I, Nathaniel A Dyment, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Biomedical Engineering.

It is entitled: Using Development and Natural Healing as a Paradigm to Improve Tendon Repair

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Using Development and Natural Healing as a Paradigm to Improve Tendon Repair

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Abstract

Tendon and ligament injuries present a significant socioeconomic problem. Tissue engineering has become an attractive option for improving repair of these injuries. Our lab has previously shown success utilizing stem-cell based therapies but we have yet to produce repairs that functionally match normal tendon mechanical properties for more strenuous activities of daily living. This dissertation focuses on using the differences between normal embryonic development and natural adult healing as a strategy to improve repair outcome of tendon injuries.

We first analyzed the biology and mechanics of natural healing in the mouse patellar tendon and how these processes compared to normal tendon development. Understanding the differences between these processes may provide therapeutic targets to modulate during the healing process. We found that natural healing of a central patellar tendon defect followed a traditional healing response with inflammation at 1 week, repair at 2 weeks, and remodeling at 3 weeks and greater. The healing tissue yielded a non-functional repair with stiffnesses and ultimate loads that plateaued at 60% of normal PT after 5 weeks of healing.

We found in the second study that the healing tendons exhibited reduced expression of known tenogenic transcription factors and fibrillogenic genes during healing. Type-I and type-III collagen gene expression was elevated during healing of both the injury and sham tendons; however, the reduced expression of tenogenic and fibrillogenic markers suggests that the matrix was not properly assembled, leading to a non-functional scar.

In order to promote tendon differentiation during repair, we then stimulated mesenchymal progenitor cell collagen constructs with myogenic (myoblasts) and tenogenic (tendon fibroblasts) signals in culture. We found that myoblast- and Achilles tendon fibroblast-conditioned media did
not promote differentiation of the constructs as gene expression of tenogenic markers was unchanged.

While the central patellar tendon defect is a reproducible model, it is not clinically relevant as it does not display degeneration, which is seen in over 90% of clinical injuries. Therefore, the next study compared the effect of prostaglandin-E2 and collagenase delivery on creating degenerative aspects in the rabbit patellar tendon. We found that collagenase yielded reduced mechanical properties and histological aspects of degeneration at 4 weeks but did not sustain these changes at 16 weeks.

The final study of this dissertation applied the methodologies used in our previous tendon healing studies to understand fracture healing in the mouse as our lab transitions into developing tissue engineered strategies for improving bone repair. We compared sub-critical vs. critical femoral defects and found that the sub-critical defects healed with successful periosteal bridging while the critical defects yielded impaired healing with capping of the ends of the bone.

Future studies need to determine the lineage of the cells that contribute to healing and if these cells can be modulated to improve repair. Mechanistic studies that alter expression of these tenogenic markers are needed to further understand the molecular signaling that drives healing. Further understanding of the healing process will provide tissue engineers with potential treatment modalities to ultimately improve repair outcome and the patient’s quality of life.
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Organization of Dissertation

This dissertation consists of a several chapters including Chapter 1 – Background, rationale, and objectives, Chapter 2 – Literature review, Chapters 3 through 7 – Manuscripts, Chapter 8 – Conclusions and recommendations.

Chapter 1 presents the overall rationale for the dissertation and specific aims with hypotheses. Chapter 2 presents an overview of the current state of the fields of tendon developmental biology, tendon healing, and clinical pathologies with gaps in knowledge that this dissertation will pursue.

Chapters 3 through 7 present five manuscripts, each with traditional sections, including the abstract, introduction, materials and methods, results, and discussion. The first manuscript in chapter 3 describes the development of a full-length, central patellar tendon defect model in the mouse, analogous to our previous work in the rabbit. Chapter 4 analyzes the expression patterns of tenogenic markers that influence normal development during healing in this model. Chapter 5 tests the effect of myogenic and tenogenic signals on the differentiation of bone marrow progenitor cells in collagen gel constructs. Chapter 6 compares cell- vs. matrix-mediated methods of producing aspects of tendon degeneration in the rabbit patellar tendon. Lastly, chapter 7 employs the same methodologies used in chapters 3 and 4 for studying tendon healing and applies it to studying healing of sub-critical vs. critical segmental femoral defects in the mouse.

Finally, chapter 8 presents an overview of the main conclusions of the dissertation and discusses the existing gaps in knowledge while offering recommendations for the future.
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Chapter 1

Background, Rationale and Objectives

Socioeconomic impact of tendon and ligament injuries

Tendon and ligament injuries present a significant socioeconomic impact to our society especially as the population ages. Injuries to tendons, ligaments, and other capsular structures contribute to nearly 50% of the 33 million musculoskeletal injuries that occur in the US each year [1]. These injuries occur with great frequency in the rotator cuff tendons of the shoulder, the Achilles tendon near the ankle, the anterior cruciate ligament (ACL) and patellar tendon (PT) in the knee, and the flexor tendons in the hand and wrist. Over 50% of the population over the age of 60 possesses degenerative injuries or other abnormalities of the rotator cuff tendons in the shoulder [2]. One in 3000 people tear their ACL each year at an estimated cost of $17,000 per reconstruction leading to an annual cost totaling $1.7 billion in the United States [3]. The frequency of injury and impaired natural healing exhibited by tendons and ligaments makes for a considerable impact on the population that requires novel treatment modalities.

Background and rationale

There are many factors that contribute to impaired healing of tendons. Tendons are primarily avascular and have low cellularity compared to other tissues [4-6]. Tendons also have to resist tremendous forces during activities of daily living, reaching loads as high as 12 times body weight [7,8]. The environment, in which a tendon resides, specifically an extra- vs intra-articular environment, can also influence the healing response following injury [9]. In addition, approximately 97% of all tendon injuries exhibit a chronic, degenerative pathology, which
impairs the healing response greatly [10]. Studying the pathogenesis of degeneration is also difficult as there are limited preclinical models available, each with limitations [11-13]. These characteristics combined with intrinsic factors such as age, obesity, and genetics all contribute greatly to the severity and variability of the healing response [4,5,14]. The clinical prevalence of tendon injuries provides a unique challenge and also opportunity for clinicians, scientists, and tissue engineers to develop novel treatments to improve repair.

Our laboratory utilizes a paradigm known as functional tissue engineering [15-17] to improve repair outcomes of tendon injuries by first understanding the normal mechanical environment required during activities of daily living and then developing design parameters based on these measurements. We have recorded forces in multiple model systems including the patellar tendon of the rabbit and goat [18,19]. Using force transducers, peak in vivo loads of 21% and 40% of normal PT failure load were recorded in the rabbit and goat, respectively [18,19]. By utilizing bone marrow-derived mesenchymal progenitor cells (bMPCs), combined with collagen-based scaffolds and in vitro mechanical stimulation, we have produced repairs that match the tangent stiffness of the normal PT failure curve up to 50% beyond the highest in vivo force recorded in the rabbit central-third patellar tendon [20]. However, we have not reached the more strenuous level of 40% of normal PT failure force recorded in the goat PT. Therefore, we have sought more “biological” strategies to improve the outcome of these repairs following surgery. One attractive strategy that we are currently employing is to use normal tendon development as a guide to promote differentiation of bMPCs in our constructs in order to improve repair outcome in vivo.
Specific aims and hypotheses

Thus, the strategy I have employed in my dissertation to advance the field of tendon tissue engineering is to compare natural adult tendon healing, a process that almost uniformly leads to scar tissue and impaired healing biomechanics [21-23], to that of normal tendon development. By doing so, I hope to broaden the mechanical paradigm that seeks to define functional tissue engineering parameters (FTEPs) by defining biological benchmarks of success [24,25]. Elucidating the differences between natural healing and normal tendon development may provide mechanisms that tissue engineers may utilize and modulate to produce improved therapies. In order to mechanistically understand the biological processes of healing and development, we will employ the murine model, which has an abundance of genetic tools available to conduct these mechanistic studies.

Therefore, the first aim of this dissertation is to establish a murine central PT defect model, analogous to the rabbit model used previously [15,26], to characterize the expression of tenogenic markers from development in the PT tendon during healing. We formulated three hypotheses for this aim.

**Hypothesis 1:** The healing response following a central PT defect in the murine PT will display decreased mechanical properties compared to normal PT.

**Hypothesis 2:** The healing tissue will exhibit histological characteristics of the normal stages of healing including inflammation, repair, and remodeling.

**Hypothesis 3:** The healing tissue will exhibit reduced gene expression of tenogenic markers from development while showing increased expression of factors that promote scar formation (ie. lower expression of scleraxis and higher expression of Col3a1).
As stated above, our strategy to further improve our repairs is to promote differentiation of bMPCs in our tissue engineered constructs (TECs) prior to implantation in vivo. Unfortunately, the genetic tools needed to assess differentiation are not available currently in the rabbit; therefore, murine bMPCs were utilized. The second aim of this dissertation is to deliver myogenic and tenogenic factors from development to our TECs in order to promote differentiation in culture. This led to our next hypothesis.

Hypothesis 4: Paracrine signals from myoblast and Achilles tendon fibroblast cultures delivered to TECs will promote increased expression of tenogenic markers and differentiation during culture.

While the central PT defect provides a reproducible injury model that allows for controlled assessment of treatment efficacy, it is not clinically relevant. Previous research has attempted to create preclinical models displaying aspects of tendon degeneration seen clinically. The limited number of animal models that succeed in creating degenerative aspects fail to sustain them for periods long enough to assess treatment. Therefore, the third aim of this dissertation is to develop an injury model with aspects of chronic degeneration such that our tissue engineered strategies can be assessed in a more clinically relevant model system. These studies will be done in the larger rabbit model, which is more reproducible and is large enough to consistently repair tendon injuries with tissue engineered constructs. Two methods will be compared to create degeneration: 1) alter the phenotype with sustained delivery of prostaglandin-E2 (inflammatory mediator that may mediate the degenerative pathology) and 2) disrupt the tendon matrix with collagenase such that the cells within the matrix alter their phenotype in response to the changes in mechanical environment. This study led to our next hypotheses.
Hypothesis 5: PGE2 will alter the resident cell phenotype and lead to histological and biomechanical alterations compared to normal PT.

Hypothesis 6: Collagenase will produce reduced mechanical properties and decreased matrix organization compared to normal PT.

Finally, our lab has translated our multifunctional tissue engineering strategies to assess healing and tissue engineered repair of problematic bone fractures. A portion of my dissertation was devoted to helping develop an injury model in the murine femur that would allow us to assess successful vs. impaired natural healing. Therefore, the last aim of this dissertation was to compare the histological and biomechanical healing response of sub-critical vs. critical-sized femoral fractures in the murine model, leading to the final hypothesis.

Hypothesis 7: Sub-critical (0.6 mm long) femoral defects will show improved histological and biomechanical healing compared to critical (1.6 mm long) defects.

Significance of proposed work

The work described in this dissertation is significant in several respects. 1) By applying knowledge gained from normal development in the murine model to the design of tissue engineered repairs, we aim to alter the natural healing process from imperfect scar formation toward tenogenesis and improved repair outcome. 2) These strategies can be translated to larger animal models where surgical interventions are more reproducible, in vivo forces are more substantial, and injury models are more clinically relevant (e.g. chronic degeneration). 3) This approach also has the potential to more rapidly lead to clinical studies with a greater potential for success in injured human patients.

The overall objective of this work is to create a multifunctional tissue engineering strategy that combines biological and mechanical benchmarks to aid the tissue engineering field
in seeking improved healing solutions. As depicted in the flowchart in Figure 1, the strategy I have helped to develop is both multi-faceted and progressive over time to build upon the past and current strengths in our laboratory.

**Functional Tissue Engineering**

**Rabbit Defect Model**

**Mechanical Stimulation of TECs**

**Development vs Healing**

- CH 3: The relationships among collagen gene expression, histology, and biomechanics following full-length injury in the patellar tendon
- CH 4: Expression patterns of tenogenic markers following a full-length patellar tendon injury

**Multi-Functional Tissue Engineering**

- CH 5: Effect of paracrine signaling on tendon tissue engineered construct differentiation in culture

**Tendon Degeneration**

- CH 6: Cell- vs. Matrix-Induced Degeneration: The Effects of Prostaglandin-E2 vs. Bacterial Collagenase on Patellar Tendon Biomechanics and Histology

**Improved Repair of Clinically Relevant Injuries**

*Fig. 1*: My dissertation aims to build upon previous functional tissue engineering successes in our lab by employing developmental biology to create a multi-functional tissue engineering strategy to improve tendon repair. First, we will compare the expression patterns of tenogenic markers during development to that of natural tendon healing. Knowledge gained from this analysis will provide benchmarks for success and potential pathways for modulation during repair. Second, we will deliver paracrine signals released from myoblasts and tendon fibroblasts to bMPC-seeded collagen gels to test the effect of these signals on tenogenic differentiation of these cells. Finally, a parallel track will test whether prostaglandin-E2 or collagenase can produce degenerative aspects in the rabbit PT in order to provide a more clinically relevant injury model for assessment of repair success. These studies all contribute to the overall goal of this work, which is to ultimately improve repair of clinically relevant tendon injuries.
Chapter 2

Literature Review

Tendon Structure and Function

Tendons, by their most basic definition, attach muscles to bone. Their main function is to resist tension such that the forces generated by the adjacent muscle can be transmitted to the bone to create movement of the skeletal system. Tendons have to transition from relatively compliant muscle to extremely stiff mineralized bone over short distances. Additionally, the cross-section of tendon is usually much smaller than that of the adjacent muscle. Consequently, the stresses developed in tendon over this short transition from compliant muscle to stiff bone can be quite high, with loads reaching up to 12 times body weight [7]. Unfortunately, tendons have a rather small safety factor of 2.5, with peak recorded in vivo forces for activities of daily living sometimes reaching 40% of ultimate failure load for the tendon [19]. These forces challenge our ability to effectively repair damaged tendons after injury.

Tendons consist of a hierarchical structure with the basic cellular unit being a tenocyte (Fig. 2). Tenocytes produce collagen, which is the key structural element of tendon [6,27]. Type-I collagen is the main structural protein in tendon making up 60% to 85% of the dry weight [6,27]. The collagen monomers form a tropocollagen triple helix, which is the building block of polymer chain [21]. The tropocollagen molecules aggregate to form fibrils, which are then crosslinked with other fibrils to form fiber bundles known as fascicles. These fibrils and fascicles form a crimp pattern that contributes to the viscoelastic behavior of this tissue [22,28]. Groups of fascicles form tertiary fiber bundles that are surrounded by endotenon, a connective tissue consisting of vessels, nerves, and lymphatics [28]. Multiple fiber bundles are surrounded by
epitenon, which is continuous with the endotenon. The surface of the tendon is then surrounded by the paratenon, which acts as sheath around the tendon and is also continuous with the epitenon [28,29].

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**Fig. 2. The anatomy of a normal tendon.** Tenocytes secrete collagen protein, which assembles into a specific hierarchy of fibrils, fibers, and fiber bundles (fascicles). The collagen fibrils display a periodicity of 67 nm with adjacent repeats containing gap and overlap regions. The fiber bundles are surrounded by loose connective tissue (endotenon) and the tendon unit itself is surrounded by epitenon. Reproduced from Banos 2008 [28].

The mechanisms of collagen production and assembly during normal tendon development can differ greatly from the healing process following traumatic injury and also the pathogenesis of chronic tendinopathies, which are seen quite frequently in patients [10]. The characterization of these differences will be the focus of this literature review and how tissue
engineering strategies can be an attractive option for improving the shortcomings of the natural tendon healing process.

Normal Tendon Development

Unfortunately, the development of normal tendon has not been studied as extensively as the development of other tissues. The limited number of tendon specific markers makes studying the development of tendon quite difficult, as there are no indicators as to when tendon differentiation initiates and how to monitor its differentiation and maturation over time. Recently, however, Schweitzer and co-workers discovered the first tendon marker – scleraxis (Scx) [30]. Scx is a basic-helix-loop-helix that is initially expressed in tendon precursor cells within the scleretome during early embryonic development. At the early embryonic stage of E10.5 in the mouse (E21 being the day of birth), Scx and Sox9 (a transcription factor highly expressed in chondrocytes and important for cartilage differentiation) are both colocalized to regions of the mesenchyme (the portion of the embryo that forms connective tissue) [31]. As the limb buds, Scx and Sox9 expression overlap until the joints condense. At this point in time, Scx is isolated to the joint while Sox9 is localized to the regions that later differentiate into the long bones. As the joint further develops and the tendons condense (E13.5), Scx labeled cells are loosely organized between the Sox9 expressing cells of the underlying cartilage/bone and the MyoD expressing cells of the differentiating muscle [32]. Proper differentiation of this connective tissue during development requires specific coordinated expression among bone/cartilage, tendon, and muscle precursor cells. These progenitors are still plastic at this stage of development. For example, when cartilage precursor cells are grafted onto tendon specific regions of the mesenchyme in the chick embryo, they differentiate into tendon cells [33]. While regional signaling factors are likely important in coordinating such differentiation of
mesenchyme, the actual signals that cause differentiation into tendon are still not fully understood.

While the factors that initiate Scx expression are not fully understood, signaling from the muscle may promote differentiation and Scx expression. For instance, Scx expressing cells that initiate in the sclerotome of chick embryonic transplant cultures require factors from the adjacent dermomyotome to differentiate properly [34,35]. Double staining for MyoD (a muscle progenitor marker) and Scx has shown that two distinct cell populations exist at the border of the sclerotome (Scx) and myotome (MyoD) [35]. Double mutants for MyoD and Myf5 (another muscle marker), which do not form muscle, abolished all Scx expression [35], suggesting that signals from the adjacent muscle are necessary for tendon development. Therefore, a potential mechanism to promote differentiation in our TECs is to utilize signals that occur in the muscle during development.

Since investigators now recognize that no one factor or signaling pathway “produces” tendon, research has focused on several pathways in development for several tendon types and model systems. Four pathways that have been studied most extensively are: 1) fibroblast growth factor (FGF) signaling, 2) transforming growth factor β (TGFβ) signaling, 3) growth differentiation factor (GDF) signaling, and 4) BMP signaling. These pathways are the focus of the review below.

**FGF Signaling**

Fibroblast growth factor (FGF) signaling occurs in muscle precursors in the dermomyotome during development [35]. Brent et. al. has shown that FGF signaling acts directly on tendon progenitors located within the sclerotome at the border of the myotome. FGF signaling, especially FGF8, activates two transcription factors (Pea3 and Erm) and this activation
is necessary for subsequent Scx expression during embryogenesis [35]. FGF4 has also been shown to regulate Pea3 activation and Scx expression in the chick embryo [36]. In the absence of FGF4, expression of Scx, tenascin, and FGF8 all decrease [37]. However, when FGF4 is reintroduced, only Scx and tenascin are upregulated, suggesting that FGF4 and FGF8 may work in a coordinated fashion to aid in cell differentiation and tendon condensation at the myotendinous junction [37]. Therefore, controlled delivery of FGF4 and/or FGF8 to mesenchymal progenitor cells in our TECs may promote tenogenic differentiation of these cells (Fig. 3).

**TGFβ Signaling**

While FGF signaling may be important in the initiation of Scx expression and tendon differentiation at the sclerotome/myotome border, TGFβ is important for recruiting and maintaining tendon progenitors during tendon differentiation (Fig. 3) [38]. TGFβs comprise a small subset of the TGFβ superfamily and their three isoforms (TGFβ-1, -2, and -3) all act through a single type II TGFβ receptor. Targeted knockouts of this receptor are sufficient to disrupt TGFβ signaling [39], resulting in severe abnormalities of tendons. Induction of tendon progenitors was not lost in these mice but tendon condensation at E13.5 was significantly disrupted [38]. This disruption was seen at a time when TGFβ2 and TGFβ3 are normally expressed in the tendon condensation between the muscle and bone. The timing of this disruption, further suggests that TGFβ is more important in tendon condensation and maturation while FGF signaling may be more important in tendon initiation.

All three TGFβ isoforms are expressed within the tendon during development and have been studied closely during the stages of tendon condensation and early maturation [40]. TGFβ1 was not seen in the tertiary bundles of the tendon but was highly expressed within the endotenon
and at the myotendinous junction. TGFβ2 was mildly expressed throughout the length of the tendon, both in the tertiary bundles as well as in the endotenon, but was more highly expressed at the myotendinous junction [40]. TGFβ3 was moderately expressed within the tertiary bundles at the early stage of condensation but reduced thereafter and like the other isoforms, was highly expressed in the endotenon at all time points [40]. Although tenocytes in the tendon midsubstance do not highly express these isoforms, release of these factors from the surrounding endotenon and adjacent muscle may help to control tendon fiber assembly and maturation during this stage of development. Future mechanistic studies are needed to test whether TGFβ signaling from the surrounding endotenon actually influences expression of other tenogenic markers within tenocytes during development. By understanding this mechanism during development, we can then attempt to drive this process during the repair process such that proper assembly occurs.

