon repair situation. They compared allogenic de-cellularized tendon matrix with allogenic re-cellularized matrix for implantation into an Achilles defect model in rats. They removed Achilles tendons from rats and de-cellularized them, either than re-seeding them with tenocytes or leaving without cells. Surgically, both Achilles tendons were operated on in each rat. Rats received either a repair using normal suture technique, de-cellularized ECM or re-cellularized ECM. Groups were assessed at 2, 4, 6, 8, 12, and 24 weeks for histopathological and biomechanical analysis. Histopathologically samples were studied for inflammatory cell concentration, cell number, cell distribution, ECM organization, collagen fiber alignment, and metaplasia. Within each week and each criterion studied the re-cellularized ECM group performed best, achieving most successful structural tendon recovery and results more closely mimicking healthy ECM. Biomechanically, re-cellularized ECM had a significantly higher UTS than de-cellularized ECM or suture repair. With any scaffold application, stiffness increased over time. This study showed the benefits of combining cells and scaffold for the repair of tendon tissue toward a healthier morphology and strength.

Stem cells are most simply defined as cells that have the capability to replicate indefinitely and differentiate toward many different final tissue types. Within the adult body, there are populations of stem cells present in various tissue types. These are known
as adult stem cells. As adult stem cells, they do have more limited differentiation potential as compared to embryonic stem cells, which are able to differentiate into all cell types. An adult stem cell is said to be pluripotent, or able to differentiate into many cell types, and can be known as a mesenchymal stem cell (MSC) or hematopoietic stem cell (HPSC). MSCs can differentiate into osteoblasts, chondrocytes, myocytes, stromal cells, fibroblasts, tenocytes, adipocytes, and dermal cells. HPSCs differentiate into blood cells and lymphocytes. MSCs are used in tendon treatment due to their differentiation potential and their homeostatic properties in tendon. The homeostatic properties most beneficial in a damaged tendon environment include anabolic effects on paracrine signaling, direct anti-inflammatory effects on surrounding cells through chemokine upregulation, chemoattraction of other local cells for repair, and anti-apoptotic effects. Commonly used sources of MSCs include bone marrow (BMSC) and adipose tissue (ADSC). Both cell types have been shown to express the requisite adult stem cell markers such as CD10, CD13, CD73, and CD105.
Figure 6: Graphic of the differentiation lineages of mesenchymal stem cells.  

BMSCs are studied frequently in literature for their differentiation capabilities towards many lineages and are also used in combination with the hematopoietic bone marrow stem cells in bone marrow transplants after radiation to regenerate the bone marrow for a source of immune cells and cells for restoration of other tissues. A bone marrow aspirate, usually taken from the iliac crest of the pelvis, is removed, centrifuged to concentrate the cells, and then either expanded in vivo to produce more or used immediately. BMSCs are very well characterized as stem cells and have been used for much longer than ADSCs. There is a limit, however, to the amount of bone marrow that can be extracted from aspiration site of which 0.01% to 0.001% of cells are BMSCs. ADSCs are isolated from adipose tissue, which is more accessible and abundant than bone marrow with 1% to 10% of cells being ADSCs. Lipoaspirate or adipose excision is taken and, in the case of the whole tissue excision, broken down by collagenase digestion. The resulting liquid is then spun using density gradient centrifugation wherein layers of differential density separate from the suspension and can be isolated. The cell pellet obtained is termed the stromal vascular fraction (SVF) and contains the ADSC population. Cells can then be plated for expansion or used immediately. Although the concentration of ADSCs within adipose tissue remains relatively low, the ability to retrieve additional tissue and reduce surgical pain compared to a bone marrow aspirate has researchers considering this option.

In tendon tissue engineering, differentiation of stem cells allows for the creation of a newly active tenocyte population. Since tenocytes become less metabolically active
as they age, creating a young tenocyte population via differentiation is a good way to increase the matrix production capacity of the tenocyte population as well as increasing the size of the population in general.58,59

In 2013 Kraus et al. showed the tenogenic differentiation potential of ADSCs by a co-culture of human flexor tenocytes and ADSCs.60 They used direct co-culture in 3:1, 1:1, and 1:3 ratios of tenocytes to stem cells, indirect co-culture in those same ratios, and media conditioning. All differentiation studies were performed over 10 days. Media for conditioning was taken from 70-90% confluent tenocytes cultured for 24 hours in said media. It was then fed at a 1:1 ratio with fresh media to ADSCs. They used IHC and PCR for tendon markers Collagen I, Collagen III, Scleraxis B, Tenascin C, Elastin, Tenomodulin, and C-FOS to determine differentiation and to determine which markers best indicated tenogenic differentiation. In direct co-culture, they saw 3.3 to 3.6 fold increase in Tenascin C (TNC) expression depending on ratio. Scleraxis B (SCX) was up-regulated 1.5 fold in 1:3 ratio. They also noted that proliferation was higher with higher ratios of stem cells to tenocytes. For media conditioned cells, TNC was up-regulated 2.5 fold and Collagen III (COL3) was up-regulated 1.4 fold. There was no up-regulation seen in other markers. In the indirect system, TNC was up-regulated 2.3 fold. Overall TNC and to a lesser extent SCX and COL3 were shown as good tendon markers, with 1:3 ratio yielding higher differentiation. However higher ADSC levels were shown to yield higher proliferation in this study.

In 2011 Gulotta et al. sought to determine if using SCX transduced BMSCs for rotator cuff augmentation would improve tendon quality in healing, specifically at the
bony insertion.\textsuperscript{61} To transduce the cells they used and adenoviral vector to add the coding for SCX to the existing genome of the BMSCs and determined that SCX expression was 115-fold higher in transduced cells than in normal cells. They performed an \textit{in vivo} rat study for repair of the supraspinatus tendon with injection of BMSCs. Animals received either transduced or natural BMSCs but there was no natural control used. Histology was performed at 2 and 4 weeks using H&E, safranin-O/fast green, and picrosirius red. At 2 weeks, there was no statistical differences seen, but at 4 weeks there was an increase in fibrocartilage at the interface. Biomechanical tensile testing was performed at 2 and 4 weeks under a 45N load cell. The samples were pre-loaded to 0.1N, loaded at a rate of 14 microns/s until repair failure. All samples failed at the tendon-bone interface. At 2 weeks there were increases in UTS and stiffness with SCX compared to non-SCX MSCs. At 4 weeks, UTS, stiffness, and ultimate load were all higher in the SCX augmented group. These results indicated that the fibrocartilage interface between the tendon and bone did increase in size over time with SCX augmentation, and that the repair strength also increased with SCX augmentation. This study used a know gene for tendon ECM protein scleraxis to directly effect BMSCs and resulted in increased strength in healing at the tendon-bone interface after 4 weeks.

In 2014, Hernigou et al. performed arthroscopic rotator cuff repair on 90 patients applying concentrated autologous BMSCs to the repair via injection in 45 patients.\textsuperscript{62} Patients were limited to those with tears of the supraspinatus alone and a full thickness tear size of 1.5 to 2.5 cm in diameter. Control patients were matched for tear size, rupture location, dominant shoulder, gender, and age. These control patients were taken from
patients who had the same repair without BMSCs prior to 2000. In the BMSC procedure 150mL of marrow was isolated and then concentrated down to 12mL. The repair was performed via a single row repair using suture anchors to the abraded lateral edge of the humeral tuberosity. 4ml of BMSCs were injected between the bone and tendon and 8mL were injected into the bone at the site of the footprint. The number of cells injected was recorded for each surgery and averaged 4300±1800 cells per milliliter. At the 10 year follow up mark, 39/45 (87%) of BMSC patients had intact rotator cuffs and 20/45 (44%) of control patients had intact cuffs. Control patients were found four times more likely to have re-tear. The BMSC group had poor or no healing in only 1 tendon (supraspinatus tendon), but the control group had 6 cases involving supraspinatus and infraspinatus that failed to heal, and 4 cases involving all 3 tendons. Within the BMSC group, the 6 failed tendons were seen to have significantly lower concentrations of BMSCs applied in the graft than the average (1500±1200 vs. 4200±1900). Size of tear, age at surgery, and time between diagnosis and repair were predictive of deficient healing in the control group but not in the BMSC applied group. There was a negative correlation found between the concentration of BMSCs within a graft and the time to obtain total healing, meaning that an increase in BMSC concentration decreased time to healing. Tears larger than 2cm required an average of 6 months, rather than 3 months for those smaller than 2cm. Regardless of tear size, control group samples took an average of 2 months longer to reach total healing than the BMSC group.

In 2015, Skoff reported on a technique for rotator cuff reconstruction using autologous biceps tendon grafts and BMSCs. He performed the surgery ten consecutive
patients with large retracted rotator cuff tears and a history of prior rotator cuff repair. The surgical technique included aspiration of iliac bone marrow (60mL) and concentration 4 to 5 fold via centrifugation (10mL). This created a concentrated solution of PRP and MSCs. LHBT was harvested and cut 50% in depth longitudinally and splayed to flatten the tendon. The graft was then incubated in the PRP/MSC solution for 60 minutes prior to implantation. The graft was attached to the bone and then to the damaged tendon. The remaining PRP/MSC solution was injected deep and superficial to the construct prior to closure. The patients reported increased in Constant, ASES, and UCLA scores for pain and functionality. At 12 months following surgery, ultrasound showed that the graft was still intact within the cuff but continuous with the adjacent tendon. At 44 months the graft was no longer visible and showed evidence of integration with the surrounding tissue. No incidence of full thickness re-tearing was reported for any of the patients in this study.

1.43 Growth Factors

Often, growth factors are indicated as necessary for the differentiation of stem cells and for the triggering of resident cells toward creating new matrix. Within tendon, this has been studied as a method for tissue engineering in which growth factors are applied to effect either the cells that are present at the application point or cells that are co-applied with the growth factors for repair. Growth factors are proteins excreted by cells in specific sequence in order to trigger surrounding cells to follow a certain biologic path. Insulin growth factor-1 (IGF-1) is a commonly studied growth factor that is
upregulated in tendon during inflammation. IGF-1 induces chemotaxis of neutrophils and fibroblasts to an injury site for repair. It also can induce collagen synthesis during the remodeling of tendon ECM.\textsuperscript{64} Basic fibroblast growth factor (FGF), is secreted by fibroblasts that arrive at the repair site and stimulates cellular proliferation as well as cell-matrix interactions.\textsuperscript{65} Additionally, growth differentiation factor (GDF) also plays a role in cellular proliferation and increase of collagen synthesis. GDF also specifically improves the alignment of collagen fibers by way of organization and assists in transcription of tendon specific genes.\textsuperscript{66}

In 1999, Kurtz et al. studied how IGF-1 contributed to tendon healing in rats in terms of healing acceleration.\textsuperscript{67} The researchers intended to use the growth factor as a therapeutic agent to positively modify the repair process. The tendon was transected and sutured closed. IGF-1 was injected or not injected depending on the study group. Biomechanical tests were performed with a 0.5 N preload at 1cm/sec to failure. Failure load, deformation, and stiffness were determined. Inflammation was determined by quantification of invading neutrophils. Mechanically, at 15 days there was no significant difference seen between failure loads but there was a trend toward increased deformation in the operated tendons compared to sham controls. Stiffness was reduced in operated groups compared to sham controls, but there was no difference between treated and untreated groups. Neutrophilic invasion was no different between groups, and IGF-1 did not affect the cellular component or the cell number of the inflammatory reaction, although it resulted in an altered tendon structure.
In 2011 Castricini et al. looked at the clinical application of platelet rich plasma (PRP) in arthroscopic rotator cuff repairs and the corresponding results seen in those patients that received PRP as part of their treatment. Platelet rich plasma is a term that is used to cover a vast array of plasma products. The materials that make up PRP are platelets, leukocytes, fibrin matrix, and growth factors. PRP is obtained from blood by spinning blood in a centrifuge, removing the pelleted solids and using the remaining fluid layer. It can often be activated into a gel when it comes in contact with thrombin that polymerized the fibrinogen in solution. These authors were looking to use the PRP as a method of growth factor augmentation at the site of a small to medium repair. They performed 88 double row arthroscopic rotator cuff repair surgeries, 45 without PRP and 43 with PRP. The methods of comparison were Constant score and MRI imaging, Constant score being a patient measurement of pain and functionality. All patients were followed up at 16 months. Within each group, there was a statistically significant increase in Constant score 16 months after surgery. However, there was not a statistically significant difference between the PRP and no PRP groups. Via MRI, it showed that 4 patients without PRP and 1 patient with PRP showed rupture, but all were satisfied with their clinical conditions. There was no difference shown between the tendon thicknesses or footprint thicknesses in the two groups. In this study, the authors showed that PRP alone was not enough to cause a significant difference between groups.
1.5 Synthesis