**GDF Signaling**

GDFs and their main isoforms (GDF-5, -6, -7, and -8) comprise another subset of the TGFβ superfamily that has been shown to influence tenogenesis (Fig. 3) [41-45]. GDF5 and GDF6 knockouts show severe disruption of tendon structure and composition, as do GDF7 knockouts to a lesser extent [41-44]. GDF5 expression is also upregulated in GDF7 knockout mice, suggesting compensatory mechanisms among these isoforms and a coordinated expression among all 3 may be needed to control tenogenesis. GDF8, also known as myostatin, is a negative regulator of muscle mass and positive regulator of tendon [45]. Myostatin knockouts exhibit small, brittle, and hypocellular tendons [45]. Myostatin has also been shown to increase proliferation and expression of Scx, tenomodulin, and type-I collagen in tendon fibroblasts [45]. The temporal expression of GDFs during tendon development has not been studied however.
Therefore, it is not clear whether GDF signaling influences certain stages of development more than others.

**BMP Signaling**

BMPs, still another subset of the TGFβ superfamily, negatively influence tendon development in the mids substance but positively regulate the formation of the tendon-to-bone insertion site [46]. In particular, BMP2 promotes chondrogenesis and Sox9 expression while inhibiting tendon differentiation at the tendon-to-bone insertion [47]. Noggin, an exogenous inhibitor of BMP signaling, promotes tendon differentiation and reduces chondrogenesis [30]. On the other hand, inhibition of FGF signaling promotes chondrogenesis in the same model system [30]. These studies suggest that an antagonistic relationship exists between FGF and BMP signaling during the stages of development when cell fate is determined between tendon and cartilage. Controlling this process during repair is extremely important for producing a functional tendon-to-bone insertion, which is one of the many problems associated with clinical repair strategies [4,48].

**Signaling Overview**

It appears that different signaling pathways at the myotendinous junction and tendon-to-bone insertion control the location of tendon differentiation. Antagonist pathways exist that regulate formation of tendon vs cartilage vs muscle at these interfaces (Fig. 3). Properly controlling these signaling pathways during tissue-engineered repair of tendon is vital to producing functional tissue. However, further mechanistic studies are still needed to test whether this intricate control seen during development can be achieved within the healing tendon of the adult.
**Fig. 3:** While the signaling events that control limb tenogenesis are not fully understood, we do have a basic understanding of the main pathways that are involved during the 3 stages of development: 1) Initiation, 2) Organization and Differentiation, and 3) Maturation. 

**Initiation.** FGF4/8 and other signals from the ectodermal ridge contribute to tendon initiation and Scx expression (green cells).

**Organization and Differentiation.** TGFβs produced by the Scx expressing cells as well as other mesenchymal cells promotes the recruitment, organization, and subsequent differentiation of cells in the tendon. The condensation process is controlled spatially via BMP signaling from the bone and FGF and GDF signaling from the muscle. 

**Maturation.** Scx and Mkx continue the maturation of the tendon by inducing expression of collagen. The collagen is then assembled with the help of decorin, fibromodulin, and other fibrillagenic proteins into mature fibers. Adapted from Schweitzer et al 2010 [49].

**Transcription Factors that Regulate Tendon Development**

Scx is not the only important transcription factor that promotes tendon development. Mohawk homeobox (Mkx), early growth response 1 (Egr1), and 2 (Egr2) are all expressed in tendon during development [29,50,51]. While shown to regulate expression of Col1 through the TSE2 promoter of the Col1α1 gene [52], Scx is not the only regulator of Col1 expression during development as Scx knockout mice still produced type I collagen in tendons [52]. Mkx positively regulates Col1 expression as evidenced by Mkx null mice that yield hypoplastic tendon due to decreased Col1 expression [50,51]. Of particular note, Scx was still expressed in tendons of Mkx null mice but the Col1 expression was still significantly reduced at later stages, suggesting that Mkx may be required for tendon maturation [50]. Mkx initially is expressed during development within the proliferative myogenic population in the dermomyotome [51]. However, it is also a
potent inhibitor of myogenic differentiation [51], suggesting that Mkx may also be essential in the initiation of tendon differentiation at the scleretome/dermomyotome interphase. Egr1 and Egr2 have been implicated in tendon differentiation as well [52]. Both FGF4 and TGFβ can increase Egr expression [52,53]. Furthermore, Egr1 and Egr2 increased expression of Scx and Col1a1 de novo [52]. Egr1 can also activate tenascin-C (Tnc) expression, another tendon marker during development and healing [54]. In summary, Scx is initially expressed within tendon progenitor cells at the location that later becomes the tendon body while Mkx is initially expressed at the myotendinuous junction then becomes more prominent in the tendon midsubstance as the tendon matures. The regulation of these transcription factors and their subsequent activity is not fully understood and requires further study unfortunately.

**Tenogenic Glycoproteins that are Highly Expressed in Tenogenesis**

Several glycoproteins are important during tendon differentiation and maturation. Tenomodulin (Tnmd) is a transmembrane protein that has been shown to be a phenotypic marker for tenocytes and is also regulated by Scx [55-57]. Tnmd has been characterized during development and is localized to avascular mesenchyme tissue with high concentrations in the tendon midsubstance [58]. Loss of Tnmd expression in gene targeted mice leads to reduced tenocyte numbers, suggesting a role of Tnmd in regulating tenocyte proliferation [59]. Tenascin-C (Tnc) is another glycoprotein shown to be involved in tendon development as well as healing but is not as tendon selective as Tnmd [60]. Tnc is predominantly expressed at epithelial/mesenchymal interphases and interacts with basement membranes to promote an anti-adhesive effect with several cell types, suggesting an important mechanism for cell migration during development and healing [61].
**Proteoglycans that Contribute to Tendon Differentiation and Maturation**

Proteoglycans that are important during development, especially during maturation and fibrillogenesis, include biglycan (Bgn), fibromodulin (Fmod), lumican (Lum), and decorin (Dcn). Biglycan and fibromodulin were recently shown to make up the niche for tendon-specific stem cells [62]. Fibromodulin, along with lumican, promote larger diameter fibrils during collagen assembly in tendon while decorin promotes smaller diameter fibrils [63-65]. Knockouts of fibromodulin or lumican alone did not significantly alter tendon morphology but double knockouts for both yielded significant alterations in fibril diameter and shape [66]. Controlling the correct spatiotemporal expression of these assembly proteins is a vital part of producing a functional tendon matrix during healing and methods to promote this assembly may be elucidated using development studies.

**Summary and Future Directions**

While expression of several markers during development has been studied and mechanistic analyses are continually being conducted, we are still not aware of what specific factors initiate tendon differentiation, maintain its differentiation, and promote its maturation into a functional load-bearing tissue. These events need to be better characterized as well as the signals that drive them. With increased knowledge on this subject, tissue engineers may then be able to better recapitulate aspects of these events in tissue engineered constructs both in culture and during repair. However, prior to taking this step, we must contrast these developmental patterns and sequences with those observed during natural tendon healing in the adult. This comparison will be extremely important if we are to identify relevant and potent therapeutic targets that can be modulated in the adult during the healing process to truly improve repair outcomes.
**Tendon Healing**

Tendon healing can be classified into three overlapping phases: 1) inflammation, 2) repair, and 3) remodeling [67]. In the initial inflammatory stage, a hematoma forms within the first 24 hours of injury [67]. Erythrocytes and inflammatory cells (ie. neutrophils, macrophages, and mast cells) march into the wound site to remove necrotic tissue while also releasing factors to attract the migration of fibroblasts into the wound site [21]. Angiogenic factors are also released to promote neovascularization. Fibroblasts move into the injury site by migrating along the provisional matrix created during this phase. Extracellular proteins, such as fibronectin and tenascin-C, aid in the migration of the fibroblasts [21]. The proliferative fibroblasts that enter the injury site then begin assembling the extracellular matrix as the healing moves into the repair stage [67]. During this stage, type-III collagen is more prominent than type-I collagen [21,23]. Water and glycosaminoglycan content also increase during this phase as infiltrating fibroblasts rebuild the surrounding matrix. Once the matrix is laid down, the remodeling stage begins. The disorganized collagen matrix that is dominated by type-III collagen during repair is slowly reorganized along the tendon axis and type-I collagen becomes more prevalent [67]. This phase also includes a decrease in cellularity and glycosaminoglycan content. Depending on the location and severity of the injury, as well as other factors, the wound site will either remodel to near normal levels over the course of a year or manifest itself as a non-functional scar [21,67,68].

Unfortunately, investigators have yet to extensively track whether those tenogenic markers expressed in development also occur during tendon healing. Loiselle, et. al. found via *in situ* hybridization that Scx was not expressed by cells within the wound site but was expressed by resident tendon cells in the adjacent native tendon [68]. Expression of Scx peaked during the repair stage and then reduced to stable levels during remodeling. GDF5 was upregulated during
the remodeling stage at four weeks following a segmental defect in the murine flexor digitorum longus tendon [68]. Tenascin-C has also been shown in multiple tissue types to be highly expressed during healing [60]. This result is somewhat expected as this protein mediates the migration of cell types in response to the mechanical environment of the surrounding matrix [60]. Biglycan is also highly upregulated during early healing but is only a transient response while decorin remains upregulated throughout all stages of healing within the murine Achilles tendon [69]. Further analysis is still required to determine what other tenogenic markers are expressed during tendon healing and whether these contribute in similar patterns to what has been observed in normal development.

Researchers continue to debate whether tendon healing is dominated by extrinsic vs intrinsic healing [21,23,70]. Extrinsic healing is based on the premise that tendon cannot heal on its own. Instead healing occurs via an extratendinous blood supply with infiltration of proliferative fibroblasts from outside the tendon to contribute to the healing response [67,70]. Intrinsic healing, on the other hand, is based on the premise that tendon can heal on its own. During this process, endotenon and epitenon cells proliferate and migrate into the wound site and drive the healing response [67]. At this point, there is no clear indication to refute one theory over the other as indications of both have been shown in preclinical models [68,71,72]. In all likelihood both mechanisms contribute to the healing response. A lineage analysis is still required to determine if the cells that contribute to matrix repair and remodeling actually originate from a population of tendon progenitors or an extratendinous cell population with no markers of tenogenic capacity. Studies that seek to answer this question between intrinsic vs. extrinsic healing will be most helpful in developing therapeutic strategies.
Tissue engineers have been using animal models to discover ways to more effectively repair tendon and ligament injuries. Using various species including rats [73], rabbits [74], and sheep [71], investigators have created simple, well-controlled yet not clinically-relevant window defects as well as more clinically-relevant tendon-to-bone insertional injuries with degenerative features [75]. However, models of tissue degeneration have been more challenging in animals, which display no natural degenerative tendon features commonly observed in patients. Still if we are to truly understand the mechanisms that contribute to the clinical healing response, which is dominated by chronic pathologies, researchers will need to develop and sustain pathologic characteristics in preclinical models.

In this regard, researchers have attempted to develop animal models of tendon degeneration by employing overuse [75-77], by injecting collagenase [78], and by delivering proinflammatory cytokines [79]. Mechanical overuse has been employed by running both rats and mice on treadmills [76,80] or by using mechanical actuators to load the tendons directly [13,81]. These models have yielded matrix disorganization and increase in proinflammatory cytokines but the effect is not sustained [76,80,81]. Collagenase has been directly injected into tendons of rats [75], rabbits [82], and horses [78]. Such treatments disrupt collagen architecture within the tissue and induce hypercellularity. However, their effects depend on injection location and concentration. PGE2 has also been injected multiple times into the rabbit PT [79]. While PGE2 disrupted the collagen organization, the study did not investigate longer-term sustainability of this disruption [83]. A controllable and sustainable model of tendon degeneration is necessary to begin assessing repairs of these degenerative injuries. However, a further understanding of the pathogenesis of chronic tendinopathies is needed prior to the
creation of these model systems. One track of my dissertation will investigate methods for inducing and sustaining aspects of degeneration in the rabbit patellar tendon.

**Pathology of Tendinopathies**

Although histologic and epidemiologic evidence tends to support the claim that tendon degeneration is a primary cause of tendon tears, the etiology of degeneration is not well understood [84-87]. Traumatic injuries in regions of tendon degeneration are extremely common and provide a challenging problem to both scientists and surgeons [4,88,89]. Studies of primary care patients indicate that 16% of the general population suffers from shoulder pain [90]. These numbers only increase with age, as approximately 50% of the population will have some form of rotator cuff tear by the age of 65 [2,91,92]. Repairs of these injuries can be highly variable and are problematic, with the rate of successful repairs being only 43% for patients in this age range [93]. Degeneration can occur within the midsubstance of the tendon (as seen in Achilles tendons) but frequently occurs at the tendon-to-bone insertion or enthesis [94].

The terminology used to describe chronic tendon pathologies is often misused. To clarify, the term tendinitis is used to refer to chronic pain within the tendon and primarily involves inflammation [95]. The term tendinosis, also referred to as tendon degeneration, involves disruption of the tendon matrix by increased matrix remodeling but does not exhibit strong inflammatory infiltration. Finally, the term tendinopathy is used for all forms of chronic tendon pathology because it doesn’t take into account the underlying pathology [96]. Tendon degeneration is the classification for the majority of chronic tendon tears seen clinically that are extremely difficult to heal and for which tissue engineered strategies may provide novel treatment modalities for improving repair.
Tendon degeneration typically results in numerous changes to the cells and extracellular matrix. These include disruption and disorganization of the collagen matrix, mucoid degeneration, glycosaminoglycan (GAG) accumulation, fatty infiltration, hypercellularity, cellular hyperplasia, neovascularization, and alterations in the production of certain matrix metalloproteinases (MMPs) and tissue inhibitors to metalloproteinases (TIMPs) (Fig. 4) [85,97]. Certain MMPs that are either over- or under-expressed in degeneration include MMP-1 (interstitial collagenase 1), MMP-2 (gelatinase A), MMP-3 (stromelysin 1), and MMP-9 (gelatinase B) [5]. These particular MMPs all proteolytically degrade different collagen types while MMP-3 also breaks down proteoglycans. Several TIMPs (TIMP-2, -3, and -4) also display altered expression [98]. MMPs and TIMPs work together to remodel the extracellular matrix of the tendon and are essential for normal matrix turnover. Unfortunately, the expression equilibrium between these enzymes becomes unbalanced in degenerative tendon, contributing to extracellular matrix disorganization and subsequent decrease in material properties, leading to tendon ruptures.
Surgeons and researchers still debate the clinical factors in tendon degeneration, including its onset, the mechanisms involved in its pathogenesis, and the most successful methods for its treatment [4,5]. Although not fully understood, most researchers agree that the etiology of tendon degeneration is multifactorial. There are three main theories that have developed in recent years that attempt to explain the etiology and pathogenesis of tendon degeneration. These mechanical, vascular, and neural theories are now briefly described [5,22,89].
**Mechanical Theory**

This theory assumes that changes in mechanical loading on isolated areas of tendon, whether increased or decreased, may have a chronic degenerative effect on the tissue. Tendons exhibit viscoelastic behavior as seen in a typical stress-strain curve (Fig. 5). At low strain levels (below 2%), the crimped collagen fibers absorb the increasing strain by elongating, which is represented by the toe region in the stress-strain curve. Once the crimp is removed, the fibers orient in a parallel manner and deform in an elastic fashion, which is demonstrated in the linear region of the curve. Researchers have found that tendons experience a maximum physiological strain of around 4% in vivo [18,99]. As strains exceed 4% strain, however, the incidence of microtraumas (failure of individual collagen fibers) within the tendon increases, which begin to deteriorate the mechanical integrity of the tendon.

![Stress-strain curve](image)

**Fig. 5:** Stress-strain curve exhibiting mechanical response of tendon to strain. With increased strain levels, the tendon risks the potential for microtraumas and complete ruptures depending on the magnitude of the strain. Reproduced from Wang 2006 [8].
Patients who maintain prolonged activity levels corresponding to stress/strain ranges above the physiological range experience multiple microtraumas, which lead to inflammatory, neural, and oxidative stress responses [88,98]. There often is inflammatory cell infiltration and the production of inflammatory cytokines, such as interleukin-1β (IL-1β), prostaglandin-E2 (PGE2), and tumor necrosis factor α (TNFα) [22,98]. These cytokines can be released by inflammatory cells and even by resident tenocytes/fibroblasts within the tendon [22,89].

As stated earlier, evidence of a traditional inflammatory response with infiltration of inflammatory cells is not seen within degenerative tendon seen clinically, classified as tendinosis [95]. Nevertheless, it has been suggested that mechanical trauma can lead to multiple acute inflammatory responses that may contribute to the degenerative process [89,100]. This process is molecular but is likely altered by changes in mechanical environment and may involve a pathway consisting of inflammatory mediators that trigger altered expression of MMPs, TIMPs, aggrecanases, and other proteases, which ultimately leads to an imbalance in matrix turner and subsequent matrix disruption [101].

When tendon cells undergo repetitive mechanical strains in vitro, representing an overuse scenario, production of an inflammatory mediator, PGE2, increases [102,103]. PGE2 decreases collagen synthesis in osteoblasts and increases matrix metalloproteinase (MMP) production in uterine cervical fibroblasts [102]. Interleukin-1β (IL-1β), a proinflammatory cytokine, enhances the conversion of PGE2 from arachidonic acid via cyclooxygenase-2 (COX-2) [104,105]. Experimentally, expression of PGE2 also relates to expression of MMP-1, -3, and -13 in human tendon cells [101]. IL-1β also increases production of these MMPs [106,107]. The mechanisms of these inflammatory mediators during the pathogenesis of tendon degeneration and how they respond to changes in mechanical loading need to be assessed further, however.
Recent publications suggest that mechanical overuse can actually lead to mechanical underuse as the cells that reside on the fibers that are damaged during microtraumatic events are underloaded [108]. It has also been shown that tendon will deteriorate when not used or stress shielded [109,110]. Stress deprivation for prolonged periods decreases the tendon’s total weight, stiffness, and tensile strength [111,112]. Therefore, nonuniform stress concentrations and abnormal loading conditions may induce stress-activated protein kinases that lead to apoptosis and possible degeneration of the surrounding tissue [113,114]. While the exact mechanism of how changes in the mechanical environment influences chronic tendon pathology is unclear, one thing is for certain – maintaining a proper mechanical environment for tendon fibroblasts is extremely important for preserving tendon homeostasis and this fact needs to be considered during tissue-engineered repairs of these injuries.

**Vascular Theory**

The pathology of tendon degeneration involves both increases and decreases in circulation. In areas of low perfusion, localized ischemia may develop and deprive resident cells of necessary nutrients [22]. The lack of nutrient delivery to the cells can then lead to apoptosis [98]. Ischemia may be a result of the tendon adapting to maximal tensile load and upon relaxation of the tendon, reperfusion can lead to the release of oxygen free radicals and tendon damage [22]. For example, there is evidence to support that an area of hypovascularity exists within the midsubstance of the Achilles tendon between 2 and 6 cm proximal to the calcaneal insertion [115]. This region of the Achilles tendon is most susceptible to both degeneration and tendon rupture. The insertion site of the supraspinatus tendon into the greater tuberosity of the humerus is also a region of low vascularity and is quite susceptible to degeneration [116]. This
theory may help explain why certain regions of tendons are more susceptible than others to degeneration.

**Neural Theory**

Tendon overuse can lead to excessive neural stimulation as well. Tendons are highly innervated with nerves and these nerve endings are in close communication with mast cells within the tissue [98]. Excessive neural activity can cause mast cell degranulation and release of neurotransmitters such as substance P and calcitonin gene related peptide [4]. High levels of the neurotransmitters glutamate and substance P have been measured in degenerative tendon regions [117,118]. Substance P is a neurotransmitter that exists in small unmyelinated sensory neurons. Substance P release has been correlated to pain in rotator cuff disease [119] and the neural theory of degenerative pathology may be closely related to the inflammatory feedback mechanism. These neurotransmitters may work in conjunction with inflammatory cytokines in producing a condition that the cells cannot recover from, leading to an unrecoverable degenerative pathway.

**Summary**

The major outcome from this literature review, which provides several gaps in knowledge for my research, is that the molecular mechanisms that drive tendon normal development, tendon healing, and pathogenesis of degeneration are poorly understood. Consequently tissue engineers could benefit greatly from improved knowledge in these areas as it will provide a guide to design and assess future novel treatment modalities.
Chapter 3

The Relationships Among Spatiotemporal Collagen Gene Expression, Histology, and Biomechanics Following Full-Length Injury in the Murine Patellar Tendon

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ABSTRACT

Tendon injuries are major orthopaedic problems that worsen as the population ages. Type-I (Col1) and type-II (Col2) collagens play important roles in tendon midsubstance and tendon-to-bone insertion healing, respectively. Using double transgenic mice, this study aims to spatiotemporally monitor Col1 and Col2 gene expression, histology and biomechanics up to 8 weeks following a full-length patellar tendon injury. Gene expression and histology were analyzed weekly for up to 5 weeks while mechanical properties were measured at 1, 2, 5, and 8 weeks. At week 1, the healing region displayed loose granulation tissue with little Col1 expression. Col1 expression peaked at 2 weeks, but the ECM was highly disorganized and hypercellular. By 3 weeks, Col1 expression had reduced and by 5 weeks, the ECM was generally aligned along the tendon axis. Col2 expression was not seen in the healing midsubstance or insertion at any time point. The biomechanics of the healing tissue was inadequate at all time points, achieving ultimate loads and stiffnesses of 48% and 63% of normal values by 8 weeks. Future studies will further characterize the cells within the healing midsubstance and insertion using tenogenic markers and compare these results to those of tendon cells during normal development.
INTRODUCTION

Tendon and ligament injuries present a considerable socioeconomic problem that requires innovative treatment solutions [1]. Those having the highest frequency include the rotator cuff tendons, the quadriceps and patellar tendons, and the Achilles tendon [4]. Such injuries can be especially difficult to repair when there is: 1) a degenerative component to the soft tissue [4] and 2) an involvement of the tendon-to-bone insertion site where mechanical stress accumulates [48]. Given the frequency and challenges with traditional repair, tissue engineers have a real opportunity to improve the healing of both acute and chronic injuries to tendon midsubstance and insertions.

Improving tendon tissue engineering designs dictates that we also better understand the natural healing process following injury. Upregulation of type-I (Col1) and type-II (Col2) collagens is important for functional healing as Col1 is the major collagen type found in the tendon midsubstance and Col2 is expressed in the insertion site during development, growth and healing [46,120]. Immunohistochemistry and quantitative real-time PCR (qPCR) have typically been used to study these collagen types during tendon healing [12,121]. However, the temporal expression of these genes during healing is still poorly understood on a spatial or cell-by-cell basis.

Natural tendon healing has been investigated in numerous species including rats [75], rabbits [122], and sheep [71]. In some studies, investigators have made simple, well-controlled yet not clinically-relevant window defects while others have created more clinically-relevant tendon-to-bone insertional injuries with degenerative features [75]. Our laboratory has been using principles of functional tissue engineering (FTE) to create repairs of central-third patellar tendon (PT) defects that match normal tangent stiffness up to 50% beyond peak in vivo forces.
recorded in the New Zealand White (NZW) rabbit during inclined hopping activities [18,20].

While the rabbit central-third PT defect allows us to create controlled injuries and reproducibly analyze tissue-engineered repairs, the lack of genetic tools in the rabbit limits our understanding of the biology of natural healing. For this reason, we bred double transgenic Col1/Col2 mice with green fluorescent protein topaz (GFPtpz) and enhanced cyan fluorescent protein (ECFP) reporters to relate spatial and temporal patterns of gene expression with biomechanics during tendon healing.

Thus, the objectives of this study were to monitor changes in: 1) spatiotemporal Col1 and Col2 gene expression patterns, 2) tissue morphology, and 3) healing biomechanics following a full-length, central PT injury in Col1/Col2 double transgenic mice and to compare these natural healing results to contralateral surgical shams and normal PT in age-matched controls. We hypothesized that: 1) Col1 expression would be greatest in the soft tissue within the defect region at earlier time points (1-2 weeks) during matrix deposition but then decrease over time as the tissue remodels; 2) Col2 expression would be greatest at the insertions at earlier time points (1-2 weeks) but also decrease over time; 3) structural and material properties of the defect tissues would be significantly less than normal PT response up to 8 weeks post-surgery.

METHODS

Experimental Design

Tissue morphology, Col1 and Col2 gene expression, and tendon mechanical properties were investigated at 6 different time points post-surgery in sixty-four (64) 20-wk old (19.5±0.2 weeks; mean±SD) double transgenic mice (Table 1). Twenty-week old pOBCol3.6GFPtpz (Col1) and pCol2-ECFP transgenic mice, whose creation and genetics were described previously [123], were chosen because the tendons at this age are large enough to create repeatable defect
injuries. Natural healing of a full-length, central PT defect injury was directly compared with healing of a contralateral sham for both histology at 1, 2, 3, 4, and 5 weeks (n=3 each) and biomechanics at 1, 2, 5, and 8 weeks (n=12-13 each). Inter-animal comparisons were also made against histology (n = 3) and biomechanics (n = 15) from eighteen (18) normal 20-wk old mice.

**Table 1.** Experimental Design – Healing tissue within full-length central PT defects were compared to contralateral shams and age-matched normals for histology and biomechanics

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**Surgical Procedure**

All animal protocols were approved by the University of Cincinnati IACUC. Each animal was anaesthetized with 4% isoflurane and both hindlimbs were aseptically prepped. Using loupes (2.5X), anteromedial skin incisions were made over each patellar tendon. Medial and lateral borders of each PT were longitudinally incised. A jewelers forceps was slipped beneath the tendon and spread to lift the tendon from the knee joint without excessive tension. A longitudinal incision in the tendon was made to create the lateral border of the defect. The jewelers forceps was inserted through the lateral incision and pushed through the tendon to create the medial border of the defect. This incision was extended by sliding the forceps from proximal to distal ends so that the central portion of the tendon could be grasped with tissue forceps and cut at each insertion. A scalpel was used to repeatedly disrupt the insertions at both the patellar and tibial ends until bleeding was induced. In the contralateral limb, a sham procedure was performed by slipping the forceps beneath the tendon without creating the defect. Both limbs
were closed with 5-0 prolene suture. Subjects were allowed full range of motion in individual cages following surgery. Each animal was euthanized by CO₂ asphyxiation and the limbs were harvested for histological or biomechanical analysis.

**Histological Analysis**

Following euthanasia, the femur and tibia were cut mid-shaft and the skin removed. Samples were fixed in 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) for 4 hours at 4°C. The limbs were decalcified in 0.5M EDTA/PBS solution (Sigma-Aldrich Corp., St Louis, MO) for 7 days at 4°C, washed 3X in 1X PBS, embedded in OCT media (Andwin Scientific, Addison, IL) and frozen on a metal platform cooled by liquid nitrogen. Sagittal sections (12µm thickness) were made within the healing region of each defect limb. Sham and un-operated control specimens were sectioned in comparable locations. Tissue sections were examined under an inverted fluorescent microscope (Axiovert 25, Carl Zeiss Inc., Göttingen, Germany). Filters for GFPtpz (Exciter - 500/25, Emitter - 545/35, XF104-2; Omega Optical Inc., Brattleboro, VT) and ECFP (Exciter - 440/21, Emitter - 480/30; XF114-2, Omega Optical Inc.) were selected to identify regions of Col1 and Col2 gene expression, respectively. Serial sections were also stained with hematoxylin and eosin (H&E) to visualize tissue morphology.

Five reviewers blindly scored three 10X H&E images from each sample according to a grading system for 1) matrix alignment and 2) cellularity. The reviewers scored each image according to the following scale: 0 indicates normal, 1 indicates mild changes, 2 indicates moderate changes, and 3 indicates marked changes. Individual scores were then averaged together to provide a grade for each criterion.

**Biomechanical Analysis**

Following euthanasia, animals were frozen at -20°C. On the day of testing, the defect
limb was placed at 45° of flexion and the medial/lateral struts were removed. A digital image was taken with a ruler in plane to quantify tendon width. The contralateral PT was then trimmed down to a similar width. The tibia-PT-patella unit was then isolated and the thickness was measured from a digital image taken in the sagittal plane. The tibia was embedded in a custom grip aided with polymethylmethacrylate (Dentsply International, York, PA) and secured with a metal staple over the tibial plateau to prevent slippage or failure at the growth plate. The tibial grip was placed in a materials testing system (100R; TestResources, Shakopee, MN) and the patella was lowered into a custom conical-shaped grip within a PBS bath at 37°C (Fig. 6C-D). Each specimen was preloaded (0.02N), preconditioned (25 cycles, 0-1% strain, 0.003mm/sec), and failed in uniaxial tension (0.003mm/sec) while recording grip-to-grip displacement and load.

**Statistical Analysis**

Comparisons against normal and comparisons among the defect groups over time were made for structural and materials properties via independent student t-tests with a bonferroni correction for multiple comparisons. A total of nine comparisons were made with a significance level of p = 0.006. All statistics were performed using SPSS 13.0 (Chicago, IL).

**RESULTS**

**Surgery and Gross Examination Following Injury**

The surgeries succeeded 89.3% of the time, with only eight animals showing lameness or ruptured tendons following the injury. Therefore, a total of 72 animals were required to complete this 64-subject study. Tendon defect width (0.63 ± 0.13mm, mean ± SD) averaged 42% of total PT width (1.50 ± 0.29mm). The natural healing site was slightly translucent at 1 week but was opaque and consistently darker compared to the surrounding native struts at all time points thereafter up to 8 weeks (Fig. 6).
Fig. 6: The full-length, central PT defect just following surgery (a) becomes discolored during healing (b). During mechanical testing, the sample was mounted in the tibial grip (c) aided with bone cement and a staple. It was then lowered into the patella grip (d) in the testing system.

**Histological Analysis**

The defect tissue at 1 week post-surgery consisted of loose granulation tissue with inflammatory cells, areas of neovascularization, marked reduction in matrix alignment and marked increase in cellularity (Figs. 7B & 8B). After 2 weeks, the ECM was denser but still highly disorganized and hypercellular (Figs. 7C & 8C). Nuclei were hypertrophic and inflammatory cells were still present within the midsubstance of the tendon (Figs. 7B-C & 8B-C). At 3 weeks, the ECM showed improved alignment with only mild to moderate changes but remained mildly hypercellular (Figs. 7D & 8D). The defect at 4-5 weeks post-surgery exhibited a more aligned ECM that appeared similar to normal PT by H&E (Figs. 7E-F & 8E-F).
Semi-quantitative histological scores for matrix and cellular alignment confirmed these qualitative observations. Compared to normal PT (0.33±0.38; mean±SD), early healing tendon matrix was quite disorganized (3.00±0 at 1 week; 2.50±0.58 at 2 weeks; and 1.42±0.63 at 3 weeks) with near normal values by 4 and 5 weeks (0.25±0.32 and 0.33±0.47, respectively). Similarly, compared to normal PT cellularity (0.42±0.50), the healing tissue was hypercellular early (2.75±0.50 at 1 week; 2.42±0.50 at 2 weeks; and 1.25±0.32 at 3 weeks) but more normal at later time intervals (0.92±0.17 at 4 weeks; and 0.17±0.33 at 5-weeks.

Gene expression and cellular morphology varied over time in the defect tendons. Compared to normal PT, Col1 expression at 1 week post-surgery was minimal within the midsubstance but elevated at the insertions (Figs. 7b & 8b,h). Col1 expression peaked at 2 weeks in the midsubstance (Figs. 7c & 8c) with cells appearing more rounded than normal tenocytes. At 3 weeks, expression was still elevated compared to normal but considerably lower than at 2 weeks (Figs. 7d & 8d). Col1 expression did not change much between 3 and 5 weeks post-surgery but cells became more aligned with the tendon axis over time (Figs. 7d-f & 8d-f). Also noteworthy were the bony nodules that formed near the insertions of the healing tissue at multiple time points (Figs. 8H-I & 8h-i). Col1 expression was highly elevated in the hypertrophic cells that lined these nodules (Figs. 8h-i). Blood and marrow also appeared within the nodule at later time points (Fig. 8i). Minimal Col2 expression was observed in these areas (Fig. 8i) and was not present in the tissue midsubstance at any time point.

Compared to normal PT, the contralateral shams were slightly hypercellular but showed a consistently aligned ECM at all time points (Fig. 9A-C). Col1 expression was elevated throughout the length of the tendon at 1 week post-surgery (Fig. 9a). The expression was reduced by 2 weeks and similar to normal PT expression at 3 weeks and thereafter (Fig. 9b-c).
Fig. 7: H&E (A-F) and Col1-GFPtpz/Col2-ECFP (a-f) micrographs from serial sagittal sections from Normal PT (A,a) and the defect region at one (B,b), two (C,c), three (D,d), four (E,e) and five (F,f) weeks of healing.
**Fig. 8:** Serial H&E and Col1-GFPtpz/Col2-ECFP micrographs from the midsubstance (A-F, a-f) and tibial insertion (G-I, g-i) from normal PT (A,a,G,g) and the defect region at one (B,b,H,h), two (C,c) three (D,d,I,i), four (E,e) and five (F,f) weeks of healing. (J,j) Col2 expression can be seen within chondrocytes in articular cartilage beneath the patella.
Fig. 9: Serial H&E and Col1-GFPtpz/Col2-ECFP micrographs of contralateral sham limbs at one (A,a), two (B,b), and three (C,c) weeks depicting an increase in Col1 expression at 1-week that reduces to normal PT levels by 3-weeks post-surgery. Four and five weeks data not shown but were consistent with the 3 week time point.
Biomechanical Analysis

Surgical treatment and time post-surgery each significantly affected the tissue’s structural and material properties. Ultimate loads transmitted by the 2-, 5-, and 8-week natural healing tissues were only 37%, 50%, and 48% of normal PT values, respectively (p < 0.006; Table 2, Fig. 10a). Similarly, stiffnesses for the natural healing tissues were 48%, 62%, and 63% of normal PT (p < 0.006). These two structural parameters appeared to reach stable asymptotic values by 8 weeks. In fact, time post-surgery (2, 5, and 8 weeks) had no effect on ultimate load or stiffness of the natural healing tissue. The mode of failure for these specimens was predominantly (94%) soft tissue failures near the distal insertion with the remainder occurring in the tendon midsubstance. Also, the failure mode did not differ among groups and had no effect on mechanical properties. It is important to note that the 1-week defect tissues were too compliant and fragile to test in uniaxial tension. These tissues were frequently damaged during dissection and therefore the 1-week biomechanical results were not reported.

The material properties of the natural healing tissues also reached asymptotic values by 8 weeks post-surgery. Ultimate stresses transmitted by the 2-, 5-, and 8-week natural healing tissues increased from 40% to 68% to 69% of normal PT values, respectively (p < 0.006; Table 2). The 2-week values were significantly less than the ultimate stresses for the 5- and 8-week time points. The moduli transmitted by the 2-, 5- and 8-week natural healing tissues also increased from 57% to 86% to 89% of normal PT, respectively, with the 8-week values being significantly greater than the 2-week moduli (p < 0.006). The modulus of the natural healing tissue at 2 weeks post injury was also the only healing time point that was significantly less than normal PT modulus (p < 0.006).

Structural properties for the contralateral sham groups were not different than normal
The material properties of the 2-week and 5-week shams were not different than normal. However, ultimate stress and modulus for the 8-week sham group was significantly higher than values for the normal PT ($p < 0.006$).

**Table 2.** Structural and material properties of the defect tissue compared to contralateral shams and age-matched normal PT (mean±SD)

<table>
<thead>
<tr>
<th></th>
<th>Ultimate Load (N)</th>
<th>Stiffness (N/mm)</th>
<th>Ultimate Stress (MPa)</th>
<th>Modulus (MPa)</th>
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<tr>
<td>Defect (2-wk, N=12)</td>
<td>1.54±0.81*</td>
<td>3.26±1.50*</td>
<td>4.67±1.99*</td>
<td>32.37±16.17*</td>
</tr>
<tr>
<td>Sham (2-wk, N=12)</td>
<td>3.85±0.92</td>
<td>5.26±0.77</td>
<td>14.17±4.06</td>
<td>60.12±20.48</td>
</tr>
<tr>
<td>Defect (5-wk, N=12)</td>
<td>2.08±0.70*</td>
<td>4.20±1.96*</td>
<td>7.90±2.53*§</td>
<td>48.85±24.39</td>
</tr>
<tr>
<td>Sham (5-wk, N=12)</td>
<td>3.76±0.76</td>
<td>7.22±0.91</td>
<td>13.80±4.93</td>
<td>79.47±23.51</td>
</tr>
<tr>
<td>Defect (8-wk, N=13)</td>
<td>2.00±0.60*</td>
<td>4.23±1.14*</td>
<td>8.01±2.28*§</td>
<td>50.35±10.79§</td>
</tr>
<tr>
<td>Sham (8-wk, N=13)</td>
<td>3.73±0.73</td>
<td>6.38±1.48</td>
<td>17.64±2.91*</td>
<td>90.57±19.18*</td>
</tr>
<tr>
<td>Normal PT (N=15)</td>
<td>4.13±0.87</td>
<td>6.78±2.05</td>
<td>11.68±3.38</td>
<td>56.51±18.29</td>
</tr>
</tbody>
</table>

* significantly different from age-matched Normal PT, § significantly different than Defect (2-wk) – bonferroni-corrected ($p < 0.006$) significant differences are noted above only for comparisons against normal and comparisons among the defect groups over time

**Fig. 10:** Average load-displacement curves. (a) The healing tissue showed significantly decreased structural properties at 2-, 5-, and 8-weeks compared to normal PT ($p < 0.05$). There were no significant differences among the healing tissues at any time point. The corresponding displacements at force levels recorded in the rabbit PT (21% of normal) [18] and the goat PT (40% of normal) [19] showed that the healing tissues were too compliant and not functional to 40% levels even after 8 weeks of healing. (b) The contralateral shams had no effect on structural
properties at any time point when compared to normal PT, except the stiffness for the 2-week sham which was greater than normal (p = 0.02). Error bars indicate SEM.

**DISCUSSION**

The objective of this study was to investigate changes in spatial Col1 and Col2 gene expression, tissue morphology and natural healing biomechanics over time following creation of a full-length, central PT injury. The natural healing tissue did not generate normal cellular or matrix organization by 5 weeks post-surgery or normal tissue biomechanics by 8 weeks postsurgery. A typical wound healing response was seen with hemostasis, inflammation, proliferation and remodeling stages [124]. We found an influx of inflammatory cells at 1 week consistent with previous work in a lamb central-third PT defect model [125]. Also consistent with our study, Sanchis-Alfonso et. al. found a disorganized matrix and hypercellular tissue at 2 weeks post-surgery with increased collagen matrix and reduced cellularity by 3 weeks. Although both studies showed further matrix remodeling and collagen fiber alignment along the primary axis of the tendon between 3 and 5 weeks, our results also revealed inferior repair biomechanics.

Most studies, including the current results, report linear stiffness and ultimate load to failure rather than repair properties in a lower-force, more functional region of loading. We have recorded peak *in vivo* forces of 21% and 40% of normal PT failure forces in the rabbit [18] and goat [19] models, respectively, for activities of daily living (ADLs) to create benchmarks for our tissue-engineered repairs. Since the extremely small size of the mouse knee prevents us from directly and accurately measuring murine PT forces, we have chosen to apply these design limits across species based on expected ADLs (Fig. 10a). **21% of Normal Failure Load (0.88N).** The 2-week natural healing tissue was functionally inadequate as it did not mimic normal tangent stiffness but required an additional displacement of 0.18mm to achieve 0.88N (p<0.05). By 5
weeks, however, the healing tissue was not significantly different than normal (p>0.05). The 8-week defect healing tissue actually matched normal PT tangent stiffness within this range. **40% of Normal Failure Load (1.65N).** The natural healing tissues at all three time points were inadequate requiring at least an additional 0.123mm of displacement (4% strain) to reach 1.65N. In fact, only 33%, 75%, and 69% of the samples at 2-, 5-, and 8-weeks, respectively, exhibited failure forces equal to or higher than this 40% level. This additional analysis further demonstrates the functional inadequacies of adult natural tendon healing in this murine model.

Multiple studies have looked at natural healing of full-length, central PT tendon defects in rabbits [122], dogs [126], and lambs [125] with varying biomechanical outcomes. Differences in surgical procedure, mechanical testing and analysis methods can contribute to these differences but other species- or size-related factors may have an even greater impact. We have attempted to compare biomechanical healing between central patellar tendon injuries in the mouse and rabbit, with average life-spans of approximately 2 years and 6-7 years, respectively. To better compare healing between these two species, we normalized time post-surgery to age at the time of surgery. Although it is apparent when mapping percent of normal force vs. normalized healing time (Fig. 11) that the mouse heals better and faster than the rabbit, several confounding factors could affect these outcomes. For instance, while the relative defect size compared to the overall tendon volume is similar in both models, the absolute defect volume in the rabbit model is 180X greater than in the mouse. As type-I collagen structure is likely homologous between species, the rabbit must produce and assemble far more ECM, potentially contributing to its slower and less complete healing response. The increased metabolism of the mouse combined with its lower tissue forces could also benefit defect healing. These comparisons are important as researchers attempt to translate tendon healing and tissue
engineered findings to larger preclinical models.

**Fig. 11:** Comparisons of ultimate load, stiffness, ultimate stress, and modulus vs. time post-surgery normalized to the age at surgery showed improved healing in the mouse model vs previous work done in the rabbit central-third defect model [122]. Error bars indicate SD.

There were limitations in this study. 1) Using a scalpel blade to create the insertional injury did not ensure that the entire insertion was removed. This limitation could have produced inconsistent spatial healing depending on where the scalpel injured the bone. This could also account for the minimal Col2 expression seen at these insertion injuries. We plan to use a surgical burr in future studies to uniformly disrupt the insertion within the central defect. This method of creating a consistent injury between species is necessary as we try to better mimic the bony trough created in our central-third NZW rabbit PT defect model [20,122,127]. 2) Due to the limited size of the tendon, we could not mark the boundaries of the defect during surgery as we
did when creating the rabbit PT defect injury [20,122,127]. Instead, we relied on the
discoloration of the defect region when removing the medial and lateral struts during dissection
prior to mechanical testing. Any remaining strut on the test specimen could have led to
overestimates of actual mechanical properties. 3) Slipping occurred within the patellar grip
during the uniaxial failure tests in a limited number of samples. The slippage typically occurred
between 1.5N and 2.5N of load. Therefore, displacement and strain at failure were not reported
and stiffnesses were calculated from the linear region prior to slipping. 4) Optical strain
measurements were not taken during the biomechanical tests, as tissue markers were difficult to
apply to the fragile healing tissue. Therefore, average modulus was reported over the full tissue
length. Future studies will utilize ink markers to measure local strains.