From the previous research done in the area of rotator cuff repairs and tissue engineering of tendon, it is clear that an ideal solution has yet be discovered. Although this is the case, it is also clear that the elements of cell delivery, scaffold material, and cell signaling must be parts of the solution. In this thesis, investigations are proposed into a tissue engineering technique for repair of torn rotator cuff tendons that combines aspects of previous investigations. More specifically, the proposed method includes the use of autologous tissue, tendon tissue as scaffold, tenocytes, and adipose derived stem cells. The combination of tailored scaffold and healthy cell source is aimed to biologically augment the torn rotator cuff and, in doing, create a more healthily structured repaired matrix. The tendon tissue scaffold will be used to deliver ADSCs and tenocytes to the repair site. In addition to being used for repair, tenocytes will also be used for their cell signaling capabilities in differentiation of the ADSCs. These cells together will then be used to deposit new matrix and reform the extracellular matrix at the repair site. Additionally, by using these materials and sourcing them autologously this method can be entirely point of care. Having a point of care method means that there would be no manipulation of materials to be implanted outside of the operating room during the surgical timeline. By reducing the manipulation of tissue and cells to the operating room potential for contamination of implant materials is reduced, time to repair is reduced, and need for a secondary surgery is eliminated.
CHAPTER TWO
PURPOSE AND AIMS

2.1 Translational Vision

The overall purpose of this project is to create a point of care biomimetic scaffold to deliver a healthy cell source to the repair site in a massive rotator cuff tear repair. The biomimetic scaffold will be made from autologous tendon, meshed to increase scaffold size, surface area for cellular attachment, and cellular infiltration. The scaffold will be seeded with autologous adipose derived stem cells, in conjugation with viable native tenocytes residing within the autologous scaffold, as the healthy cell source. This construct will be fabricated during the surgical timeline using entirely autologous cells and tissues, making it a true point of care therapy. The tendon scaffold (i.e. its ECM and resident tenocytes) will be used to promote differentiation of the ADSCs toward a tenocyte-like phenotype. The differentiated stem cells and tenocytes will then be capable of creating a tendon matrix to improve tendon quality at the rotator cuff repair site. By using native tendon as a scaffold, the tenocytes and stem cells will have a template for creation of a properly structured tendon matrix repair.

In order to achieve our long-term goal; the feasibility of our proposed approach needed evaluation. Thus, basic point of concept studies were undertaken to evaluate the following;

1. The ability to reliably form macro-porous (meshed) scaffolds from a tendon source
2. The viability of tenocytes within scaffolds
3. The mechanical properties of the scaffold

4. The ideal autologous tenocyte source for differentiation of hADSCs

5. The ability of the scaffold-stem cell construct to integrate with underlying tissue

Therefore, within this thesis the research presented will provide an *in vitro* examination of construct properties that will impact the eventual efficacy of this model for potential implementation in an *in vivo* situation.

### 2.2 Aim 1 Description

1. Reliably form macro-porous (meshed) scaffolds from a tendon source.

2. Determine viability of tenocytes within scaffolds.

3. Evaluate mechanical properties of the scaffold.

The first goal of the overall project is to characterize the tendon autograft scaffolds. For the investigation of this goal in this thesis, the focus was placed fabrication of the scaffold by attempting to identify an optimal meshing/perforating system to create the scaffold in the form of meshing. It was first necessary to determine if meshed scaffolds could be reliably created of an appropriate size to cover a massive rotator cuff tear repair site. Additionally, the questions of the effects of meshing were a major focus. In terms of these meshing effects, studies were performed to determine the effects of meshing on cellular viability and scaffold mechanical properties. These questions comprise the first aim of this thesis, which is: “Characterize meshed tendon scaffolds”.
2.3 Aim 2 Description

1. Determine the ideal autologous tenocyte source for differentiation of hADSCs.

The second goal of the overall project is to determine optimal autograft scaffold to drive stem cell differentiation. For the investigation of this goal in this thesis, it was necessary to determine the ideal human autologous tendon source for differentiation of adipose derived stem cells. The tendons under investigation for use in the eventual scaffold construct include long head biceps tendon and semitendinosus tendon. Long head biceps tendon is commonly removed during rotator cuff tear repairs through a simultaneous biceps tenotomy. This offers a commonly discarded tissue that can be found within the operative shoulder and could potentially be used as a tissue source for the proposed construct. However, LHBT regularly exhibits pathology in RC repair patients.\textsuperscript{70} Such pathology consists of macroscopic degeneration, chronic inflammation, tendon dislocation, and tendon adhesions.\textsuperscript{18,71} Semitendinosus tendon would also represent a potential scaffold and cell source as it is commonly used in anterior cruciate ligament reconstruction and lacks rotator cuff pathology. These two potential sources must be compared for their hADSC differentiation potential. Therefore, the second aim for this thesis is to “determine the optimal cell source for tenogenic differentiation of hADSCs”.