Given its genetic power, the murine full-length PT defect injury serves as a potent tool
for biological and biomechanical study of natural tendon healing. While its limited size may
prevent examination of certain novel tissue engineering treatments, advances made in the murine
model could conceivably be translated into larger models where repeatable surgeries and design
benchmarks are possible. Developing such analogs is particularly attractive if similar
biomechanical and/or biological responses to injury can be identified.

Just as functional tissue engineers have been developing mechanical success criteria,
corresponding biological success criteria will be needed as well. Murine injury models allow
measurement of such biological success criteria by comparing the expression of tenogenic
markers in natural healing tissue to normal adult tendon as well as normal tendon during
embryonic and early post-natal development. These comparisons are expected to provide multi-
functional design benchmarks for tissue-engineered therapies. Such strategies have the potential
to promote tenogenesis and zonal insertion regeneration rather than non-functional scar
formation and inferior repair biomechanics.

ACKNOWLEDGMENTS

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Chapter 4

Expression Patterns of Tenogenic Markers Following a Full-Length Patellar Tendon Injury

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ABSTRACT

Tendon injuries remain a significant socioeconomic burden due to their impaired healing capacity. Several preclinical injury models, including the murine central patellar tendon defect used in this study, have shown impaired natural healing. However, the expression patterns of important tenogenic markers during tendon development have not been studied extensively during natural healing. This study aims to classify the expression patterns of known tenogenic markers at 1, 2, and 3 weeks following tendon injury in the murine patellar tendon. We found that the healing tissue showed increased expression of fibrillar collagens (Col1a1 and Col3a1) but showed decreased expression of tenogenic transcription factors (scleraxis and mohawk homeobox) and collagen assembly genes (fibromodulin and decorin). In contrast, early growth response 1 and 2 were upregulated in these tissues along with tenasin-C. These data suggest that although tenogenic factors are expressed during healing, they are not expressed at the appropriate levels and therefore, are not producing a functional tendon. While Col1a1 is being produced, it may not be assembled properly outside of the cell due to the decreased expression of the fibrillogenic proteins (fibromodulin and decorin) needed for proper assembly. Future studies examine the assembly of the collagen matrix during healing and determine the lineage of cells that contribute to the healing response in order to develop mechanisms for attracting appropriate cells to the wound site and drive tendon differentiation during repair.
INTRODUCTION

Tendon injuries remain a significant socioeconomic problem that requires innovative treatment strategies for repair [1]. Unlike other tissues, the normal development and natural healing processes within tendon are not fully understood. Recently, the mechanisms for normal tendon development have begun to be elucidated. While there are no known markers that are truly tendon-selective, several genes have been shown to influence normal development [28-32]. However, the expression of these markers during natural healing has not been studied extensively. Determining the differences between natural, impaired adult tendon healing and normal tenogenesis may provide guidance for improved tissue-engineered repair by augmenting the healing process with therapies that promote tenogenesis.

Tendon healing has been studied in a wide variety of preclinical models including several defect and rupture models in the patellar, Achilles, rotator cuff, and flexor tendons in a number of species (mice, rats, rabbits, dogs, sheep, etc) [13,68,69,71,126,128]. Tendons typically experience a normal wound healing response to injury with inflammatory, repair, and remodeling stages [23]. Previous work in our lab has shown that these stages exist at 1 (inflammation), 2 (repair), and > 3 weeks (remodeling) post-surgery following the creation of a full-length, full-thickness, central patellar tendon defect in a murine model [129]. A substantial, yet transient peak in Type-I collagen (Col1) gene expression was seen during the repair stage at 2 weeks. However, the healing tissue only achieved ultimate loads and stiffnesses of 50% and 62% of normal values by 5 weeks and did not further improve by 8 weeks [129]. The mechanisms of this healing response are still unknown and the expression of tenogenic markers by cells within the wound site needs to be elucidated.

Expression of tenogenic markers is beginning to be studied during healing as they are
discovered during development studies. Loiselle et. al. [68] found that scleraxis (Scx) was expressed in the native tendon tissue surrounding the wound site following segmental defects of the flexor digitorum longus tendon in the mouse. Scx was not found within the wound site however, suggesting that these cells are likely not tenogenic in origin. Understanding the expression of tenogenic markers as well as the lineage of cells that express these markers during healing may provide mechanisms for tissue engineers to attract the appropriate cells to wound site in order to yield a functional repair.

This study will measure expression of numerous potentially relevant tenogenic markers. These include scleraxis (Scx), mohawk homeobox (Mkx), early growth response 1 (Egr1) and 2 (Egr2), six homeobox 1 (Six1), biglycan (Bgn), tenomodulin (Tnmd), fibromodulin (Fmod), decorin (Dcn), tenascin-C (Tnc), type I collagen (Col1a1), and type III collagen (Col3a1). These markers consist of transcription factors, glycoproteins, proteoglycans, and collagens that drive tenogenesis and fibrillogenesis in tendon. Genetic knockouts of most of these markers have shown impaired tendon formation [32,49,69,130] but investigators have not fully explored the expression of these markers during healing and how they differ from normal development.

To augment and accelerate the natural healing process, it is now imperative that tissue engineers understand the lineage of cells that enter tendon wound sites following injury. Therefore, the objective of this study is to measure the expression patterns of tenogenic markers following full-length, central PT injury in the transgenic reporter mice at 1, 2, and 3 weeks post-surgery. These expression patterns will be measured via qPCR, GFP reporters, and immunohistochemistry. The wound site will be compared to age-matched normal PT and contralateral shams. We hypothesize that the expression of tenogenic markers will be decreased in the wound site compared to normal PT and contralateral shams.
METHODS

Experimental Design

Tissue morphology, fluorescent reporter expression (Col3.6GFPtpz, Col2ECFP, and ScxGFP), immunohistochemistry (IHC), and qPCR were investigated at 3 time points post-surgery in sixty-seven 20-wk old (20.5±1.6 weeks; mean±SD) mice (Table 3). Real-time qPCR (n = 12 each) were analyzed in double transgenic pOBCol3.6GFPtpz (Col1) and pCol2-ECFP double transgenic (Col1/Col2 DT) mice, whose genetics were described previously and were used previously in this injury model [123,129]. Additional histology, immunohistochemistry, and two-photon imaging (n = 5 each) were also done in ScxGFP mice, graciously provided to us by Dr. Ronen Schweitzer [131]. Natural healing of a full-length, central PT defect injury was directly compared with healing of contralateral shams for both histology/IHC (n = 5) and qPCR (n = 12) at 1, 2, and 3 weeks. Inter-animal comparisons were also made against histology/IHC (n = 4 each) and qPCR (n = 12) from 16 normal 20-week old mice.

Table 3. Experimental Design – Healing tissue within full-length central PT defects were compared to contralateral shams and age-matched normals for histology/IHC and qPCR

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mouse Strain</th>
<th>Response Measure</th>
<th>Time Post-Surgery (weeks)</th>
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<td>Defect</td>
<td>ScxGFP</td>
<td>Histology/IHC</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Col1/Col2 DT</td>
<td>qPCR</td>
<td>12*</td>
</tr>
<tr>
<td>Sham</td>
<td>ScxGFP</td>
<td>Histology/IHC</td>
<td>5</td>
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<tr>
<td></td>
<td>Col1/Col2 DT</td>
<td>qPCR</td>
<td>12*</td>
</tr>
<tr>
<td>Normal</td>
<td>ScxGFP</td>
<td>Histology/IHC</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Col1/Col2 DT</td>
<td>qPCR</td>
<td>12*</td>
</tr>
</tbody>
</table>

* Twelve animals were pooled together in 3 samples of 4 tendons each for qPCR
**Surgical Procedure**

All animal protocols were approved by the University of Cincinnati IACUC. The surgical procedure was performed as previously described [129]. Briefly, the animals were anesthetized and aseptically prepped. The tendon was accessed through the skin and longitudinal incisions were made on the medial and lateral edges of the tendon. Jewelers forceps were then slid under the tendon and spread open. The lateral edge of the defect was created with a scalpel and then the jewelers forceps were placed into the incision and pushed through the tendon to create the medial edge of the defect. The central strip of tissue was cut away at the proximal and distal insertions. The distal insertion was then debrided with a fine-tooth jigsaw blade (T118G; Bosch, Farmington Hills, MI) that was filed down to 300 µm wide so that it could fit into the defect. The limbs were closed with suture and the subjects were allowed full range of motion in their cages following surgery. Each animal was euthanized with CO₂ asphyxiation and the limbs were harvested for their respective response measure.

**Sample Preparation for Histology and Immunohistochemistry**

Following euthanasia, the femur and tibia were cut mid-shaft and the skin removed. Samples were fixed in 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) for 6 hours at 4°C. Limbs to be imaged using two-photon microscopy were incubated in PBST (0.1% Trion X-100 in PBS) overnight at 4°C. The whole limb was then stained with 1X 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO) for 1 hour at room temperature. Limbs were transferred to PBS and imaged for ScxGFP and DAPI using the two-photon microscope. Following the multi-photon imaging, limbs were decalcified in 0.5M EDTA/PBS for 9-10 days at 4°C and then embedded in OCT media (Andwin Scientific, Addison, IL). The tendon healing region was then cut in sagittal sections (10-12 µm) and prepared for either
immunohistochemistry or fluorescence reporter analysis.

**Two-Photon Imaging**

Limbs harvested from ScxGFP mice that were stained with DAPI were mounted on the multi-photon microscope (Zeiss LSM510 NLO) stage such that the anterior surface of the patellar tendon was pressed against the glass coverslip on the stage. ScxGFP was imaged at varying depths by exciting with a wavelength of 900 nm and DAPI was imaged at an excitation wavelength of 740 nm. The emission filters for GFP and DAPI were 500-590 nm and 400-485 nm, respectively. Images were taken at 5 µm slices up to 100 µm deep within the defect region as well as in the adjacent native struts in the midsubstance of the tendon.

**Immunohistochemistry**

Frozen sections in either sagittal or coronal plane were incubated in blocking solution (10% donkey or goat serum, 0.1% Triton X-100, and 4% BSA in PBS) for 1 hour at room temperature. The sections were then incubated in anti-fibromodulin (1:200; AbCam) primary antibody overnight at 4°C. The next day, sections were washed 3X in PBST and PBS before being incubated in the secondary antibody.

**Quantitative Real-Time PCR (qPCR)**

Following euthanasia, the defect, sham, and normal tendon tissues were isolated and stored in RNALater® (Ambion) solution at -20°C. RNA was then isolated from the samples using the RNAqueous®-4PCR kit (AM1914; Ambion) following the manufacturer’s protocol. Briefly, samples were transferred to a fresh tube with lysis/binding solution and then disrupted with a pestle and vortexed vigorously. The lysate was captured in the glass filter, washed, and eluted. RNA was quantified using the Qubit® RNA assay kit (Invitrogen) and converted to cDNA using the High Capacity RNA-to-cDNA kit (4387406; Applied Biosystems). Real-time reactions were
performed using Taqman® Gene Expression Mastermix (4369510; Applied Biosystems) and Taqman® probes for Scx, Mkx, Egr1, Egr2, Six1, Tnmd, TnC, Fmod, Dcn, Bgn, Lum, Colla1, and Colla1 (Table 4). The relative amount of mRNA for each gene of interest was computed using the comparative C_T method (ΔΔC_T) with ΔC_T values normalized to the average of 18S and Gapdh and ΔΔC_T values normalized to normal 20-wk old patellar tendon controls.

Table 4. Taqman probe assay ID for each gene of interest.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>Assay ID</th>
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<td>Eukaryotic 18S rRNA</td>
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<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>Type III collagen, alpha 1 chain</td>
<td>Colla1</td>
<td>Mm01254476_m1</td>
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**Statistical Analysis**

Delta C_T values for the defect and sham groups at 1, 2, and 3 weeks were compared for each gene of interest via two-way MANOVA analysis with surgical treatment and time post-surgery as fixed factors (p < 0.05). Comparisons were also made among the defect, sham, and normal groups at each individual time point (1, 2, and 3 weeks) via one-way MANOVA with surgical treatment as the fixed factor (p < 0.05).

**RESULTS**

**Surgery and Gross Examination Following Injury**

Surgeries succeeded 86% of the time with only 9 animals presenting with lameness or
ruptured tendons. Grossly, the healing tendons appeared similar to a previous study using this injury model [129] with the healing tissue being darker than the adjacent struts at all time points. There were no apparent differences in the gross appearance between healing tissues in the ScxGFP and Col1/Col2 DT mice.

**Two-photon imaging**

Imaging both ScxGFP and DAPI fluorescence at 5µm increments into the tissue showed that none of the cells within the superficial paratenon/epitenon expressed Scx for any group at any time point (data not shown). However, once in the body of the tendon, cells expressed Scx in the struts and even in the defect tissue (Fig. 12). At 1 week, few cells expressed Scx in the defect region but cells within the adjacent struts displayed high ScxGFP signal (data not shown). By 2 and 3 weeks post-surgery, however, cells in the wound site were expressing more Scx than at 1 week. The healing region was hypercellular compared to the sham and normal PT controls at these later time points with poor cell alignment.
Fig. 12: Two-photon micrographs depicting ScxGFP expression (green) and DAPI nuclear staining (blue) with the defect tissue and contralateral shams at 1, 2, and 3 weeks post-surgery. The healing tissue at all three time points were hypercellular and Scx expressing cells increased with time. The contralateral shams had similar Scx expression compared to the normal PT controls. All images taken at a depth of 40 µm.

**Immunohistochemistry**

At 1 week post-surgery, coronal sections across the width of the tendon in the ScxGFP mice revealed decreased Scx expression within the defect region compared to the adjacent native struts (Fig. 13). Cells near the edge of the defect region showed greater Scx expression and alignment than cells in the center of the defect. Tissue at the defect/strut border and within the native strut showed greater fibromodulin staining than in the center of the wound site (Fig. 13). Similar to the two-photon imaging results, the number of Scx-expressing cells within the defect increased at 2 weeks compared to 1 week. However, the cells were disorganized and
hypercellular compared to the adjacent strut, contralateral sham, and normal PT (Fig. 13). Fibromodulin staining became more intense within the defect at 2 weeks; however, it was still more concentrated at the defect/strut border.

**Fig. 13:** Coronal sections across the width of the tendon show that Scx gene expression and FMOD protein production were reduced in the defect region (D) compared to the adjacent struts (S). Within the defect region, Scx and FMOD were decreased in the center of the defect compared to the defect/strut interface. Scale bar equals 100 μm.

**qPCR**

Although time post-surgery did not affect gene expression (p > 0.05), surgical treatment (defect vs. sham vs. normal) did significantly affect expression at each time point post-surgery (p < 0.05). Results for the genes of interest have been categorized into 3 groups: 1) fibrillar collagens, 2) transcription factors, and 3) proteoglycans and glycoproteins.

1) **Fibrillar collagens.** Defect and sham tissues showed elevated Col1a1 and Col3a1 gene expression compared to normal tissues (p < 0.05; Fig. 14). There were no differences between the defect and sham groups for either Col1a1 or Col3a1 expression. However, Col1a1 expression did reduce to normal levels at 3 weeks while Col3a1 remained elevated.
2) Transcription factors. The surgery groups produced variable changes in transcription factor expression (Fig. 15). Compared to shams, both Scx and Mkx were significantly reduced in the defect tissue ($p < 0.05$). Compared to unoperated normals, both of these genes showed a decreasing trend in the defect tissue, with Mkx displaying a significant 4-fold decrease in expression at 1 week ($p < 0.05$). Creating the defect also increased Egr1 and Egr2 expression compared to normal ($p < 0.05$) but not when compared to sham tissues ($p > 0.05$). Six1 expression did not change for either the defect or sham tissues compared to normal ($p > 0.05$).

3) Proteoglycans and glycoproteins. Bgn and Tnc showed increased expression in both the defect and sham groups with Bgn being increased during the early time points and Tnc being elevated throughout healing, with expression levels approaching a 32-fold increase ($p < 0.05$; Fig. 16). Creating the defect also decreased Fmod, Tnmd, and Dcn compared to normal ($p < 0.05$) but not compared to shams ($p > 0.05$). In particular, simply creating a defect produced a consistent 4-fold decrease in Dcn expression for all time points (Fig. 16).
Fig. 14: Fibrillar collagens exhibited similar expression between defect and sham tissues. Both Col1a1 and Col3a1 gene expression were upregulated in the defect and sham tissue compared to normal PT controls. The gene expression of all experimental groups was normalized to normal 20-week old PT (* significantly different than normal PT; # significantly different than defect/sham at corresponding time point).
Fig. 15: Scx and Mnx expression were down-regulated in defect vs sham tissues. While Egr1 and Egr2, known regulators of Scx expression [52], were upregulated in both the defect and sham tissues. Six1, a transcription factor that controls trunk tendon differentiation [132], did not change with either group (* significantly different than normal PT; # significantly different than defect/sham at corresponding time point).
Fig. 16: Fmod, Tnmd, and Dcn were down regulated in defect tissues. Fmod and Dcn, which are important in regulating collagen fibril assembly, were both down regulated. Tnmd was also down regulated in the defect tissue early. Bgn showed similar expression between the defect and sham tissues while Tnc was upregulated to a greater extent in the defect than the sham tissues (* significantly different than normal PT; # significantly different than defect/sham at corresponding time point).

DISCUSSION

Previous work by our lab showed that a full-length central patellar tendon injury in the Col1/Col2 DT mouse produced an impaired healing response with abnormal cellular and matrix organization and reduced mechanical properties up to 8 weeks post-injury [129]. Based on these findings, we next sought to discover if the impaired healing was in part due to the fact that the cells that contributed to the healing response were not properly differentiated and therefore were not expressing the appropriate tenogenic markers. Our current findings show that tenogenic markers are, in fact, expressed within the wound site during healing but at reduced levels
compared to normal aged-matched controls suggesting that they did not properly differentiate and did not produce a biomechanically functional repair with organized tissue structure.

Creating both the injury and the surgical sham resulted in differing effects on tenogenic gene expression. The surgical defect increased Col1a1 and Col3a1 expression levels, two of the most prominent tendon fibrillar collagens. However, the surgical defect also downregulated tenogenic transcription factors known to regulate Col1a1 expression (Scx and Mkx) [29,50,133] in the defect tissue compared to the contralateral shams, suggesting the Col1a1 expression is being modulated via other mechanisms during healing. Creating the surgical defect also downregulated fibromodulin and decorin, two genes important during tendon fibrillogenesis. This decreased expression may help to explain the poor mechanical integrity of the repairs seen previously [129]. These combined results also suggest that the main fibrillar collagens within normal and healing tendons were being produced but weren’t necessarily being assembled properly. Disrupted collagen assembly due to decreased production of fibrillogenic proteins outside of the cell is one likely mechanism for this impaired healing and future studies will need to more closely investigate the collagen assembly process.

It appears that scleraxis and mohawk homeobox are not regulating the healing response within the wound site in this model system. However, early growth response 1 and 2 influence both development of tendon [52] and healing of several tissues, including tendon [134]. Both Egr1 and Egr2 gene expression were increased in the defect and sham tissues. Egr1 was shown previously to increase Col1 gene expression following stimulation with TGFβ via Smad3 signaling in fibroblasts [53] and both of these transcription factors are implicated in promoting fibrosis [53,135]. Egr1 also regulates Tnc expression in response to mechanical stresses [136,137] and these genes may be involved in wound contraction during the repair and
remodeling stages at 2 and 3 weeks in this model system. Further analysis is needed to determine the signaling mechanisms that regulate this response and if these correspond with decreased expression of the two fibrillogenic genes we studied (Fmod and Dcn).

Researchers still debate whether extrinsic or intrinsic mechanisms drive tendon healing [21,23,70]. We have shown in this study that cells that migrate into the wound site express tenogenic markers to a certain extent but expression varies spatially across the defect width. The healing tissue near the adjacent struts shows higher expression of tenogenic markers and the matrix aligns earlier than what is observed in the center of the defect. The fact that the strut tissue is also hypercellular and less organized than normal tendon suggests that the injury influences the cellular phenotype within the struts and these cells may contribute to the healing response via paracrine signaling and/or direct migration into the wound site. This result suggests a potential intrinsic mechanism for healing in this model system, which we intend to study further.

This study did have a limitation. Due to the limited size and frailty, it was difficult to isolate the healing tissue away from the adjacent native struts for qPCR. Therefore, contaminating tissue from the struts may have influenced the gene expression results. However, the ScxGFP mouse showed that Scx was elevated in the adjacent struts compared to the wound site. Consequently, the qPCR gene expression of tenogenic markers within the defect samples may actually be overestimated due to strut contamination.

Understanding the expression of tenogenic markers during healing is an important initial step in classifying the degree of tenogenic differentiation of the cells that contribute to tendon healing. However, the mechanisms that control tendon healing and how they differ from normal tenogenesis in utero still need to be determined. Future studies will investigate the lineage of the
cells that contribute to the healing response via conditional knockouts of relevant tenogenic markers. Only upon understanding the lineage of the cells, where they come from, and to what extent they differentiate during healing, can we expect to formulate therapeutic strategies for improving healing of these repairs.

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Chapter 5

Effect of Paracrine Signaling on Tendon Tissue Engineered Construct Differentiation in Culture

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³ This manuscript is currently in preparation for Tissue Engineering.
ABSTRACT

Unfortunately, the natural tendon healing process often results in non-functional scar tissue and traditional repair of these injuries leads to inconsistent results depending on the location and severity of the injury. The challenge that scientists face when developing therapeutic strategies for tendon repair is that the signals required to stimulate the recruitment and differentiation of tenogenic cells to the wound site in order to produce a functional matrix are poorly understood. Our lab is using developmental biology as a strategy to guide our mesenchymal progenitor cell (bMPC) tissue engineering strategies. Signals from the adjacent muscle and signals from tenocytes within the tendon release factors during development that promote the differentiation of tendons. Therefore, the objective of this study was to test the effect of paracrine signaling released from myoblast and Achilles fibroblast cultures on expression of tenogenic markers in bMPC collagen cell constructs. Unfortunately the myoblast- and AFB-conditioned medias did not alter the tenogenic markers that we monitored (scleraxis, mohawk homeobox, fibromodulin, and tenomodulin). The myoblast media did increase the expression of the myogenic marker MyoD while both media types increased expression of Col3a1. The myoblast and AFB medias also stimulated proliferation of bMPCs within the constructs. These data suggest that the preconditioned medias are promoting a proliferative phenotype as opposed to inducing tenogenic differentiation, which is characterized by reduced proliferation.
INTRODUCTION

Tendon injuries represent a significant socioeconomic problem that worsens as the population ages [1,2]. Unfortunately, traditional repair strategies for these injuries yield inconsistent results depending on the location and severity of the injury [4,6]. One of the biggest challenges that scientists and clinicians have yet to overcome in tendon repair is delivering the appropriate factors/cells during healing such that the cells that participate in the repair process differentiate properly and subsequently assemble a functional tendon matrix. An approach utilized currently in our lab to solve this problem is to elucidate the factors that drive tendon differentiation and maturation during development and then employ these mechanisms in our tissue engineered strategies.

Several researchers, including those in our lab, employ cell-based strategies for improving tendon repair [15,26,138,139]. The cell source for these approaches can vary from mature adult tendon cells to immature mesenchymal progenitors [15,26,138,139]. Our lab has experience delivering bone marrow mesenchymal progenitor cells (bMPCs) into collagen-based scaffolds and providing a mechanical stimulus in culture to drive the progenitor cells to differentiate [20]. This approach has yielded 12-week repairs in the New Zealand White rabbit patellar tendon that match normal tendon tangent stiffness up to 50% beyond the peak in vivo load recorded for activities of daily living in the rabbit [18,20]. Unfortunately, forces as high as 40% of normal tendon failure force have been recorded in other model systems [19]. We therefore strive to produce repairs that match normal to this level and beyond.

In order to improve repair outcome, we are now using normal tendon development as a guide for our tissue engineering strategies. Understanding the signaling factors that drive tenogenesis and the markers that identify the different stages of the tenocyte lineage is of upmost
importance as we try to regenerate tissue as opposed to creating a non-functional scar, which occurs naturally. During development, signaling at the myotendinous junction controls tendon differentiation and Sex expression [37,49,140]. Factors released from the tendon cells within the developing tendon also influence the differentiation and maturation of the tendon [40]. Therefore, this study will test the effect of paracrine signals released from myoblasts and tendon fibroblasts on the differentiation of mesenchymal progenitor cells in collagen gel constructs.

The objective of this study is to test the effect of paracrine signaling from 1) C2C12 myoblasts and 2) Achilles tendon fibroblasts on tenogenic gene expression (scleraxis, mohawk homeobox, tenomodulin, fibromodulin, and type I collagen), construct stiffness, construct contraction, and cell proliferation of murine bMPC tissue engineered constructs. We hypothesize that myoblast and fibroblast signaling will both increase expression of tenogenic factors. Furthermore, we hypothesize that the myoblast signals will also increase early myogenic markers (ie. MyoD) and the fibroblast signals will also increase type III collagen (Col3) expression.

**METHODS**

**Experimental Design**

Gene expression, construct contraction, and construct linear stiffness were measured in bMPC-collagen gel tissue engineered constructs (TECs) at multiple time points in culture. The treatment groups consisted of 3 different media types: 1) 10% serum supplemented controls, 2) myoblast preconditioned media, and 3) Achilles fibroblast preconditioned media. Gene expression (n = 5 per group) and construct linear stiffness (n = 3 per group) were measured at three time points: 1) D0 – the beginning of the first day of stimulation, 2) D6 – after six days of stimulation, and 3) D12 – after twelve days of stimulation. Construct contraction was measured at D3, D6, D9, and D12 of stimulation (n = 8 per group). Cell proliferation was also measured at
Materials

The constructs were seeded with murine bone marrow mesenchymal progenitor cells (GIBCO® Mouse Mesenchymal Stem Cells; Invitrogen) that have been shown to be multipotent and express cell surface markers CD29, CD44, CD34, and Sca1. Prior to construct creation, these cells were cultured in MesenCult® murine proliferation media (STEMCELL, Technologies) supplemented with 1% antibiotic-antimycotic (Invitrogen). C2C12 myoblasts (CRL-1772; ATCC) were used to produce the myoblast preconditioned media. The Achilles tendon fibroblasts (AFBs) were isolated from one-year-old New Zealand White rabbits by removing the paratenon with a scalpel blade, then slicing the tendon into ~ 1-2 mm wide strips, and allowing the cells to migrate from the tissue over the course of two weeks. The myoblasts and Achilles tendon fibroblasts were cultured in ADV-DMEM (Invitrogen) supplemented with 1% GlutaMAX (Invitrogen), 1% antibiotic-antimycotic (Invitrogen), and 10% fetal bovine serum (premium quality; Atlanta Biologicals). PureCol® bovine type-I (Advanced Biomatrix) collagen product was used to make the collagen gel constructs.

Construct Creation

The bMPCs were subcultured to ~75% confluence at passage 2. The cells were then trypsinized and counted. The cells were mixed with PureCol® collagen gel adjusted to a final collagen concentration of 2.6 mg/ml and a final cell concentration of 0.5 x 10^6 cells/ml. Once mixed thoroughly, the cell-gel solution was pipetted into wells of custom silicone dishes [15,26,141] and allowed to gel in an incubator (37°C, 5% CO₂, 95% relative humidity). The constructs were given two days to equilibrate prior to the start of stimulation.
**Construct Stimulation**

On the day after the constructs were created, the myoblasts and AFBs were passaged into T175 flasks at seeding densities of $5.7 \times 10^3$ cells/cm$^2$ and $20 \times 10^3$ cells/cm$^2$, respectively. These seeding densities were chosen such that both cell lines were at similar cellular densities at each passage. After two days of incubation, the constructs were stimulated with control 10% FBS media, myoblast preconditioned media or AFB preconditioned media. The construct media was changed twice a day with either 1) 10% serum supplemented media (control), 2) myoblast preconditioned media, or 3) Achilles fibroblast preconditioned media. To do this, half of the media from the myoblast and AFB cultures was used to stimulate the TECs. Preconditioned media removed from the myoblast and AFB cultures was replaced with equal volumes of fresh ADV-DMEM supplemented with 10% FBS. The myoblast and AFB cultures reached confluency every three days and therefore were passaged on days 3, 6, 9, and 12 of stimulation.

**Quantitative Real-Time PCR (qPCR)**

Collagen constructs were harvested at the beginning of the first day of stimulation (D0) and after 6 (D6) and 12 (D12) days of stimulation. They were placed in TRI Reagent®, disrupted with nuclease free pestles, and frozen at -80°C. Total RNA was isolated from the samples according to manufacturer’s protocol (Ambion) and quantified using the Qubit® RNA assay kit (Invitrogen). The RNA was converted to cDNA using the High Capacity RNA-to-cDNA kit (4387406; Applied Biosystems). Real-time reactions were performed using Taqman® Fast Universal PCR Master Mix (2X; Applied Biosystems) and Taqman® probes for 18S, Scx, Mkx, Tnmd, Fmod, Col1a1, Col3a1, and MyoD (Table 5). The relative amount of mRNA for each gene of interest was computed using the comparative $C_T$ method ($\Delta\Delta C_T$) with $\Delta C_T$ values normalized to the average of 18S and $\Delta\Delta C_T$ values normalized to D0 control constructs.
Table 5. Taqman probe assay ID for each gene of interest.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eukaryotic 18S rRNA</td>
<td>18S</td>
<td>Hs99999901_s1</td>
</tr>
<tr>
<td>Scleraxis</td>
<td>Sex</td>
<td>Mm01205675_m1</td>
</tr>
<tr>
<td>Mohawk homeobox</td>
<td>Mkx</td>
<td>Mm00617017_m1</td>
</tr>
<tr>
<td>Fibromodulin</td>
<td>Fmod</td>
<td>Mm00491215_m1</td>
</tr>
<tr>
<td>Tenomodulin</td>
<td>Tnmd</td>
<td>Mm00491594_m1</td>
</tr>
<tr>
<td>Type I collagen, alpha 1 chain</td>
<td>Col1a1</td>
<td>Mm00801666_g1</td>
</tr>
<tr>
<td>Type III collagen, alpha 1 chain</td>
<td>Col3a1</td>
<td>Mm01254476_m1</td>
</tr>
<tr>
<td>Myogenic differentiation 1</td>
<td>MyoD</td>
<td>Mm00440387_m1</td>
</tr>
</tbody>
</table>

Biomechanical Evaluation of Constructs

Collagen constructs were harvested at the beginning of the first day of stimulation (D0) and after 6 (D6) and 12 (D12) days of stimulation and then frozen at -80°C. On the day of testing, the constructs were thawed and rehydrated in PBS at room temperature. The constructs were secured in grips and loaded into the mechanical testing system (TestResources, Inc). The constructs were failed in uniaxial tension at a strain rate of 10%/sec while load and grip-to-grip elongation were continuously recorded. Linear stiffness was then measured from the resultant load-displacement curves.

Construct Contraction

On days 3, 6, 9, and 12 of stimulation, digital images through the bottom of the silicone dishes were taken to quantify the total area of the constructs. Images were loaded into ImageJ (NIH Image, National Institutes of Health), converted to a binary image, and pixel area was converted to square millimeters. The percent of initial area was then calculated for each construct prior to performing the statistical analysis.

Cellular Proliferation (AlamarBlue)

AlamarBlue® (Invitrogen) was delivered to the constructs on days 6 and 12 of
stimulation to quantify the proliferation of cells within the constructs. The alamarBlue solution was diluted 1:10 in the respective media types and delivered to the constructs. The constructs were incubated for 3.5 hours prior to transferring the media to a 96-well plate. The absorbance was then read at 570 nm and 600 nm according to manufacturer’s protocol in a microplate reader (SpectraMax M2; Molecular Devices).

Statistical Analysis

Delta C_\text{T} values for constructs in each treatment group were compared for each gene of interest via two-way MANOVA analysis with media stimulation (DMEM, AFB, and Myo) and time in culture (D0, D6, and D12) as fixed factors (p < 0.05). TEC linear stiffness, construct contraction, and cell proliferation were also compared amongst the same treatment factors and levels via two-way ANOVA (p < 0.05).

RESULTS

Construct Gene Expression

Both days of stimulation and media type had significant effects on the gene expression of certain genes tested (p < 0.05). Effect of days of stimulation. Both the AFB media and control media increased fibromodulin expression over time (p < 0.05; Fig. 17). In fact, the control media produced a 13-fold increase in Fmod at day 12 (p < 0.05). The AFB media yielded a significant 5-fold increase at day 12 as well (p < 0.05). The myoblast media yielded a 3-fold decreased in tenomodulin expression at day 12 compared to the earlier time points (p < 0.05; Fig. 17). The TECs stimulated with AFB and myoblast media exhibited increased Col3a1 expression at days 6 and 12 compared to D0 controls (p < 0.05; Fig. 18). In addition, the myoblast media significantly increased MyoD expression over time while the AFB stimulated TECs showed a 13.5-fold decrease in expression at day 12 (p < 0.05; Fig. 19). Days of stimulation had no significant effect
on Scx and Mkx expression for any media type (p > 0.05; Fig. 20). Effect of media type. The AFB media reduced fibromodulin expression at day 6 while the myoblast media reduced its expression at day 12 compared to controls (p < 0.05; Fig. 17). The AFB conditioned media produced significant increases in Col1a1 expression at day 12 compared to both the control and myoblast stimulated constructs (Fig. 18). Both myoblast and AFB treatments increased Col3a1 compared to the controls (p < 0.05; Fig. 18). MyoD was considerably higher in the myoblast stimulated constructs than either the controls or AFB stimulated constructs at both days 6 and 12 as well (p < 0.05; Fig. 19). Media type did not affect Scx expression but the myoblast media did produce a 2-fold decreased in Mkx expression day 12 (p < 0.05; Fig. 20).
Fig. 17: Fibromodulin (Fmod) and Tenomodulin (Tnmd) gene expression (n = 5). Fibromodulin increased with stimulation time for the AFB and control media types while Tnmd was reduced in the myoblast-conditioned TECs at day 12. Error bars indicate ± SD. Bars indicate statistical significance (p < 0.05). * denotes difference from D0 (p < 0.05).
**Fig. 18:** Type-I (Col1a1) and Type-III (Col3a1) gene expression (n = 5). The AFB increased Col1a1 expression compared to the control and myoblast media types. However, both the myoblast and AFB media increased Col3a1 over time while the control TECs did not change. Error bars indicate ± SD. Bars indicate statistical significance (p < 0.05). * denotes difference from D0 (p < 0.05).
**Fig. 19:** Myogenic differentiation 1 (MyoD) gene expression (n = 5). The myoblast media increased expression of MyoD over time while the AFB decreased it. Error bars indicate ± SD. Bars indicate statistical significance (p < 0.05). * denotes difference from D0 (p < 0.05).
Fig. 20: Scleraxis (Scx) and Mohawk homeobox (Mkx) gene expression (n = 5). None of the media types had an effect on Scx expression while the myoblast media reduced Mkx expression at 12 days. Error bars indicate ± SD. Bars indicate statistical significance (p < 0.05).
**Construct Linear Stiffness**

Days of stimulation had a significant effect on construct linear stiffness ($p < 0.05$). However, media type had no effect. The construct stiffness increased from day 0 to day 6 in all constructs ($p < 0.05$). Also, while not significant, there were increasing trends in stiffness in the myoblast and AFB stimulated constructs compared to the controls.

![Graph showing construct linear stiffness over time](image)

**Fig. 21:** Construct linear stiffnesses recorded following uniaxial failure tests demonstrate an increase in stiffness from day 0 to day 6 ($n = 3$). There were no significant differences in stiffness with type of media stimulation. Error bars indicate ± SD. * denotes difference from D0 ($p < 0.05$).

**Construct Contraction**

Construct contraction was calculated from digital images at 3, 6, 9, and 12 days of stimulation. Both days of stimulation and media type had a significant effect on construct contraction. The initial area of the constructs decreased from 47.4%, 48.4%, and 50.1% at day 3 to 32.0%, 29.9%, and 32.8% at day 12 for the control, myoblast, and AFB media, respectively ($p < 0.05$; Fig. 22). In fact, the construct area was significantly reduced from the previous time
point for each day of stimulation (p < 0.05). There were differences between the media types at each time point, with the control constructs being smaller at day 9 and the myoblast constructs being smaller at day 12 (p < 0.05), but no patterns emerged across time points.

**Fig. 22:** Construct contraction increased with each day of stimulation for all treatments (n = 8; p < 0.05). While the control constructs were contracted more at day 9 and the myoblast stimulated constructs were contracted more at day 12, none of these changes persisted over multiple time points. Error bars indicate ± SD. Bars indicate statistical significance (p < 0.05).

**Cell Proliferation**

The number of cells within the construct was measured at day 6 and day 12 of stimulation with alamarBlue®. Both days of stimulation and media type had significant effects on cell proliferation. Cell proliferation increased by 15%, 65%, and 60% from day 6 to day 12 in the control, myoblast-stimulated, and AFB-stimulated constructs, respectively (p < 0.05). In addition, both the myoblast and AFB preconditioned constructs proliferated more than the controls during this period (p < 0.05).
The myoblast and AFB stimulated constructs showed increased cellular proliferation compared to controls at 12 days of stimulation as shown by relative absorbance units during an alamarBlue assay (n = 3). All media types increased in cell number from day 6 to day 12 (p < 0.05; * denotes significance from day 6). Error bars indicate ± SD. Bars indicate statistical significance (p < 0.05).

DISCUSSION

The objective of this study was to test the effect of paracrine signaling released from myoblast and Achilles tendon fibroblast cultures on the tenogenic differentiation of murine bMPC-collagen gel constructs. Our hypotheses were that signals released from a myoblast would increase expression of tenogenic markers by mimicking the signaling that initiates tendon differentiation at the myotendinous junction during development [37,49,140,142] while the Achilles tendon fibroblasts would promote differentiation by releasing tendon specific signals, since these are mature tendon cells. Unfortunately, we found that myoblast-preconditioned media promoted a considerable upregulation of the myogenic marker MyoD while reducing expression of known tenocyte markers, Mkx and Tnmd. On the other hand, the AFB-preconditioned media increased expression of both fibrillar collagens (Col1a1 and Col3a1) but did not alter any other
tenogenic markers (Scx, Mkx, Fmod, Tnmd). In addition, both the myoblast- and AFB-preconditioned medias promoted the proliferation of the bMPCs in the constructs over time. These data suggest that the preconditioned medias in this current configuration are not promoting the tenogenic differentiation of the progenitor cells in the constructs and instead are promoting a proliferative myogenic and scar fibroblast phenotype, respectively.

Currently, the factors that initiate and promote differentiation of mesenchymal progenitors towards tenogenesis are not fully understood. Developmental studies suggest that the initiation of tendon differentiation is highly influenced from signaling from the adjacent muscle [142]. Specifically, fibroblast growth factor 4 (FGF4) and 8 (FGF8) induce expression of Scx during development [37] via myogenic transcription factors Pea3 and Erm [35]. Therefore, C2C12 cells were used in this study as they have been shown to express high levels of Pea3 in culture [143]. The likely case is that these cells may not be releasing these FGF factors as originally thought and therefore, would not promote expression of Scx and other tenogenic markers. Likewise, tendon fibroblasts show increased expression of TGFβ1 compared to other cells types [144] in culture and therefore, could potentially increase expression Scx and other tenogenic factors as seen during development [49]. Further analysis is needed to determine the factors released by these cells and to optimize the concentrations of appropriate factors needed to promote tenogenesis.

This study was not without limitations. The cells and constructs in this study were cultured in media supplemented with 10% FBS. This level of serum was chosen to prevent the differentiation of the C2C12 myoblasts into myotubes, which occurs at low serum levels [145]. Media was changed twice daily in the myoblast and fibroblast cultures and then delivered to the constructs. There is a possibility that the serum levels were too high and the media was changed
too quickly to precondition the media enough to alter expression of these tenogenic markers in the constructs. Therefore, the high serum level may have been masking an effect that could exist at lower serum levels. Future studies will investigate this question by using 5% FBS supplemented media.

As tendon tissue engineers continue to develop novel strategies for improving tendon repair, we must further understand the factors needed to promote differentiation of the progenitor cells that contribute to the repair process. As we begin to further understand these mechanisms, we will then be able to develop strategies to induce this differentiation during healing in anticipation of promoting the proper matrix assembly seen during tenogenesis. The signals that drive this process must happen in a highly coordinated fashion consisting of the appropriate factors being delivered to the appropriate cells at the appropriate times. Strategies that stimulate this process whether through chemical, mechanical, or cell-based therapies may yield functional repair outcomes clinically.

ACKNOWLEDGMENTS

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Chapter 6

Cell- vs. Matrix-Induced Degeneration: The Effects of Prostaglandin-E2 vs. Bacterial Collagenase on Patellar Tendon Biomechanics and Histology

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4 This manuscript is currently in preparation for the Journal of Orthopaedic Research.
ABSTRACT

Controllable, repeatable, and sustainable animal models for tendon degeneration are needed for accurate repair assessment of clinically relevant injuries. This study was designed to contrast cell-mediated vs. extracellular matrix (ECM)-mediated strategies to create histological and biomechanical aspects of tendon degeneration within the rabbit patellar tendon. The cell-mediated model consisted of delivering prostaglandin-E2 (PGE2) from poly(lactic-co-glycolide) films into the midsubstance of the NZW rabbit patellar tendon. The ECM-mediated model consisted of injecting bacterial collagenase into the distal insertion of the tendon. PGE2 displayed a sigmoidal release pattern from PLGA films in saline at 37°C for 24 days. Delivery of PGE2 did not yield histological changes resembling that of degenerative tendon at 4 weeks post-surgery. A single injection of 200IU collagenase produced collagen matrix disorganization, hypercellularity, and neovascularization without inflammation at 4 weeks. Biomechanically, the PGE2-treated tendons were not significantly different than normal tendon while the collagenase-treated tendons displayed significantly reduced structural and material properties at 4 weeks. The long-term response to the collagenase injection was also investigated at 16 weeks. These tendons continued to display hypercellularity but the matrix began to reorganize such that structural properties were not significantly different than normal tendon. We conclude that the collagenase injection produces aspects of degeneration in the short-term but does not sufficiently alter cell phenotype in the long-term (3 month) to prevent tendon matrix restoration. A possible approach for achieving sustainability of these degenerative changes may be a combination of altering ECM using collagenase while also disrupting cell phenotype by other means.
INTRODUCTION

Chronic tendon pathologies, particularly within tendon-to-bone insertion sites, contribute significantly to the overall number of musculoskeletal injuries [89]. In fact, 97% of tendon injuries involve chronic degeneration [10]. These injuries occur with high frequency to the rotator cuff (supraspinatus and infraspinatus tendons in the shoulder), the quadriceps and patellar tendons, and the Achilles tendon [4,5,88]. The frequency of these injuries increases with age as well with 50% of the population over the age of 60 exhibiting degeneration of the rotator cuff [2].