\textsuperscript{70} Personal communication from Jadranko Semelj, MD, PhD, University of Manitoba, 2018.
2.4 Aim 3 Description

1. Evaluate the ability of the scaffold-stem cell construct to integrate with underlying tissue.

The third aim of the overall project is to evaluate the efficacy of engineered constructs to support tendon regeneration in vivo. For this thesis, all research performed was on a preliminary level and done in vitro. However, in order to understand the potential for bonding of the construct to underlying tissue and the differences that may occur with and without stem cells prior to in vivo testing, integration testing was performed in an in vitro model. This study sought to illuminate the matrix fabrication timeline in vitro, so that it may hopefully be applied to planning future in vivo studies. Therefore, the third aim for this thesis is: “Evaluate the construct integration with un-meshed tendon in vitro”.
CHAPTER THREE

AIM ONE

3.1 Materials and Methods

3.11 Specimen Procurement – Porcine tendon

Porcine forelimbs were obtained from a local abattoir. All whole tissue was washed in alternating solutions of phosphate buffered saline (PBS) + 2% antibiotic and antimitotic (ABAM) and Betadine. Flexor and extensor digitorum tendons were dissected out from the forelimbs and incubated at 37°C in Dulbecco’s Modified Eagle Medium with 10% fetal bovine serum and 2% ABAM (DMEM +10%FBS +2%ABAM) for 24 hours before use. Samples were then incubated in DMEM +10%FBS +1%ABAM for 3 days and to monitor fungal infection prior to use.

3.12 Specimen Procurement – ST + LHBT

Long head biceps tendons and semitendinosus tendons were obtained from patients at the time of surgery with informed consent under IRB protocol number #Pro00031185. The long head biceps tendons were harvested from patients undergoing rotator cuff tear repair with biceps tenotomy and the semitendinosus tendons were harvested from patients undergoing anterior cruciate ligament reconstruction with autologous hamstring grafting. All samples were incubated at 37°C in DMEM +10%FBS +2%ABAM for 24 hours prior to use in order to watch for contamination of the tissue.
3.13 Graft Scaffold Formation Optimization

Meshing optimization was performed on porcine forelimb tendon samples. Initial testing was performed using the Zimmer™ Skin Graft Mesher with 2:1 expansion ratio cutter and the Brennen Skin Graft Mesher with a 6:1 expansion ratio cutter.

Figure 7: Zimmer™ Skin Graft Mesher with 2:1 expansion ratio cutter and 1.1mm thick autoclavable polypropylene carrier.
All non-tendinous tissue was removed including fat, muscle, and loose connective tissue. Each tendon was cut down to approximately 2mm in thickness and measured for actual thickness using digital calipers. Samples of varying test conditions including compression and meshing conditions were then tested to determine the optimum meshing conditions. Samples were tested as is in the Brennen mesher by running them through the mesher. Conditions tested in the Zimmer mesher are noted in Table 2. Briefly, compression coincides with running the sample through the mesher with the comb down and without a carrier. Manual meshing coincides with running the sample through the mesher with the comb up and with a carrier on 1.1mm thickness, wherein the user must
remove the tendon from the cutter with forceps. Combed meshing coincides with running the sample through the mesher with the comb down and with a carrier of 1.1 mm thickness. Sample dimensions were taken before and after each step using digital calipers.

Table 2: Combinations of meshing and compression techniques tested for meshing optimization.

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3.14 Cellular Migration

Samples of porcine tendon were cut to 1 cm x 1 cm sized pieces and either meshed or left un-meshed. The meshing protocol used was determined by the previous meshing optimization study (test 3 as seen in table 2). Tissues were cultured in 6 well plates for 14, 21, and 28 days in 3 mL DMEM +10%FBS +1%ABAM which was refreshed every 3 days. Every 7 days, the tissue was moved to another well plate and any cells attached to the plate were trypsinized and counted to determine the number of cells that had migrated from the tissue. At each end-point tissues were fixed in 10% neutral buffered formalin for 24 hours, embedded in paraffin, sectioned, and stained using hematoxylin and eosin (H&E).
3.15 Viability Testing

Semitendinosus tendon samples of 1cm x 1cm were separated into three groups (n=2): meshed, un-meshed, and ethanol treated. Ethanol treated samples were soaked in 100% ethanol for 30 minutes prior to staining to be used as a positive control for cell death. All samples were incubated in a live/dead working solution of 2mM calcein AM and 4mM Ethd-1 at room temperature for 30 minutes in order to dye live cells green and dead cells red.

3.16 Confocal Microscopy

Live/dead stained samples were imaged via laser confocal microscopy (Leica SPE Confocal Microscope). Lasers used were at 488nm and 635nm wavelengths. Images were averaged from three captures and taken at 600Hz speed. A 50µm deep Z-stack was imaged for each sample. 3-D images and slice images were compiled from the resulting fluorescent data. Three representative images from each sample were used to determine the live/dead ratios.

3.17 Mechanical Testing

Meshed and un-meshed samples of porcine tendon measuring 30mm x 10mm were prepared (n=9) and clamped longitudinally into a physiological saline bath mounted on an MTS machine using a 100 N load cell. The meshing protocol used was determined by the previous meshing optimization study (test 3 as seen in table 2). The distance between clamps was measured with digital calipers and used as the gauge length of the
tissue for strain calculations. The samples were pre-conditioned to 10% strain for five cycles and then put under tensile stress at 10mm/min until failure. The data was recorded using the testing software (Testworks 4) in Excel and processed for elastic modulus, ultimate tensile strength (UTS), and stiffness.

Figure 9: Image of mechanical testing set up while loading a meshed tendon sample.

3.18 Statistical Analysis

All data was presented as a mean +/- SEM (standard error of the mean). Statistical analysis was completed using John’s Macintosh Package (JMP) software using a one-way test for variance (ANOVA) and an $\alpha$ of 0.05.
3.2 Results

3.2.1 Meshing Optimization

Meshing of the scaffold was performed to increase the size of the tissue to fit the size of a massive rotator cuff tear. The minimum length from anterior to posterior of a massive rotator cuff tear is 5 cm, meaning that the width of the scaffold should be at least that size to cover the entirety of the repair footprint. The length of the tear corresponds to the width of the scaffold because the tendon tears from side to side and the scaffold will be placed on the repair with fibers oriented in the same direction as the underlying tendon.