What constitutes degeneration in patients, the factors that influence the onset, the mechanisms involved in the pathogenesis, and the most successful methods for treatment are still debated [4,5,88]. Mechanical overuse is thought by many to be a primary factor in the etiology [4,5,88]. Mechanical overuse leads to microtrauma, eliciting both local inflammation and oxidative stress to resident tenocytes [22,98]. The tendon’s response to multiple microtraumatic events may cause an imbalance in the matrix turnover process [5], leading to altered production of several matrix metalloproteinases (MMPs). This imbalance is then believed to lead to collagen matrix disruption and changes in cell phenotype, ultimately resulting in degenerative characteristics such as collagen matrix disruption/disorganization, tenocyte hypercellularity, tenocyte nuclear hypertrophy and rounding, mucoid degeneration, neovascularization, and small nerve ingrowth (Fig. 24) [22,85,98].

Although microtrauma elicits local inflammation, inflammatory cells are not seen histologically in degenerative tendons [22,23]. Since clinical specimens are usually taken from end-stage degenerative regions, it does not rule out the role of inflammation in the pathogenesis. Based on mechanical overuse models demonstrating increased expression of inflammatory
mediators as well as MMPs [75,102], it has been suggested that multiple acute inflammatory responses may drive the pathogenesis of degeneration [4,5,89]. For instance, mechanical overloading of tendon explants and tendon fibroblasts in culture increases expression of MMP-1, MMP-13, interleukin-1β (IL-1β), and PGE2 [102,106]. PGE2 delivery to tendon fibroblasts in culture produces decreased collagen synthesis and cellular proliferation [146]. Multiple injections of PGE2 into the midsubstance of the rabbit patellar tendon (PT) induce matrix disorganization and hypercellularity as well [79]. Furthermore, administration of IL-1β increases MMP production in numerous tissues including tendon [106,107]. A potential feedback mechanism likely exists where mechanical overuse leads to the release of inflammatory mediators which in turn leads to alteration in MMP production and, over time, matrix disruption.

Fig. 24: Schematic of potential mechanisms leading to tendon degeneration. Figure reproduced from Shearn et. al. 2011 [147].

Preclinical models are needed to better understand chronic tendon pathologies as well as to assess novel treatment modalities. Unfortunately, the majority of animal models of tendon
injuries do not recreate the degenerative condition. Instead, these models mimic the uncommon situation in which an acute traumatic injury or tear occurs to healthy tendon. Researchers have attempted to develop animal models of tendon degeneration by employing overuse [75], collagenase injections [78], and cytokine treatments [79] to better reproduce aspects of degenerative tendon injuries. Collagenase has been directly injected into tendons of rats [75], rabbits [82], and horses [78]. This treatment disrupts collagen architecture within the tissue and induces hypercellularity, and depends on the injection location and concentration. PGE2 has also been injected multiple times into the rabbit PT, producing disruption of the collagen organization but the study did not investigate long-term sustainability [79]. A controllable and sustainable model representative of tendon degeneration is needed to begin accurately assessing repairs of these clinical injuries.

This study aims to investigate two potential mechanisms to induce a chronic degenerative tendon. The objective of this study is to determine the extent to which PGE2 delivered from a poly(lactic-co-glycolide) (PLGA) film and bacterial collagenase delivered via injection into normal rabbit patellar tendons induce changes in histology and biomechanics consistent with aspects of tendon degeneration. We hypothesize that PGE2 will alter cell phenotype and therefore produce more prolonged degenerative aspects while collagenase will significantly alter the matrix initially but the cells will be capable of repairing the damage over time.

MATERIALS AND METHODS

Experimental Design

PGE2/PLGA Model. Prior to implantation into the tendon, the release of 750 ng of PGE2 from the PLGA films (n = 4) was measured in 0.9% PBS at 37°C for 24 days in vitro to measure the total amount, duration, and pattern of the release. PLGA films containing 750 ng of PGE2
were inserted into patellar tendons of twelve skeletally mature, 1-year-old, female New Zealand White (NZW) rabbits (Myrtles Rabbitry, Thompson Station, TN). PLGA films containing 0 ng of PGE2 were inserted into the contralateral tendons as a control. The tendons were harvested at 4 weeks post-implantation for histological (n = 3) and biomechanical (n = 9) analysis.

**Collagenase Model.** A single injection of bacterial collagenase (200IU in 0.05ml saline) was injected unilaterally in the distal end of the patellar tendon of ten skeletally mature, 1-year old female NZW rabbits. Six rabbits were assigned for histological (n = 3) and biomechanical (n = 3) analysis at 4 weeks post-surgery, while eight rabbits were assigned for histological (n = 3) and biomechanical (n = 5) analysis at 16 weeks post-surgery. The contralateral limbs received no injection and served as the unoperated normal PT group. A group receiving an injection of equal volume saline was not used as this has been previously shown to not affect histological nor biomechanical properties [82].

**Cell-Mediated (PGE2/PLGA) Model**

**Casting of PGE2/PLGA films.** PGE2 (≥99%, Sigma Aldrich, St. Louis, MO) in absolute EtOH was evaporated in glass vials. Then 50:50 PLGA (M_w = 51 kD, M_w/M_n = 2.04, Lakeshore Biomaterials, Birmingham, AL) and chloroform were added to form a 20% w/v mixture. The mixture was cast in aluminum weigh dishes, air dried for 72 hours, and vacuum dried for 24 hours. Elliptical samples (8 x 4 x 0.45 mm) were punched from the films under sterile conditions and either placed in glass vials (15 x 45 mm; Thermo Fisher Scientific, Inc., Waltham, MA) containing 1 ml of 0.9% PBS (HyClone, Logan, UT) for the in vitro release experiment or implanted at surgery.

**In Vitro Release of PGE2 from PLGA films.** The glass vials containing PGE2-incorporated PLGA films in 1 ml of PBS were placed on an orbital shaker (Model #22406A,
Daigger, Vernon Hills, IL) at 60 rpm at 37°C. The one milliliter volume of PBS was replaced with an equivalent volume every 3rd day until day 9 and then daily for the remainder of the experiment (24 days). Samples were removed, vacuum dried, and weighed every 9 days (n=4). Release of PGE2 into the PBS was measured via competitive-binding ELISA following manufacturer’s protocol (R&D Systems, Minneapolis, MN).

**PGE2/PLGA Film Insertion at Surgery.** All animal protocols were approved by the University of Cincinnati Institutional Animal Care and Use Committee. Each rabbit was anesthetized and the hind limbs were aseptically prepped. The PT was accessed via an anteromedial incision. Full-width coronal incisions were made through the midsubstance of the tendons, creating a pocket to insert the film. The PGE2/PLGA or PLGA only films were inserted into the pocket, which was then closed on both the medial and lateral sides using suture. The skin was closed with simple interrupted sutures. The rabbits were returned to normal cage activity following surgery. The rabbits were euthanized at 4 weeks post-surgery and the hind limbs were harvested and assigned to histology or biomechanics.

**ECM-Mediated (Collagenase) Model**

*Collagenase injections at surgery.* Bacterial collagenase (Sigma Aldrich, St. Louis, MO) was dissolved in saline the morning of surgery and kept on ice prior to use. Each rabbit was anesthetized, limbs were aseptically prepped, and PTs were accessed via anteromedial 1 cm long incisions. The distal insertion of the PT was palpated and collagenase was injected using a 25G needle into the center of the tendon. The rabbits returned to normal cage activity following surgery. The rabbits were euthanized at 4 or 16 weeks post-surgery and assigned to either histology or biomechanics.
**Histological Analysis**

Tendons were fixed in 10% neutral buffered formalin, processed through a gradient of alcohols, and embedded in paraffin blocks. Sections (4 µm thick) were made through the location of the PGE2 and collagenase delivery. Sections were stained with Hematoxylin and Eosin.

**Biomechanical Analysis**

Hind limbs were dissected down to patella-PT-tibia units. The tendon’s width was cut to its central-third such that comparisons could be made with previous PT tissue engineering studies. Length and width measurements of the pre and post-cut tendons were made with vernier calipers. Thickness measurements were made with a light force (<0.15 N) digital indicator (IDC-type Mitutoyo Digimatic Indicator, MTI Corp., Aurora, IL). The patella and tibia were held in custom grips aided with polymethylmethacrylate. Samples were placed in a tank of 0.9% PBS at 37°C mounted on a mechanical testing system (Model 8501, Instron, Inc., Canton, MA). The samples were preconditioned between 0 and 3% strain at 1 Hz for 50 cycles. The load was removed and the tendon was failed in uniaxial tension at a strain rate of 20%/sec. Grip-to-grip displacements and loads were acquired throughout the duration of the tests to create average load-displacement curves and to calculate structural (ultimate load, displacement at failure, and stiffness) and material (ultimate stress, ultimate strain, and modulus) properties.

**Statistical Analysis**

Structural and material properties of PGE2/PLGA and collagenase treatment groups were compared to the normal PT group via independent, two-tailed student t-tests with Bonferroni correction for multiple comparisons. The PT load-displacement failure curves were also compared between these groups using repeated measures ANOVA with Tamhane T2 post-hoc
comparisons due to heteroscedasticity. The significance level was set at \( p < 0.05 \). All statistics were performed using SPSS 13.0 (Chicago, IL).

RESULTS

**In Vitro Release of PGE2 from PLGA Films**

PGE2 released from the film in a sigmoidal fashion over the course of 24 days (Fig. 25). The films experienced significant swelling beginning at approximately day 9 and lasted through day 21, which corresponded to a dramatic increase in PGE2 release. The release plateaued around day 21, which corresponded to \(-40\%\) of the original film weight. The cumulative release of PGE2 was \(420.4 \pm 60.3\) ng (mean±SEM) for the four samples, which accounted for 56% of the total amount of PGE2.

![Fig. 25](image)

*Fig. 25:* The PGE2 released from the 750ng PLGA films in a sigmoidal fashion over the course of 24 days in vitro. A dramatic increase in the rate of release corresponded to periods of film swelling and breakdown starting after day 6. Error bars indicate ± SEM (n=4).

**Short-term Degenerative Response**

*PGE2/PLGA Model.* Four weeks after implantation of the PGE2/PLGA films, there was no histological difference between those limbs receiving PGE2/PLGA films and those receiving
PLGA films without PGE2 (Fig 26a-b). The incision line used to create the pockets for the films can be seen in the histological micrographs; however, no tissue response is observed that can be attributed to the release of PGE2. The release of PGE2 from the PLGA film had no significant effect on either subfailure or failure biomechanical properties of the tendon at 4 weeks post-surgery when compared to the contralateral control containing a PLGA film without PGE2 (Fig. 27, Table 6). The ultimate stresses for both groups were significantly less than that of normal PT (p < 0.05) but all other properties were not different than normal (Table 6).

**Collagenase Model.** At 4 weeks, a single 200IU injection of collagenase into the distal insertion of the PT elicited severe matrix disorganization, hypercellularity, nuclear hypertrophy, and neovascularization. Furthermore, there were no histological signs of inflammation (Fig. 26c). The severe matrix disruption observed histologically corresponded with inferior biomechanical results at 4 weeks. The ultimate load and stiffness for the collagenase-treated tendons at 4 weeks post-surgery were 57% and 48% less than normal PT values, respectively (p < 0.01, Fig. 27, Table 6). Sixty-seven percent of the collagenase-treated tendons failed at the site of the injection compared to zero percent of the normal control tendons (p < 0.05).

**Long-term Degenerative Response**

**Collagenase Model.** At 16 weeks post-injection, the tendon was hypercellular with hypertrophic nuclei compared to control tendons (Fig. 26d). However, compared to the response seen at 4 weeks, the cells were more elongated and the surrounding matrix was more aligned along the tissue axis. The improved matrix realignment observed histologically was consistent with the biomechanical results. The ultimate load and stiffness of the 16-week collagenase-treated specimens were 22% and 18% lower than normal PT values, respectively (p > 0.05; Fig. 27, Table 6).
**Fig. 26:** H&E micrographs depicting location of (a) PLGA and (b) PGE2/PLGA film within the midsubstance of tendon at 4 weeks post insertion. (c) The collagenase injection produced matrix disruption and hypercellularity at 4 weeks and (d) hypercellularity and nuclear hypertrophy at 16 weeks following the collagenase injection. (e) Normal PT histology is shown for comparison. All sections stained with H&E (scale bars = 50 µm).

**Table 6.** Structural and material properties for tensile failure tests (Mean ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>PGE2/PLGA Film</th>
<th>PLGA Film</th>
<th>Collagenase (4 weeks)</th>
<th>Collagenase (16 weeks)</th>
<th>Normal PT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultimate Load (N)</td>
<td>435.7±33.6</td>
<td>473.0±34.6</td>
<td>204.1±16.4*</td>
<td>372.1±30.0</td>
<td>476.8±29.6</td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>216.8±18.9</td>
<td>218.4±11.6</td>
<td>93.5±15.8*</td>
<td>146.2±13.5</td>
<td>178.2±9.9</td>
</tr>
<tr>
<td>Displacement at Failure (mm)</td>
<td>2.83±0.14</td>
<td>2.90±0.12</td>
<td>2.90±0.27</td>
<td>3.58±0.22</td>
<td>3.45±0.23</td>
</tr>
<tr>
<td>Ultimate Stress (MPa)</td>
<td>66.7±6.4*</td>
<td>63.4±3.8*</td>
<td>40.8±3.7*</td>
<td>68.8±6.5</td>
<td>93.9±6.6</td>
</tr>
<tr>
<td>Modulus (MPa)</td>
<td>653.5±75.9</td>
<td>575.7±25.9</td>
<td>356.4±51.0*</td>
<td>531.2±49.0</td>
<td>676.3±46.7</td>
</tr>
<tr>
<td>Ultimate Strain (%)</td>
<td>14.5±0.7</td>
<td>14.9±0.7</td>
<td>15.0±1.7</td>
<td>18.2±1.1</td>
<td>18.3±4.1</td>
</tr>
</tbody>
</table>

* indicates significant difference compared to normal (p<0.05)
Fig. 27: Average load-displacement uniaxial failure curves showing significant reductions in subfailure and failure properties for the 4-week collagenase group (n = 3) compared to results for normal PT (n = 8; p < 0.05; see Table 6). No significant reductions were seen in structural properties for the 16-week collagenase (n = 5) and PGE2/PLGA (n = 9) film groups compared to normal PT (p > 0.05). Error bars indicate ± SEM.

DISCUSSION

Chronic degenerative tendon injuries are a significant clinical problem with many questions still left unanswered regarding the pathogenesis and proper treatment of the condition. By contrasting cell and ECM-mediated techniques for inducing degeneration, this study yielded several major findings. 1) A single injection of bacterial collagenase delivered to the distal insertion of the rabbit PT yielded appreciable matrix disruption/disorganization, tenocyte hypercellularity, and neovascularization, without inflammatory infiltration 4 weeks post-injection. This is relevant as these changes are consistent with the degenerative condition seen clinically [85]. 2) The severe histological changes observed 4 weeks following a single collagenase injection of 200IU did not persist up to 16 weeks. Although at 16 weeks the tissue was hypercellular compared to normal, matrix alignment appeared closer to normal tissues. We
conclude that matrix disruption caused by a single injection of collagenase is insufficient to chronically alter cell phenotype, thus allowing cellular recovery and extracellular matrix repair. 3) PGE2 delivered at a concentration of 750ng did not produce appreciable histological or biomechanical changes to the PT, suggesting that altering the concentration or release duration may be needed to elicit even an acute degenerative effect.

The histological changes seen at 4 weeks after the collagenase injection are consistent in appearance with previous animal studies. Our findings are similar to reports showing increased cellularity, vascularity, and matrix disruption at an equivalent time point following a collagenase injection into the midsubstance of the rabbit PT [82]. These histological changes are also consistent with findings by Carpenter et al. employing a Sprague-Dawley rat supraspinatus tendon model that combined collagenase injection with overuse running on a declined treadmill for 1 hour/day, 5 days/week [148]. Cell number increased, cell shape changed from spindled to round, and matrix disruption at 4 weeks was similar to the changes we observed in the current study (Fig. 26c).

Our PGE2 findings did not correspond to those found previously in which multiple injections of PGE2 were delivered to the midsubstance of the rabbit PT [79]. In this study, 500 ng of PGE2 were injected four times (2,000 ng total) over the course of four weeks into the PT. The authors noticed areas of hypercellularity and tendon disorganization at 1 week following the last injection. This study suggests that our concentration of 750 ng PGE2 may not be sufficient to elicit an effect.

There were limitations to this study. We did not measure the in vivo release of PGE2 from the PLGA films. However, we found no evidence of the films at 4 weeks post-surgery when examined grossly, in paraffin-embedded sections, and in frozen sections (data not shown).
This strongly suggests that the full dose of PGE2 was released in 4 weeks. Also we measured a full three weeks of release in vitro (Fig. 25), suggesting that the in vivo release period was longer than that of a bolus but within 4 weeks.

Delivering PGE2 or collagenase alone was unsuccessful in creating a sustained degenerative injury that lasted 3 months. PGE2 did not appreciably alter the matrix or cellular behavior and may in fact not be the most potent mediator for effectively altering cell phenotype. IL-1β has been delivered to tenocytes in culture resulting in increased expression of both MMP-1 and MMP-3 [106]. IL-1β has shown similar effects on MMP expression in chondrocytes as well [149]. A single injection of collagenase, while producing appreciable changes at 4 weeks that were consistent with aspects of degeneration seen clinically, was unable to sustain these changes long term because it did not sufficiently alter cell phenotype. These approaches alone are most likely inadequate for creating sustained degeneration of the rabbit PT. A combination of these techniques may ultimately be needed to not only disrupt the collagen matrix but also alter cell phenotype sufficiently such that the tendon cannot recover from the altered matrix produced by the collagenase injections.

ACKNOWLEDGEMENTS

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Chapter 7

The Effect of Defect Size on Collagen Expression and Healing Capacity of Murine Femoral Defects

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PREFACE

This chapter is the result of a collaborative effort between our lab and Dr. Goldstein’s lab at the University of Michigan, Dr. Awad’s lab at the University of Rochester, and Dr. Rowe’s lab at the University of Connecticut. This study was not the primary focus of my dissertation and consequently, I had a smaller impact on its design and execution. However, this study utilizes similar methodologies that I employed in chapter 3 and 4 to study tendon healing and is an extension of the BRP grant. I contributed to the design of this study and helped Dr. Grawe develop and execute, with guidance from Dr. Goldstein, the surgical procedure used to consistently create sub-critical (0.6mm) and critical-sized (1.6mm) defects. Dr. Awad’s group performed all biomechanical assays and Dr. Rowe’s group performed all histological assays. I assisted Dr. Aditya Chaubey, who is the primary contributor to this study, with preparation of the samples for biomechanics and histology as well. The findings of this study are presented in this chapter in my own words as it relates to the tendon natural healing studies that have been the primary focus of my dissertation.
INTRODUCTION

Previous chapters of this dissertation, specifically chapters 3 and 4, have focused on understanding the processes of tendon healing following full-length, central patellar tendon defects in GFP reporter mice. One of the mice used was a double transgenic Col3.6GFptpz (Col1) and Col2ECFP mouse [129]. This Col1/Col2 DT mouse contains fluorescent reporters for two important structural collagens in both the midsubstance (Col1) and tendon-to-bone insertions (Col1 and Col2). Using this mouse, we found that Col1 expression is highly elevated at 2 weeks of healing, which corresponded to the repair stage where fibroblastic cells were producing matrix to fill in the wound site. While Col1 and Col2 gene expression are important during tendon healing, they are also important in other musculoskeletal tissues – for instance, bone. This chapter will apply similar strategies used in chapters 3 and 4 for studying tendon healing and apply it to segmental defect healing in the femurs of the Col1/Col2 DT mouse. The purpose of this study is to test the effect of defect size on the degree of histological and biomechanical healing in the femurs of Col1/Col2 DT mice over time.

New bone is formed through two processes: 1) intramembranous ossification and 2) endochondral ossification [150,151]. This chapter will focus on the process of endochondral ossification and how it relates to fracture. Endochondral ossification is the main process that drives the formation of long bones (i.e. femur) during development, the lengthening of these long bones during growth, and the natural healing of bone fractures [150,152,153]. Bone initially starts as cartilage in this process and the chondrocytes form a primary center of ossification [154,155]. The cells secrete extracellular matrix causing the center to grow. The outside perichondrium of this center becomes the periosteum of the bone while the chondrocytes within the center hypertrophy and stop producing collagen and proteoglycans and start producing
alkaline phosphatase [155]. This enzyme promotes ossification of the matrix and the hypertrophic chondrocytes apoptose [155,156]. Osteoprogenitors then migrate into the voids left from the chondrocytes and differentiate into osteoblasts, which then secrete and assemble the osteon of normal bone [154-156].

During functional fracture healing, the bone goes through a similar process with several stages. The initial stage is inflammation where inflammatory cells (i.e. macrophages, leukocytes, and mast cells) migrate into the wound site to remove necrotic tissue and secrete factors that attract osteoprogenitors to the site [157]. In the second stage, a soft or cartilaginous callus forms around the fracture site and create a rudimentary bridge that span the fracture [158]. This callus then mineralizes in the third phase to form a hard callus. Finally, the callus remolds to a functional state [156-158].

Like tendon, bone can spontaneously heal depending on the severity of the injury. Relative to tendon, bone actually has a much higher healing capacity and is capable of healing large defects. However, like tendon, large segmental or “critical” sized defects (i.e. longer than the diameter of the bone) do not heal and are quite problematic clinically.

The objective of this study is to compare the stabilized fracture healing of a sub-critical and critical-sized transverse defect in the femurs of 10-week-old Col1/Col2 DT mice. We hypothesize that the critical (1.6mm) defect would yield non-functional healing while the sub-critical (0.6mm) defect would bridge properly, leading to a functional response.

**METHODS**

**Experimental Design**

Four treatments were tested using bilateral surgeries in femurs of thirty-six double transgenic Col3.6GFPtpz and Col2ECFP reporter mice [123,129]: 1) sub-critical fracture
(0.6mm long) consisting of a transverse fracture with internal plate fixation, 2) critical fracture (1.6mm long) consisting of a transverse fracture with internal plate fixation, 3) contralateral sham consisting of internal plate fixation without fracture, and 4) contralateral unoperated controls (Table 7). The treatment groups were equally weighted among the animals such that an equal number of intra- and inter-animal comparisons could be made for each comparison. Histological and immunohistochemical analyses were performed at 2 and 5 weeks post-surgery (n = 3 per group) while µCT and biomechanical analyses were performed at 5 weeks (n = 12 per group).

**Table 7.** Experimental Design

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Response Measure</th>
<th>Time Post-Surgery (weeks)</th>
</tr>
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<tr>
<td></td>
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<tr>
<td>Sub-critical Defect</td>
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<td>Critical Defect</td>
<td>Histology/IHC</td>
<td>3</td>
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<tr>
<td>Sham</td>
<td>Histology/IHC</td>
<td>3</td>
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<tr>
<td></td>
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</tr>
<tr>
<td>Normal</td>
<td>Histology/IHC</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Biomechanics</td>
<td>12</td>
</tr>
</tbody>
</table>

**Surgical Procedure**

All animal protocols were approved by the University of Cincinnati IACUC. Each animal was anesthetized with 4% isoflourane and each limb was aseptically prepped. A lateral, longitudinal incision was made posterior to the vastus lateralis muscle along the length of the bone to expose the femur. Forceps were used to move the muscle away from the bone and a custom tapered, low contact titanium plate (courtesy of S. Goldstein at the University of Michigan) was fitted onto the bone and fixed with 4 self-tapping stainless steel screws (Fig. 28).
To create the sub-critical or critical defects, the surgeon (BG) used 0.6mm and 1.6mm diameter high speed steel drill bits, respectively, to cut through the bone. The muscle was pulled over the plate and the skin was closed with 5-0 prolene suture. In the sham group, the plate was fixated to the bone but no defect was created. No surgery was performed in the unoperated control group. Following skin closure, the animals were given an intraperitoneal injection of buprenorphine at 2 mg/kg and allowed free range of motion in their cages post-op. At either 2 or 5 weeks, the animals were euthanized at prepped for either histology or biomechanics.

**Fig. 27:** Controlled segmental defects with internal fixation. A centrally tapered customized plate (developed by Dr. Goldstein) was used to stabilize the fractures (A-C). The plate is fixed to the bone with 4 stainless steel self-tapping screws (D-E) and then either a sub-critical (F) or critical defect (G) is created using a 0.6mm or 1.6mm diameter drill bit, respectively.
**Histological Analysis**

Histology specimens were sent to Dr. Rowe at the University of Connecticut. Femurs were excised, trimmed of skin, and fixed in 10% neutral buffered formalin solution (Sigma-Aldrich) at 4°C for 2-3 days. The specimens were then radiographed at 24 kV for 8 seconds with a digital X-ray (Faxitron LX 60, USA). After the X-ray was recorded, the femur were soaked overnight in 30% sucrose/PBS solution and frozen in Neg-50 embedding medium (Richard-Allan Scientific, MI). Longitudinal full length 5 µm cryosections of the femur were taken either in line with or in cross section to the screw holes (Leica CM3050S Cryostat, Germany).

Images of each section were taken using a Zeiss Mirax Midi scanning fluorescent microscope (Carl Zeiss, Thornwood, NY). The bone mineralization was detected with a DAPI filter (Chroma, #49000ET), Col2ECFP with a CFP (blue) filter (cat no#49001ET), and Col3.6GFPTpz with a YFP (yellow-green) filter (cat no #49003 ET). After a section was imaged for endogenous signals, the cover slip was removed by brief soaking in PBS and then processed for additional stains. Osteoclasts were identified using the fluorescent ELF-97 phosphatase (TRAP) substrate (E6589, Invitrogen) and reimaged a second time with a yellow filter optimized for tetracycline (Chroma Technology Custom HQ409sp, 425dcxr, HQ555/30, set lot C-104285). Next the coverslip was removed and the slide was stained for alkaline phosphatase and imaged using the fast red substrate and a TRITC filter (Chroma 49005 ET). Hematoxylin staining was performed on the same slides after the fluorescent staining and imaging was completed. The slides were mounted with 50% glycerin in PBS and cover slipped for imaging.

**Micro Computed Tomography (CT) Imaging and Biomechanical Analysis**

At 5 weeks post-surgery, the femurs were excised, trimmed of skin and muscle, and frozen at -20°C. The samples were then shipped to Dr. Awad at the University of Rochester. On
the day of mechanical testing, the femurs were thawed and scanned by µCT (VivaCT 40; Scanco Medical, Bassersdorf, Switzerland) at 70 kVp, 145 µA, 300 ms integration time. Planar x-rays were also taken (5s exposure at 20kV, Faxitron X-Ray LLC, Lincolnshire, IL) before and after testing. Specimens were hydrated and potted in poly (methyl methacrylate) bone cement (PMMA; DePuyOrthopaedics, Inc., Warsaw, IN) in a custom jig to ensure axial alignment and a constant gauge length. Prior to testing, the titanium plates were cut perpendicularly through the center using a hand Dremel fitted with a stainless steel, diamond coated disc (Part # 011960U0, Brasseler USA Dental Instrumentation, Savannah, GA). Specimens were tested in torsion at 1°/s until failure using an EnduraTecTestBench system (200 N.mm torque cell; Bose Corporation, Minnetonka, MN). Torque data were plotted against rotational deformation (normalized by the gauge length and expressed as rad/mm) to determine torsional rigidity, yield torque, and ultimate torque.

Statistical Analysis

Biomechanical properties from the sub-critical defect, critical defects, contralateral shams, and normals were compared using two-way MANOVA with Tukey’s HSD post hoc comparisons (p < 0.05). Statistical significance was determined using SPSS 13.0 (Chicago, IL).

RESULTS

Histological Analysis

Non-decalcified longitudinal sections from the unoperated controls showed typical bone activity for a 10-week old mouse (Fig. 29). The Col3.6GFP positive cells were isolated to the metaphyseal bone. The growth plates were not fused in these animals and scattered Col2 expression was seen within the cartilaginous cells of the growth plate. Little osteoclastic or osteoblastic activity was seen in the diaphysis and DAPI nuclear staining identified mature
osteocytes within the cortical bone. Hematoxylin staining displayed the marrow space and vasculature throughout the bone.

The sham procedure depicted the effect of the low-contact plate and cortical set screws. In some cases, the screws produced a strong periosteal response that included the formation of an outer cortical shell and remodeling (Fig. 30-31). In the areas around the screws, there was an increase in osteoblast (AP) and osteoclast (TRAP) activity. Bone formation also occurred within the grooves of the screws through the width of the bone marrow space but not all of the screws produced this response. By 5 weeks, the intensity of the remodeling had diminished and was only present at the new bone in the outer cortical shell (Fig. 31).

The sub-critical defect (0.6mm) produced a greater periosteal response than the sham controls (Fig. 32-33). The periosteal activity extended along the length of the bone, not just isolated to the defect site. The sub-critical fracture showed good bridging even at 2 weeks (Fig. 32). There was a mild periosteal response at this time point but DIC imaging revealed new bone formation across the defect. Inwarding (inward growth of new bone and resorption of old bone) was also present with osteoblastic AP staining on the endosteal surface (Fig. 32D). At 5 weeks, remodeling was still occurring with increased AP and TRAP staining near the injury site (Fig. 33C-D).

The bone in the critical (1.6mm) group did not bridge the defect and there was an influx of inflammatory, fibroblastic, and myogenic cells within the defect space (Fig. 34-35). A strong periosteal response was seen and the bone progenitors attempted to span the defect but were incapable and ended up capping the end of the bone (Fig. 34C-D).
**Fig. 29:** Normal femur at 5 weeks shows no osteoblastic activity in the diaphysis as seen via Col3.6GFP and Col2ECFP fluorescence (C), TRAP (C), and AP (D) staining. X-ray (A), DIC imaging (B), and hematoxylin (E) staining of the femur show mineralization, new bone formation, and marrow space, respectively. Dr. Rowe’s lab performed all histological analysis.
Fig. 30: Femurs that were plated, but no defect was created (Sham) at 2 weeks post-surgery. (B) DIC image reveals new periosteal bone throughout the femur length. (C-D) Fluorescent imaging reveals osteoblastic (GFPTpz and AP) and osteoclastic (TRAP) activity near the 2 distal screw holes (left). (E) The marrow space appears healthy as indicated by Hematoxylin staining. Dr. Rowe’s lab performed all histological analysis.
**Fig. 31**: Femurs that were plated, but no defect was created (Sham) at 5 weeks post-surgery. (B) DIC imaging reveals periosteal thickening of the bone. (C-D) Fluorescent imaging shows the expression of endogenous Col1 (GFP) and relatively low AP and TRAP activity. (E) Hematoxylin staining indicates the healthy quality of the marrow. Dr. Rowe’s lab performed all histological analysis.
**Fig. 32:** Sub-critical defect (0.6mm) at 2 weeks post-surgery shows adequate healing of the defect. (A) Bone bridging is evident by X-ray. (B) Modest periosteal response is seen using DIC. (C-D) Fluorescent imaging indicates bone formation (AP) on the periosteum adjacent to the plate. (E) Hematoxylin staining shows variable quality of the marrow and the presence of debris. Dr. Rowe’s lab performed all histological analysis.
**Fig. 33:** Sub-critical defect (0.6mm) at 5 weeks post-surgery shows adequate healing of the defect. (A-B) Bone bridging is seen in the X-ray and DIC images. (C-D) Fluorescent imaging reveals new bone formation via AP staining at the wound site and also high rates of resorption to remove the old bone and debris. (E) Hematoxylin staining reveals variable quality of the marrow space. Dr. Rowe’s lab performed all histological analysis.
Fig. 34: Critical defect (1.6mm) at 2 weeks post-surgery shows absolute lack of healing of the defect. (A-B) No mineralization is seen via X-ray and DIC and the proximal edge of the defect has capped. (C-D) New bone formation can be seen at this capped end via fluorescence imaging. (E) Hematoxylin staining shows fibrous tissue within the defect space. Dr. Rowe’s lab performed all histological analysis.
Fig. 35: Critical defect (1.6mm) at 5 weeks post-surgery shows inadequate healing. (A-B) The fracture is capping at both ends of the defect as seen by X-ray and DIC. (C-D) Fluorescence imaging shows strong AP staining near the edge of the defect where the bone is capping. (E) Significant amount of debris is also seen in the defect space via hematoxylin staining. Dr. Rowe’s lab performed all histological analysis.

**Torsional Biomechanical Analysis**

Surgical treatment significantly affected torsional properties ($p < 0.05$). Unoperated and sub-critical defects showed no difference in torsional rigidity (Fig. 36). However, the critical defect exhibited significantly reduced rigidity while the sham actually produced a 45% increase
in rigidity (p < 0.05; Fig. 36). In fact, only two of the specimens within the critical defect group produced any measurable torsional resistance. Ultimate torque was significantly reduced in both sub-critical and critical defects with the values of 62% and 5% of the unoperated normal controls, respectively (p < 0.05). However, the sham group was not different than normal. Finally, the yield torque was only significantly reduced in the critical defect while the sham and sub-critical defect were not different than normal.

**Fig. 36:** Biomechanical properties of critical defects are significantly reduced while the sub-critical defect exhibit properties that match normal except for ultimate torque. Error bars indicate SD. * represents values significantly different from normal unoperated controls (p<0.05). Dr. Awad’s lab performed all biomechanical analysis.

The volumetric µCT imaging correlated to the biomechanical data. Qualitatively, we found that sub-critical defects (Fig. 37C) healed, characterized by dense bone formation and bridging of the defect. In comparison, the critical defect samples showed no bridging or
mineralized bone in the defect site (Fig. 37D). Examination of the two Critical defect samples that had shown measurable, but low mechanical properties revealed marginal continuity and inadequate healing by CT analysis (Fig. 37E-F).

**Fig. 37:** Representative µCT images of (A) Normal, (B) Sham, (C) Sub-critical and (D) Critical defects. (E and F) µCT images of Critical defect samples that displayed measurable, but markedly inferior, biomechanical properties. Dr. Awad’s lab performed all µCT analysis.

**DISCUSSION**

The objective of this study was to histologically and biomechanically assess the effect of segmental defect length on healing in the mouse femur. The sub-critical (0.6mm) and critical
(1.6mm) lengths were chosen to produce a union and non-union repair, respectively. These sizes indeed yielded these outcomes and there were significant differences in the healing response between these two scenarios. In both scenarios, a strong periosteal response was seen but due to the size of the defect (and possibly other factors) the cells could not span the critical defect (Fig. 34-35) and did not create the outer cortical shell that was seen often in successful healing of the sub-critical defects (Fig. 32-33). Instead, fibrosis occurred in this space with an influx of inflammatory and fibroblastic cells, while the ends of the bone capped the marrow space.

Preclinical models that are reproducible and display aspects of the clinical condition are extremely important for assessing treatment efficacy. Molecular mechanisms that lead to non-union fractures are still not fully understood. Several studies have shown that the periosteum is extremely important for successful fracture healing and helps form the callus and outer cortical shell – all of which were seen in this study [152,158,159]. The murine model, as shown in this chapter and previous chapters, provides a multitude of genetic tools that allows researchers to further understand the biology that occurs during healing and to characterize potential mechanisms. Utilizing different reporter mice as done in this study or phenotypically altered strains of mice will allow us to delve deeper into the mechanisms that drive the healing processes, whether good or bad.

If we can better understand the molecular processes that lead to a functional outcome (ie. sub-critical defect) vs. a non-functional outcome (ie. critical defect), we could then discover potential therapeutic targets that can be modulated with treatment modalities. For instance, a gap in knowledge exists from the results of this current study in that it is still unclear whether the initial healing response was similar in both the sub-critical and critical defects. It appears that the cells were incapable of spanning the critical defect due to its size so they simply capped the bone
but were their molecular mechanisms driving this response? If the geometry of the defect was altered and a scaffold was placed in the wound site just following injury, would the cells form a bridge? It is likely not that simple but the size of the defect alters the functional outcome of a healing response in a variety of musculoskeletal tissues including bone, tendon, skin, and cartilage [160-162].

Homologies likely exist between the mechanisms of healing in hard and soft tissues. The cells that are involved and the molecular signals may differ but the process itself of stages of inflammation, repair, and remodeling are similar. Therefore, comparable approaches can be used in both to better understand the healing mechanisms and how pathological conditions develop. The use of reporter mice and phenotypically altered mice that are tailored towards specific aspects of the healing process may provide tremendous impact towards the understanding of the natural healing process. Only by first understanding the natural healing process and what mechanisms drive inadequate healing, can be hope to improve repair as tissue engineers. Our ultimate goal is to produce clinically efficacious repair strategies for these types of injuries in both hard and soft tissues by letting the inadequacies of natural healing and the adequacy of normal development drive our therapeutic strategies.
Chapter 8

Conclusions and Recommendations

My dissertation is part of the larger NIH Bioengineering Research Partnership (BRP) between our lab at the University of Cincinnati and Dr. Chris Wylie’s lab in the Division of Developmental Biology at Cincinnati Children’s Hospital Research Foundation that aims to work at the interface of developmental biology and tissue engineering to further improve tendon repair. The overall working hypothesis of the BRP is that recapitulating aspects of normal embryonic development in our tissue engineered constructs (TECs) will improve repair outcome of tendon injuries. In order to test this hypothesis and reach our ultimate goal of improving tendon repair, we needed to first understand the normal development process that produces functional tendon and compare it to the natural healing process in the adult that produces non-functional scar tissue.

While Dr. Wylie’s lab at Children’s has and continues to study the normal developmental processes that drive patellar tendon initiation, differentiation, and maturation in the embryonic and early post-natal mouse, my focus was to further define the biological processes that contribute to natural healing in the mouse patellar tendon. Chapters 3 thru 4 of this dissertation have described the initial studies to 1) develop a full-length, central patellar tendon defect model in the mouse, analogous to our previous work in the rabbit [15,20,26,122,163] and to 2) analyze the expression patterns of tenogenic markers that influence normal tendon development during healing in this model. By comparing the outcomes among these development and natural healing studies, we can then focus our tissue engineering efforts to promote signaling pathways that lead to differentiation in our constructs while inhibiting pathways that lead to scar formation. This
transitioned to chapter 5, where paracrine signals produced by myoblast and Achilles tendon fibroblast cultures were used to stimulate the differentiation of bone marrow-derived mesenchymal progenitor cells (bMPCs) in collagen gel constructs. The hypothesis of this study was that promoting differentiation in our constructs would ultimately lead to an improved repair outcome of the central-third patellar tendon defect model. The central PT defect model is advantageous in that it is highly reproducible for studying TE repair and is an initial step in showing treatment efficacy; however, the central-third PT defect is not clinically relevant. Therefore, chapter 6 was aimed at comparing two methods for producing aspects of tendon degeneration seen clinically within the rabbit patellar tendon, which is large enough for us to conduct reproducible tissue engineered repair studies. Finally, the methods used in chapters 3-4 to understand the natural healing process in tendon, specifically fluorescent reporter for Coll1 and Coll2, were applied to natural healing of sub-critical and critical size bone defects in chapter 7 as an extension of the work in the BRP. In this model, we compared functional vs. non-functional healing by varying the size of a segmental bone defect. This paradigm of comparing functional vs. non-functional healing is an extension of our developmental paradigm and will be explained further in this chapter.

There were several significant findings that lead to gaps in knowledge and direction for future studies in this dissertation. These findings will be reviewed below with addition discussion for each and recommendations for future studies to address the existing gaps in knowledge. A flowchart is provided below as an overview of this discussion (Fig. 38).
CH3: Murine central PT defect produced non-functional healing

CH4: Tenogenic markers are NOT expressed at appropriate levels in wound site

What is the lineage of the healing cells?

Can we modulate healing tissue to produce a functional matrix?

Is the central PT defect healing response similar to other injury types?

Future Study
Lineage analysis using Scx conditional knockout

Stimulate resident cells
Are resident cells capable of regeneration?

Future Study
Compare healing of MRL mouse with wild type

Replace cells w/bMPC-constructs

Modulate bMPCs to induce tendon differentiation in culture

CH5: Myoblast- & AFB-conditioned media increased proliferation of cells but did not promote differentiation in TECs

CH6: Collagenase injection in the rabbit PT did not sustain degenerative aspects

CH7: Size affects healing of murine femoral defects

Future Study
Multiple collagenase injections with avulsion

Fig. 38: Flowchart of major findings, gaps in knowledge, and future directions from my dissertation work.
Chapter 3: A full-length, central PT defect in the Col1/Col2 double transgenic reporter mouse produced a non-functional healing response that displayed inflammation, repair, and remodeling stages of healing with a peak in Col1 gene expression at 2 weeks following injury.