Samples were tested using the Zimmer and Brennen meshers. The Brennen mesher was tested simply by running the samples through the mesher. The Brennen mesher comes with no comb and is a carrier free system, so this was the only method for use of this mesher. Samples did not achieve perforation using the Brennen mesher. Additionally, portions of the tendon tore from the sample and caught between the blades of the cutter subsequently blocking the blades in that area from pressing into the tissue. Since the Brennen mesher caused more destruction to the tissue than beneficial expansion, the remaining tests and determination of expansion ratios were performed on the Zimmer mesher. Samples were tested at each of the technique combinations shown in Table 2. Not all of the methods provided perforation through the full thickness of the tendon. As seen in Figure 8, test 1 (combed meshing) did not cause full perforation of the tendon. Test 1 consists of running the sample through the mesher with the comb down and with a carrier of 1.1mm thickness. Additionally, tendon caught in the comb during
meshing causing many of the attempts to mesh a tendon to fail due to clogging of the teeth. In order to ensure clogging did not occur, the tendon was pulled through using forceps. Test 1 yielded an average of 15% expansion along the length of the tendon.

![Figure 10: Tendons before and after (left to right) meshing test 1 (Table 2).](image1)

Test 2 (compression only) did not cause perforation of the tendon but did show some increase in tendon width at the distal end, which is shown at the bottom of the image. Test 2 consisted of running the sample through the mesher with the comb down and without a carrier. Width was initially 60mm, but after test 2 width was measured at 110mm. This represents an expansion of 83.3%.

![Figure 11: (left to right) Tendon before and after meshing test 2 (Table 2).](image2)
Test 3, compression followed by manual meshing resulted in full perforation of the tendon as well as expansion. Average width expansion seen using test 3 was 187%. Test 3 consisted of running the sample through the mesher with the comb down and without a carrier followed by running the sample through the mesher with the comb up and with a carrier on 1.1mm thickness.

Figure 12: Images of (from left to right) un-meshed, compressed, and meshed porcine tendons as obtained from meshing test 3 (Table 2).

The average expansion ratios for each test are shown in figure 11. The only test that created both full perforation in the scaffold and surpassed the 100% expansion expected for the 2:1 cutter was test 3. Although the results were variable in expansion ratio, the lowest expansion ratio seen with test 3 was still higher than the advertised expansion ratio of the mesher. The higher than advertised expansion indicates that there is a potential to increase the expansion even further if needed with the use of higher expansion ratio cutters. This is beneficial in fabricating scaffolds for massive rotator cuff tears, as they do not have a maximum size but there is theoretically a maximum tissue volume that one patient can give as autograft (as determined by the surgeon).
3.22 Cellular Migration

After meshing the tendons to create scaffolds, it was necessary to determine the effects that said meshing had on the cells within the tendon. Therefore, initial viability assessments were performed in the form of this cellular migration study. Histologically, all day 14 samples showed well-dispersed nuclei throughout the tissue regardless of meshing. By day 28, the nuclei had concentrated at the edges of the tissue. This pattern followed in both meshed and un-meshed tissues. Cells therefore were migrating towards the edges or the outsides of the tissue in both meshed and un-meshed groups.

Cellular migration out of meshed and un-meshed tendons was compared at 14, 21, and 28 days. There were no statistically significant differences seen between meshed and un-meshed at any time point. The highest number of cells counted on the plates occurred...
in both groups at day 14 and decreased in number over time following that time point. Although the difference between cell counts was not statistically significant, values did trend toward lower cell numbers when the tissues were meshed.

Figure 14: Top: cell migration after meshing compared to un-meshed porcine tendon as seen in histological imaging. Pink areas in histological images show extracellular matrix and dark blue dots are cell nuclei. Nuclei remain dispersed in both samples at day 14 but focus along the tissue edge at day 28. Bottom: graph indicating the number of cells vacating the tissue (per unit volume of tissue) for meshed and un-meshed tissue at days 14, 21, and 28.
3.23 Direct Assessment of Viability

Following cellular migration studies, which indicated a potential cellular decrease in meshed tissue, a direct assessment of cellular viability in the form of a live/dead assay was required. Using the confocal images taken of live/dead stained cells, viability was determined. Live cells were stained green while dead cells were stained red. All ethanol samples, as a positive control for cell death, resulted in 100% cell death. Un-meshed and meshed tendon samples had averages of 31% and 61% death respectively. All differences in cell death were statistically significant. Between un-meshed and meshed tendon samples, there was a 30% decrease in cellular viability seen. Through the 3-D rendering of the confocal images, it is clear that live/dead ratios remained approximately the same throughout the density of the tendon samples.
Figure 15: Single section (A, B, C) and 3-D (D, E, F) confocal images of semitendinosus tendon stained for viability. Green cells are live and red cells are dead. A, D: Un-meshed tendon showed the highest percentage of live cells, with some dead cells still present. B, E: Meshed tendon showed a higher concentration of dead cells than un-meshed tendon. C, F: All cells within the ethanol treated samples were stained as dead.
3.24 Mechanical Testing

Tendons are capable of withstanding high amounts of stress prior to failure in normal conditions, however the meshing of a tendon to create a scaffold alters the connectivity between the fibers of the extracellular matrix. This alteration was likely to alter the mechanical properties of the tendon; therefore it was necessary to perform mechanical testing on meshed and un-meshed tendon tissues to determine the change seen due to meshing. Stress/strain graphs were created for each tensile test performed. Meshed samples exhibited lower ultimate tensile stress than un-meshed samples, with graphs peaking at lower values on average (0.56 MPa for meshed, 2.38 MPa for unmeshed). Conversely, meshed samples exhibited increased deformation prior to breakage than un-meshed tissues. Meshed tissues yielded at 60.6% strain and un-meshed tissues yielded at 28.4% strain. Elastic modulus and stiffness were also calculated from...
the stress/strain curves. The elastic modulus of un-meshed tendon was significantly higher than that of meshed tendon with calculated values of 37.17 MPa and 4.72 MPa respectively. There was not a statistical difference between the stiffness values calculated for meshed and un-meshed tendon. This indicates that stiffness is the only material property of tendon that remains unchanged due to meshing.
Figure 17: Representative stress-strain curves for meshed and un-meshed tendon samples under tension.
Figure 18: Graph of elastic moduli of meshed and un-meshed tendons. Un-meshed tendons have a statistically larger modulus than meshed tendons.

Figure 19: Graph of stiffness values of meshed and un-meshed tendons.
Figure 20: Graph of ultimate tensile strength (UTS) values of meshed and un-meshed tendons. The UTS of un-meshed tendon is significantly higher than that of meshed tendon.

3.3 Discussion

3.3.1 Meshing Optimization

In order to fabricate this construct, the ideal meshing technique must be determined. The ideal meshing technique was determined by finding a technique that required few manipulations of the tendon, created a clean mesh, and created a reliably expanded mesh. Using a skin graft mesher to perforate a tendon scaffold requires alterations of the normal use protocol. A skin graft mesher was chosen because it is already approved by the FDA for manipulation of skin tissue, is easily cleanable and sterilizable, and is designed to create expandable meshes from soft connective tissues. For example, the normal use of the mesher called for leaving the comb in place to remove
tissue from the teeth of the cutter. However, when meshing a tendon the comb caused back up of meshed tendon within the teeth leading to shredding and clogging of the mesher. By meshing with the cutter and without a carrier to force the tissue into the blades, only compression of the tissue was achieved. This compression did expand the width of the tissue, but it did not allow for perforation by the cutter blades. The third method tested, using compression to initially increase the width of the tissue followed by meshing without the comb to allow for easier extraction, yielded the best-constructed and most highly expanded scaffolds. The advertised expansion ratio of 2:1, or 100% expansion, was surpassed by the third method with an average of 187% expansion. This increase in expansion likely occurred due to the combination of the expansion from compression, which under test 2 caused an average of 83.3% expansion, and the perforation from mechanical meshing, which likely added the advertised 100% expansion. This will allow for larger scaffolds from less tissue, so that a massive tear of 50mm could be covered by a sample of the initial width of 17.4mm.

3.32 Cellular Migration

Following meshing optimization, it was next necessary to determine the effects of the chosen meshing method on the scaffold. Tenocytes within the meshed tendons and un-meshed tendons migrated from the scaffolds. Within both groups, the pattern of migration appeared at the same times histologically. Although the numbers of cells that migrated from the tendons were similar, there was a trend towards a decrease in cell number after meshing. This was seen as an indicator of potential cell death and therefore
a need to perform direct viability studies. This, however, is not a measurement of cellular viability as cellular mobility and proliferation may have contributed to the overall cell numbers.

Since tendon is a dense connective tissue, it is likely that the media was unable to fully penetrate the tissue. Therefore, tenocytes likely migrated to reach areas of higher nutrient concentration. This cellular crawl-out has been used by many researchers as a method for isolation of tenocytes.\textsuperscript{58,72,73} This information could be used to further study the potential to direct the location of cells within the construct via nutrient concentration, potentially using a nutrient dense biologic such as PRP. This would be beneficial for the project’s next steps because mobilizing tenocytes increases the potential number of cells acting at the surgical repair interface and increases the likelihood of hADSC to tenocyte contact.

3.33 Direct Assessment of Viability

Meshing of ST tendon causes a notable decrease in viable tenocytes within the tendon scaffold but does not kill all of the cells within the scaffold. There is a 30% decrease in cellular viability between meshed and un-meshed tendons. However, it can be seen from the confocal images in figure 10 that the morphology of meshed tendon tenocytes remain aligned and spindle shaped.

The concentration of cells within a tendon is low compared to other tissue types (<5% of the total volume), and further decreased by the meshing process it remains to be seen if the remaining concentration will be sufficient for the differentiation of hADSCs.
and the fabrication of ECM.\textsuperscript{32} Future studies within this overall project goal should focus on determining the ideal concentrations of hADSCs and tenocytes for tendon hADSC differentiation and ECM regeneration.

### 3.34 Mechanical Testing

Many tendon scaffolds are used as mechanical augmentation for a repair site.\textsuperscript{41,74} However, with the significant decrease in ultimate tensile strength (UTS) and young’s modulus seen with meshing this proposed construct must only be used as a biological augmentation. This limitation of mechanical strength may lead to the potential use as a biological augmentation to be used with current mechanical augmentations in use. It is also yet to be seen how cellular activity affects the mechanical properties of the meshed scaffold. In order to determine the biological augmentation benefits of the scaffold, later mechanical testing should be done to determine if cellular culture would increase the mechanical properties measured herein. In ECM were to be deposited by resident and applied cells, it could change the mechanical properties from those reported.
4.1 Materials and Methods

4.11 Specimen Procurement – ST + LHBT

Long head biceps tendons and semitendinosus tendons were obtained from patients at the time of surgery with informed consent under IRB protocol Pro00031185. The long head biceps tendons were harvested from patients undergoing rotator cuff repair with tenotomy and the semitendinosus tendons were harvested from patients undergoing anterior cruciate ligament reconstruction with autologous grafting. All samples were incubated at 37°C in Dulbecco’s Modified Eagle Medium with 10% fetal bovine serum and 2% antibiotic antimitotic (DMEM +10%FBS +2%ABAM) for 24 hours prior to use in order to watch for contamination of the tissue.

4.12 Cell Isolation

Semitendinosus and long head biceps tendon tissues were cut to 10mm x 10mm x 2 mm, meshed using a skin graft mesher (Zimmer) as determined by the meshing optimization trial (test 3 as seen in table 2). The tissue samples were then cultured in a 6 well plate in 3 mL DMEM +10%FBS +1%ABAM that was refreshed every 3 days. Adherent tenocytes were harvested after 14 days. Samples were left in culture and additional adherent cells were harvested again at day 21. Cells were then expanded in monolayer culture for further use. Human adipose derived stem cells (hADSCs) were purchased from Invitrogen. Passage 4 tenocytes and stem cells were used in all studies.
4.13 Indirect Co-Culture

Indirect co-culture of human tenocytes and hADSCs was performed via conditioned media transfer. 2x10^5 long head biceps tenocytes or semitendinosus tenocytes, and hADSCs were cultured in individual T25 flasks. Tenocytes were cultured with 5mL of DMEM +10%FBS +1%ABAM that was removed and refreshed every 3 days. Media taken from the tenocyte cultures every 3 days was sterile filtered and given to the hADSCs in a 50:50 volumetric ratio with fresh media (n=3) to a total of 5 mL. This method transferred any cellular excretions from the tenocytes to the hADSCs through the media.

4.14 RT-PCR

RNA was isolated from hADSCs using an RNEasy Mini Kit (Qigen) in accordance with the protocol provided by the manufacturer and then quantified after 14 and 21 days of culture (n=3). Quantification of RNA was performed using a nanodrop spectrophotometer. A sample of un-conditioned hADSCs at day 0 was used as a control. A tenocyte gene transcript profile was evaluated using the target specific primers in table 3 was evaluated for use as a housekeeping gene. Reverse transcription and real time polymerase chain reaction (RT-PCR) was performed using a RETROscript and QuantiTect SYBR green PCR kit in a Rotogene 3000 thermocycler. Data was analyzed using the 2^-ΔCT method with GAPDH as an internal control. Normalized expression was determined by using 2^-ΔCT of the sample / 2^-ΔCT of the GAPDH control.
Table 3: Target specific primers for tenocytes with their corresponding forward primer sequences.

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<th>Primer</th>
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<tr>
<td>Collagen I (COL I)</td>
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<td>Collagen III (COL III)</td>
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<tr>
<td>Scleraxis (SCX)</td>
<td>5’ ACACCCAGCCCAAACAGA 3’</td>
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<td>Tenascin C (TNC)</td>
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<td>GAPDH</td>
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</table>

4.15 Statistical Analysis

All data was presented as a mean +/- SEM (standard error of the mean). Statistical analysis was completed using John’s Macintosh Package (JMP) software using a one-way test for variance (ANOVA) and an α of 0.05.

4.2 Results

Expression of collagen I, collagen III, scleraxis, and tenascin C markers were compared for hADSCs differentiated in semitendinosus or long head biceps tendon tenocyte conditioned media. hADSC gene expression was measured at days 14 and 21 as compared to a day 0 undifferentiated hADSC control. Semitendinosus conditioned cells showed an increasing trend in all markers over time, whereas long head biceps tendon conditioned cells held a relatively steady expression over time. Results found were not statistically significant in any marker group. There was a trend towards higher expression over time in the ST group for all markers.
Figure 21: Graphs of tenocyte gene expression seen in hADSCs (collagen I, collagen III, scleraxis, and tenasin C) with respect to GAPDH and an un-differentiated control.

4.3 Discussion

Autologous tendon sources that are available for use for rotator cuff repair are relatively limited. Authors have previously used long head biceps tendon for its proximity to the surgical site and frequent removal after transection during biceps tenotomy.\textsuperscript{18,48} However, the noted pathologies seen in the biceps tendon in patients with rotator cuff injuries such as macroscopic degeneration, chronic inflammation, tendon dislocation, and tendon adhesions can be a cause for reconsideration of this tendon
The semitendinosus tendon is commonly used as both autograft and allograft for ACL reconstruction and comes from outside the damaged rotator cuff environment. Therefore, I compared these two tendon sources to determine if the reported pathologies of the long head biceps tendon would affect their potential to differentiate hADSCs. Co-culture between tenocytes and hADSCs for the differentiation of hADSCs has been shown as feasible by many investigators. Kraus et al. demonstrated the feasibility of direct, indirect, and media-based co-culture methods between tenocytes and stem cells.

The resulting gene expression data showed that expression of tenogenic markers in hADSCs differentiated using long head biceps tendons remained static over time. Those hADSCs differentiated by semitendinosus tendons, however, showed an increasing trend over time in each of the markers measured. This difference in expression combined with the potential for macroscopic degeneration of the biceps tendon indicated that semitendinosus tendon would likely be the ideal tendon scaffold for this project.

Future studies with gene expression would include determination of the ideal tenocyte to hADSC ratio for differentiation as well as the effects of the scaffold itself on the gene expression of hADSCs.
CHAPTER FIVE

AIM THREE

5.1 Materials and Methods

5.11 Cell staining

Adipose derived stem cells were stained with PKH26 red fluorescent marker (cell linker kit from Sigma-Aldrich). Cell staining began by pelleting $2 \times 10^6$ hADSCs via centrifugation and re-suspending them in 100 µL Diluent C, from the cell linker kit. Following re-suspension, the dye solution was made by combining an additional 100 µL Diluent C with 1 µL of the ethanolic dye solution and vortexing them to combine. The dye solution was then added to the cell suspension, mixed via pipetting, and then incubated at room temperature away from light for 5 minutes. After 5 minutes of incubation, 200 µL FBS was added, mixed via pipetting, and incubated away from light for 1 minute to bind excess dyes. The cell solution was then centrifuged down to a pellet, re-suspended in 10 mL complete media, and re-centrifuged to remove any remaining dyes. The stained cells were then seeded onto meshed porcine tendon scaffolds and cultured for 21 days. After 21 days, the samples were imaged under fluorescent microscopy to determine cellular attachment and alignment with scaffold matrix. PKH26 is a lipophilic membrane marker that has excitation and emission peaks at 551nm and 567nm respectively. Therefore, the fluorescent filter for red fluorescent protein (RFP) was used to visualize the cells.
5.13 Integration Study

Porcine tendons were meshed to create scaffolds and cut to 10mm x 10mm in size. The meshed scaffolds were then soaked in 49% DMEM + 50% FBS + 1% ABAM for two hours. Following the findings from the cellular migration study in Aim 1, artificially increasing the nutrient content within the scaffold by soaking it in a higher concentration of FBS should keep the cells from migrating out of the tissue to reach the surrounding media. The scaffolds were then seeded with $2.0 \times 10^5$ human adipose derived stem cells. Un-meshed tendon of 10mm x 10mm x 2mm were submerged halfway in FBS for 2 hours to create a higher nutrient content in the tissue than would be present within the surrounding media. This was done, based on the findings from the cellular migration study in Aim 1, to drive the cells within the tissue to the interface. Each scaffold was then sutured to the FBS soaked face of an un-meshed tendon using a single simple suture at each end. The construct was plated in a 6 well plate with the scaffold on top of the un-meshed tendon and cultured for 14 or 21 days (n=3) in DMEM +10%FBS +1%ABAM. Media was refreshed every 3 days. Control samples were constructed as previously described but without the addition of stem cells (n=3).

![Figure 22: Schematic of integration study constructs. Meshed tendon is fixed on top of un-meshed tendon. Blue circles represent tenocytes and red triangles represent hADSCs. Sizes of components are not to scale.](image)
Figure 23: Images of experimental constructs used to determine suture placement and fixation method in integration study. Samples are 4x length of the final scaffolds, two pieces of 2mm thick porcine tendon, and connected using a single simple suture at each end.

5.14 Histology

Samples were fixed in 10% neutral buffered formalin for 24 hours, embedded in paraffin, sectioned, and stained using hematoxylin and eosin (H&E) and picrosirius red. Samples were imaged at 100x and 200x under a light microscope in order to locate the integration line.

5.2 Results

PKH26 stained hADSCs were seen to migrate into the meshed tendon scaffold onto which they had been seeded. Figure 17 shows the hADSCs aligned within the tendon matrix.
Figure 24: Fluorescent image of PHK26 stained hADSCs within a tendon matrix after 21 days of culture.
Figure 25: Histological images of meshed and un-meshed tendons at their integration sites stained with H&E. ECM is stained pink and nuclei are stained dark blue. A, B) Meshed tendon with hADSCs at day 14 (50x, 200x). C, D) Meshed tendon without hADSCs at day 14 (50x, 200x). E, F) Meshed tendon with hADSCs at day 21 (50x, 200x). G, H) Meshed tendon without hADSCs at day 21 (50x, 200x). Cells at the integration zones are noted with black arrowheads in 200x images.

When imaged, the integration border between meshed and un-meshed tendon is visible in all histological samples, though less easily discerned in those samples seeded with hADSCs stained with H&E. At day 14, hADSC seeded samples show a line of higher tissue density between two distinct tissue textures, suspected to be the interface. At the top of this interface, where the tissues come together, a collection of cell nuclei are seen between the two edges. Additionally, in the area where the edges of the two tissues have not met, cell nuclei are seen to line the borders of each tissue. At day 21, hADSC seeded samples show a less discernable border as there is no line of higher cell or tissue density where the tissues connect. The tissue fibers appear to blend the tissues together without a distinction between the two. There is still an area where the two tissues do not connect, but this is likely due to poor fixation during culture allowing space between the scaffold and underlying tendon.

In those samples that did not receive hADSCs a border can be seen between a very dense un-meshed tissue and a more loosely fibrous meshed tissue day 14. The two tissues do appear close together at their borders but there does not appear to be
integration between the two tissues at the edge. Along the un-meshed edge there is a higher density of cells, but they do not appear to be interacting with the adjacent tissue. At day 21, tissues without stem cells had a significantly difference appearance from other tissue groups. There are clear lines where the edges of tissue used to be, appearing more similarly to the day 14 stem cell seeded group, but missing the line of higher density matrix. Cells seen at the edge of the un-meshed tissue do appear to be creating bonds with the scaffold, as thin lines of matrix can be seen extending from a cell on the un-meshed tissue toward the scaffold.

5.3 Discussion

This study aimed to determine how our proposed scaffold system would potentially fuse with native tendon in an *in vitro* model. Integration, or the combining of the scaffold in the body of the native tendon, is essential because it represents both cellular activity in reconstruction of tendon and a physical augmentation at the repair site. Stained cells were seeded onto meshed tendons in order to determine if they would migrate into the scaffold and align properly with the matrix. This migration is important because the goal for the seeded hADSCs is for them to inhabit the entirety of the scaffold or underlying tendon and eventually create a bond with the injured tendon surface.

For this study we sought to determine how stem cells at the interface of the repair site would interact with the scaffold and the tendon. Therefore, in this study, the hADSC-seeded surface was in contact with the un-meshed tendon. Additionally, for this study we soaked meshed scaffolds in an increased concentration of FBS prior to seeding. We
determined in previous studies that cellular crawl-out of the tissue was likely due to low nutrition at the core and higher nutrition within the plate as a whole. Therefore, by soaking the tendon in FBS prior to seeding, we simulate an environment in which the tendon is properly supplied with nutrients during the migration process. Upon seeing histological results, they indicated that cells were driven to the interface by day 14 in both stem cell seeded and un-seeded groups by the FBS soaking method employed. The use of FBS in this study led us to consider the further use of PRP in our model as a method of concentrating cells at a given surface area and drawing stem cells and tenocytes from the scaffold to the injury site for repair purposes. Since PRP gelates in vivo, it would remain in place at least temporarily as a signaling guide for the movement of cells within the tissue.

The integration of the two tissues was relatively similar between groups, but it did seem as though the groups containing hADSCs reached a more complete level of integration faster than those without hADSCs. This indicates that hADSCs may not be strictly necessary for the integration of scaffold and tissue, but they do increase the speed of the process. Additionally, the day 21 samples without stem cells seemed to be very weakly connected. This follows the pattern that tenocytes initially heal tendon by using insufficiently strong scar tissue. At day 21, the samples with hADSCs appeared to have similar ECM structure throughout which would not cause a weak spot within the tendon. This could be tested in future by repeating the study and performing mechanical testing on the resulting integrated scaffold constructs.
CHAPTER SIX

CONCLUSIONS

Within this thesis several initial elements of a long-term project on the biological augmentation of rotator cuff repair were investigated. Optimization the fabrication of a meshed tendon scaffold for biological augmentation was performed using a skin graft mesher. The resulting construct was then investigated for mechanical properties and determined to have a significant change in those properties due to meshing. It was demonstrated that ST tendon is capable of maintaining limited cellular viability and mobility towards nutrients after meshing for the formation of an autologous scaffold. Additionally gene expression results indicate that tendon sources from differing areas of the body have varying hADSC differentiation potential. Due to a pattern of increases in hADSC differentiation and sufficient viability after meshing, ST tendon has been chosen as the autologous tendon source for use in this proposed application. Finally, the integration patterns of meshed scaffolds with un-meshed tendons follow the hypothesis that hADSCs would increase the quality of tendon tissue at a repair site \textit{in vitro}.

Future studies for this project should include mechanical testing of \textit{in vitro} integration models as well as implementation of an \textit{in vivo} model for integration of this scaffold at a repair site. For a point of concept of tendon integration, this \textit{in vivo} study could be performed on any tendon not necessarily in the rotator cuff. Additionally, studies into the ideal hADSC to tenocyte ratio should be performed to determine ideal repair conditions.
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