In chapter 3 we showed that creating a full-length, central PT defect in the murine PT produced inflammation at 1 week, repair at 2 weeks, and remodeling at 3 weeks and greater. The defect consisted of loose granulation tissue at 1 week with an influx of inflammatory cells and minimal collagen expression as shown through Col3.6GFp7 fluorescence. By 2 weeks, the defect was filled with disorganized matrix consisting of an increased number of cells that were expressing Col1. Inflammatory cells, such as mast cells, still remained in the defect at this time and these cells were not expressing Col1. At 3 weeks and after, the defect tissue became more organized and appeared normal via H&E at 5 weeks. Col1 expression was also decreased to normal levels by this time point. Biomechanically, the healing tissue at 1 week was too compliant to dissect during testing. By 2 weeks, the ultimate loads and stiffnesses of the healing tissue were 37% and 48% of normal properties, respectively. The healing response did not improve mechanically between 5 and 8 weeks with ultimate loads and stiffnesses only reaching 48% and 63% of normal by these time points, respectively. Additionally, we compared the amount of displacement required to reach the highest in vivo loads that we’ve recorded in a preclinical model (goat) for activities of daily living [19], which corresponds to 40% of normal PT ultimate load. It required at least an additional 0.123 mm of displacement (or 4% strain) for the healing tissues to reach this level. In fact, only 33%, 75%, and 69% of the samples at 2, 5, and 8 weeks exhibited ultimate loads above this 40% level.
Thus, we characterized through histology, Col1 gene expression, and biomechanics that the patellar tendon displayed an inadequate healing response following this injury. However, we still didn’t have a complete understanding of the phenotype of the cells that contributed to the healing response. Did these cells express tenogenic markers that control normal tendon development? What factors were regulating the Col1 expression seen at 2 weeks? In order to answer these questions, we studied the expression of several tenogenic factors using qPCR, GFP reporter mice, and immunohistochemistry at 1 (inflammation), 2 (repair), and 3 (remodeling) weeks post-surgery in chapter 4.

**Chapter 4:** Tenogenic markers are not expressed at appropriate levels in the central PT defect during healing and this may lead to impaired collagen assembly and non-functional biomechanics.

In this chapter, we quantified gene expression via qPCR for tenogenic transcription factors (Scx, Mkx, Egr1, Egr2, and Six1), proteoglycans (Bgn, Fmod, Dcn), glycoproteins (Tnmd, Tnc), and fibrillar collagens (Col1a1 and Col3a1). We found that Col1a1 and Col3a1 expression levels were similar between the defect and contralateral sham tissue, which were both increased relative to normal age-matched controls. This suggests that these two important collagens during tendon development and healing were expressed at appropriate levels since the sham tissue showed no biomechanical difference compared to normal PT at any time point tested. However, we found that Scx and Mkx, important regulators of tendon differentiation and maturation during development [49,50], both showed decreased expression compared to the contralateral sham. This suggests that the cells that contribute to the healing response are not differentiating properly. It also suggests that both Scx and Mkx, which regulate Col1a1 expression [50,133], are in fact not regulating the collagen expression seen in this model system.
While the healing tissue expressed elevated levels of Col1a1 during these time points of healing up to 3 weeks, expression of fibromodulin and decorin were decreased compared to both normal PT and the contralateral shams. Fmod and Dcn are important collagen assembly proteoglycans [63-65] and decreased expression of these genes suggests that the collagen being released by the cells wasn’t being assembled properly. This is a significant failure during the healing process that likely contributed greatly to the impaired biomechanics. However, this needs to be investigated further. First, we should section the healing tendons in the transverse plane, such that we can see where fibromodulin and decorin are expressed in relation to the collagen fibrils using immunohistochemistry. This may provide us with better insight into the interaction between these fibrillogenic proteins and the collagen fibrils being assembled within the wound site.

Along with not understanding the spatial distribution of fibromodulin and decorin within the tendon during healing, we still do not have a complete understanding of the level of impairment to the assembled collagen fiber bundles. Even though histologically the organization of the healing tissue at 5 weeks appeared normal, it was still significantly impaired with structural properties reaching only 60% of normal PT (Fig. 10). Using transmission electron microscopy we can assess the shape and quantify the size and distribution of the collagen fibrils during healing. Collagen fibril distribution in combination with altered fibromodulin and decorin expression may lead to a potential mechanism for the impaired biomechanics. In fact, a model has recently been developed to explain this [164] in which these assembly proteins become increasingly important at larger strains to prevent “fibril slipping.” This is not the case at lower strains (< 2%) where collagen fibril stretching accounts for the tendon deformation. Therefore, the production of a “tendon-specific” matrix is imperative for yielding mature mechanical
properties of the repair and we should focus on increasing expression of these assembly proteoglycans within our tissue engineered repairs.

While we showed that Scx and Mkx were not regulating Col1a1 expression following injury, we did find that both early growth response 1 (Egr1) and early growth response 2 (Egr2) were increased during healing. These transcription factors are implicated in fibrosis and scar formation and regulate Col1a1 expression indirectly [53,135]. Egr1 is a Smad-independent regulator of TGFβ signaling and TGFβ isoforms 1 and 2 are known “scar promoting” growth factors [40,165,166]. Egr1 and Egr2 are also sensitive to the mechanical environment and the changes resulting from the creation of the PT defect may drive the early expression of these transcription factors [135]. The altered mechanical signals are likely also inducing the increased expression of tenascin-C seen in this study [61,167-169]. However, the relationship between Egr1/2 and Tnc is unknown.

The findings within chapter 4 suggest that a combination of signals involving Egr1, Egr2, and Tnc are promoting scar tissue formation while decreased expression of Scx, Mkx, Fmod, and Dcn suggest that the cells within the wound site are not properly differentiated and are not producing a functional tendon matrix. These findings lead to several research questions that transitioned into later chapters in this dissertation as well as suggested potential future studies to investigate. These include: 1) **What is the lineage of the cells that contribute to the healing response and from where do they originate?** 2) **Can we modulate these healing cells to produce a functional matrix or can we insert constructs that are preconditioned to produce this matrix?** and 3) **Is the healing response from the central PT defect similar to other injury types including avulsion and degenerative tears?**
**1) What is the lineage of the cells that contribute to the healing response and from where do they originate?** Scleraxis is required for tendon differentiation and tenogenic cells express this transcription factor at an early progenitor stage and maintain its expression throughout the differentiation of the cell [30,31]. This characteristic makes Sex a clear candidate to study the lineage of cells that contribute during healing. Cre recombinase enzyme-mediated lineage tracing is a tool that allows researchers to track cells in vivo by permanently marking them without altering cell phenotype [170]. This method allows for both spatial and temporal control over the cell tracking. Spatial control is done via a tissue-specific promoter [170]. In our case, the tissue-specific promoter would be for Sex (Fig. 39a). Temporal control is achieved by attaching an inducible version of Cre to the Scx promoter [170]. One example of inducible Cre utilizes an altered hormone-binding domain of the mouse estrogen receptor ER<sup>TAM</sup>, which responds to tamoxifen and in turns activates the Cre enzyme (Fig. 39). Cre recombinase functions by translocating to the nucleus, recognizing loxP binding sites (red arrows in Fig. 39) in the DNA sequence, and cleaving the DNA between these binding sites [170]. This is highly useful as the Cre can be used to cleave a stop codon that is upstream of a reporter gene (RFP in this case) and downstream of a ubiquitously-expressed Rosa26 promoter (Fig. 39b). Therefore, by using the inducible Cre construct in combination with the ubiquitously expressed reporter construct, we can permanently mark Scx-expressing cells with RFP by delivering a single dose of tamoxifen. We could then cross this mouse with the ScxGFP mouse, which would allow us to monitor normal Scx expression (GFP) as well as tag Scx-expressing cells (RFP) using tamoxifen at a specific time (in a normal adult mouse prior to injury).

By utilizing the ScxGFP mouse crossed with the Scx inducible Cre mouse, we can study whether the cells that migrate into the wound site following injury come from a population of
cells that was expressing Scx prior to the injury. Prior to injury, we can deliver tamoxifen to these mice to tag the cells that normally express Scx with RFP. Following the injury, we can then delineate four populations of cells within the wound site: 1) Scx+/RFP+ – these are Scx expressing cells within the wound site that originated from Scx expressing cells prior to the injury, 2) Scx+/RFP− – these are Scx expressing cells that were not expressing cells prior to the injury, 3) Scx−/RFP+ – these are non-Scx-expressing cells that were expressing Scx prior to the injury, and 4) Scx−/RFP− – these are non-Scx-expressing cells that were also not expressing Scx prior to the injury.

**Fig. 39:** Scx cell lineage tracking analysis using Cre recombinase. This method allows for both spatial control, via Scx cell-specific promoter, and temporal control, via tamoxifen-inducible Cre-ER, of cell lineage tracking. In the absence of tamoxifen, the Cre enzyme is inactive (A). When tamoxifen is delivered (B), Cre becomes activated via tamoxifen-estrogen receptor (ER) binding, translocates to the nucleus, and cleaves the stop codon from the ubiquitously expressed reporter construct (R26-RFP). Therefore, a single dose of tamoxifen will permanently tag Scx-expressing cells with RFP (red fluorescent protein). Adapted from Greco et. al. 2010 [170].
Understanding the lineage of the cells that contribute to natural healing is extremely important for tissue engineers. This knowledge will guide our therapeutic strategies. For instance, a potential outcome of this study is that the cells that contribute to healing are Scx+/RFP- cells. This outcome suggests that the cells that migrate into the wound site differentiated from an early, non Scx-expressing, progenitor population. This progenitor population could potentially come from the epitenon or surrounding tendon sheath. The cells that reside in this region of the tendon do not express Scx normally. In addition, the epitenon and tendon sheath proliferate in the response to injury [171] and this response may promote the migration of proliferative progenitors that differentiate into Scx expressing cells during the healing response. A potential mechanism to improve healing based on this outcome would be to expedite the recruitment and differentiation of these cells following injury. Along with providing guidance towards potential therapies, this type of study would also improve our basic science understanding of natural healing and the mechanisms that drive the response.

2) Can we modulate these healing cells to produce a functional matrix or can we insert constructs that are preconditioned to produce this matrix? This question brings us closer to a tissue engineered therapy, which is the ultimate goal of the BRP. Two tracks exist that are potential methods to answer this question. The first track is that we can stimulate the native cells that migrate into the wound site to differentiate properly and produce a functional matrix. However, it is debatable whether the cells that contribute to tendon repair in the adult are capable of producing such an outcome. While these cells differentiate to a certain extent, it is still unknown whether regeneration is possible when only using resident cells. The extent of the injury contributes largely to this response as shown in the chapter 7 where a subcritical-sized fracture healed successfully while a critical-sized fracture did not. Also, small cuts or lacerations
of the skin typically heal without scar while larger injuries will produce a scar [160]. It appears that the body has a set healing capacity for certain tissue types and larger critical injuries require a response beyond what the body can regenerate; therefore, it produces a scar to fill the space.

An interesting development occurred recently from a group studying systemic lupus erythematosus, an autoimmune disease. Researchers working on this disease developed a mouse strain known as the Murphy Roths Large (MRL) mouse [172]. The researchers noticed upon creating punch defects in the ears of these mice for identification purposes that the ears would regenerate from the injury with no signs of scarring [172]. These mice healed by forming a blastema or mass of progenitor cells at the wound site in a similar fashion to an amphibian regenerating a limb [172]. The blastema then promoted the regeneration of the tissue. Upon further genetic analysis, it was found that the healing phenotype of these mice was distributed over 20 loci on multiple chromosomes [173], suggesting multiple factors that may impact the regenerative phenotype. However, later studies have investigated the roles of p21 and p53 using these mice [174,175]. The cyclin-dependent kinase inhibitor, p21, is a potent cell cycle regulator while p53 is a tumor suppressor protein. During the cell cycle, p21 can arrest the cell in the G1 phase and it can mediate the tumor suppressor response of p53 through G1 arrest [174]. However, the MRL mouse exhibits reduced expression of p21 and cells that contribute to healing in this mouse show an accumulative G2/M phenotype [174]. In addition, p21-null mice show similar regeneration during healing of these ear defects while p53-null mice do not [175]. The suggestive mechanism of regeneration is that p21 functions through abrogation of the TGFβ/Smad pathways [175].

The MRL mouse presents an interesting opportunity for studying natural healing in our lab. While these mice exhibit improved healing in several tissue types including the ear [172],
articular cartilage [176], and limb bud [175], they also show differential healing in the skin [177] and heart [178]. Therefore, we should first examine the extent of regeneration in the PTs of these mice following a full-length, central defect. If these mice show improved healing through regeneration as opposed to scar formation, then this could lead to another branch of the BRP. Our initial aim of the BRP was to use normal embryonic development as a guide for improving tendon repair in the adult. However, recapitulating certain aspects of embryonic development in the adult may be quite difficult if not impossible. Therefore, having a model that exhibits regeneration in the adult would provide another comparison and potential guide for our tissue engineering strategies.

If we find improved healing and regeneration in these mice following injury, then the next step would be to characterize the healing response in a similar fashion to what has been done in chapters 3-4 of this dissertation. We would need a spatiotemporal characterization of the healing response to determine if this mouse exhibits tenogenesis comparable to embryonic development or if another mechanism exists that does not include embryonic-like tenogenesis. If we find that a blastema forms during healing of the PT defect, then the next step would be to isolate both blastema cells and bMPCs from these mice and characterize them in our TECs in culture to determine if the blastema cells show a higher degree of tendon differentiation. Finally, we should repair the murine central PT defect and the segmental femoral defect in wild type mice using TECs seeded with either blastema cells or bMPCs from the MRL mouse. The purpose of these studies would be to compare the regenerative capacity of the blastema cells vs bMPCs, which has been our cell source of choice for tissue engineered repairs.

While the MRL mouse may provide us with insights into improved healing through regeneration, the normal adult still doesn’t have the healing capacity to regenerate a critical
wound. Therefore, can tissue engineers use cell-based therapies to increase the regenerative capacity in the adult? We have shown previous success using bMPC-collagen constructs [15] to improve tendon repair. However, we still have not produced repairs that match normal tangent stiffness up to peak in vivo loads of 40% of normal failure force [19]. Thus, our focus currently is to initiate tenogenic differentiation of these cells in our TECs in culture prior to implantation in vivo. Therefore, these cells will have the appropriate cues to produce a functional matrix during the repair process in vivo. This strategy transitions into chapter 5 where the objective was to use signaling factors from myoblast and Achilles tendon fibroblast (AFB) cultures to stimulate tenogenic differentiation of bMPCs in collagen constructs.

**Chapter 5: Myoblast- and AFB-conditioned media increased proliferation of cells in the constructs but did not promote tendon differentiation.**

In chapter 5 we aimed to promote differentiation of bMPCs in collagen constructs by delivering signals released by myoblast cultures and by Achilles tendon fibroblast (AFB) cultures. The rationale for using the myoblast culture was that signals released from these muscle cells might be analogous to embryonic signals that initiate tendon differentiation at the myotendinous junction [37,140]. On the other hand, AFBs, being a mature tendon cell, may release factors that would promote the tenogenic differentiation of a mesenchymal progenitor cell. We found that in fact neither one of these media types promoted differentiation as Scx, Mkx, Tnmd, and Fmod gene expression were either unchanged or reduced in these TECs (Fig. 17 & 20). We did find that these media types increased the proliferation of cells within the TECs, which further suggests that these cells are not differentiating, as tendon cells typically do not proliferate when they differentiate.
While we did not find that these media types under this current configuration promoted
differentiation of our TECs, we should still investigate what signaling factors can promote the
tenogenic differentiation of bMPCs in culture. Promising growth factors to test include FGF4,
FGF8, GDF7, and GDF8. FGF4 and FGF8 have been shown to initiate tendon condensation and
differentiation during early embryonic development in the several tendons [35,36]. Also, GDF7
and GDF8 are capable of producing ectopic tendon tissue [179,180]. In fact, Lee et. al.
stimulated bMPC-seeded scaffolds with recombinant GDF7 in a recent study [179]. GDF7
increased Scx and Tnmd expression in these constructs and also produced improved repair of
Achilles tendon defects compared to unstimulated controls. These growth factors along with
other signals that may be discovered by Dr. Wylie’s lab during their studies should be
investigated in our tissue engineered studies.

As stated previously, the central PT defect is a reproducible model that allows for
consistent assessment of tissue engineering therapies. This model is also unique to other tendon
injury models in that the defect tissue is stress-shielded by the adjacent native struts. The
mechanical environment as well as the tendon being extra-synovial may alter the healing
response compared to other injury types, leading into the next gap in knowledge.

3) Is the healing response from the central PT defect similar to other injury types
including avulsions and degenerative tears? The central third defect is quite unusual compared
to the tendon injuries seen clinically. Clinical tendon injuries typically involve a traumatic
rupture within the midsubstance or an avulsion of the tendon off of the bone [4,10,21-23,181].
Tendon degeneration often accompanies and might even cause these traumatic tears, which
further complicates the repair strategy. The healing response following a central-third defect may
be quite different than a rupture or avulsion and in all likelihood is significantly different than the
mechanism involved in healing degenerative injuries. The mechanics involved in a full-width rupture or avulsion may differ greatly from a partial-width defect that is stress-shielded by native strut(s).

Therefore, now that we have a much better characterization of the spatiotemporal expression patterns that occur during healing of the central PT defect in the mouse, we should attempt to create more clinically relevant models. Within the mouse model, we could reproducibly create a partial transection injury in either the midsubstance of the patellar tendon to study soft tissue healing or at the tendon-to-bone insertion to study insertional healing, which occurs more frequently in the clinic. Due to the differences in mechanics of a partial transection and the fact that less volume of tissue needs to be filled during the healing process, there may be a considerable difference in the healing response.

Creating preclinical models with aspects of the degenerative condition seen clinically is still needed by our lab to effectively assess the efficacy of our repairs. Our initial attempt to do this was in the rabbit as seen in chapter 6 and summarized below, but creating degenerative aspects in the mouse is not out of the question despite its limited size. Several researchers have used overuse in several model systems including rabbits, rats, and even mice to generate tendon fatigue and indications of the degenerative phenotype [12,13,80,128]. Recently Zhang et. al. used an intense treadmill regimen to induce elevated levels of PGE2, an inflammatory mediator that may mediate the progression of tendon degeneration, in the mouse patellar and Achilles tendons [80]. The benefit of the murine model with its abundance of genetic tools may yield additional insight into the pathogenesis of degeneration that is so difficult to study in patients and other animal models.
CH6: A single collagenase injection yielded degenerative aspects at 4 weeks but did not sustain these changes to 16 weeks, while PGE2 did not alter biomechanics or histology of the rabbit patellar tendon. The objective of chapter 6 was to compare the histological and biomechanical response of PGE2 vs collagenase delivery to the rabbit patellar tendon. The goal was to create aspects of the degenerative condition while also sustaining these aspects up to 16 weeks to provide a sufficient time frame to assess the efficacy of tissue engineered repairs. We found that PGE2 delivery created a mild inflammatory response at 4 weeks but there were no differences in mechanical properties compared to normal PT. On the other hand, a 200IU injection of type-I collagenase to the tendon yielded severe matrix disruption, disorganization, and hypercellularity at 4 weeks, which is consistent with the disorganization and hypercellularity seen in degenerative tendons (Fig. 26-27). The structural properties were also significantly reduced at 4 weeks, only reaching 50% of normal (p < 0.05). However, by 16 weeks the matrix had realigned and the structural properties were 75% of normal (p > 0.05), suggesting that the phenotype of the tendon fibroblasts was not sufficiently altered by the collagenase and could eventually repair the matrix damage.

This chapter compared a method to alter the phenotype of the resident tenocytes in PGE2 vs. a method to accelerate the matrix breakdown in collagenase. These methods alone were not enough to sustain aspects of the degenerative phenotype, suggesting that a combination of methods is required to sustain the condition. However, we did show degenerative aspects following a collagenase injection. Therefore, would delivering multiple injections disrupt the matrix to a point where the resident cells would not be able to recover? We have seen previously that the severity of the injury contributes to the body’s healing response. Therefore, we should test the hypothesis that multiple injections of collagenase would disrupt the matrix to the extent
that the resident tenocytes would not be able to functionally repair the tendon, producing an impaired healing response.

If this hypothesis is accepted, then we must characterize the phenotype of the cells within the injection site to determine if they exhibit similar characteristics to the degenerative phenotype seen clinically. Such a phenotype includes elevated MMP production, diminished TIMP production, increased GAG content, and increase Type-V collagen production [147]. If the cell phenotype exhibits a number of these characteristics, then we would have a novel preclinical model system to test healing of traumatic injuries to degenerative tendon.

Upon the characterization of this degenerative model, a traumatic injury should be fabricated that shares characteristics with the clinical condition. As stated previously, traumatic tendon injuries typically occur at the tendon-to-bone insertion. Our lab has previous experience in developing such a model with the help of Dr. Gregory Boivin at Wright State University and Dr. Marc Galloway at Cincinnati Sportsmedicine and Orthopaedic Center. In this model, we created a surgically induced avulsion of the central-third region of the rabbit patellar tendon at the distal insertion and preliminary data show reduced structural properties at 16 weeks that are comparable to properties produced by a collagenase injection at 4 weeks (Fig. 40). Therefore, the creation of a surgically induced avulsion within a degenerative region of the rabbit PT would provide a significant improvement in clinical relevancy.
Fig. 40: A central-third surgical avulsion at the distal insertion of the rabbit patellar tendon yields reduced biomechanical properties that are similar to those produced at 4 weeks following a collagenase injection.

The focus of my dissertation in the beginning was to use the differences between normal tendon development and natural healing in the adult as a guide for our tissue engineering repair strategies. While we are making significant strides on this front, we still acknowledge that promoting tenogenic differentiation within our TECs in culture may not lead to an improved repair outcome when implanted into a skeletally mature, adult animal. Therefore, we should consider studying other aspects of the healing response that may not include tenogenesis but may also be important. The MRL mouse provides a model system to pursue some of these mechanisms as this model may allow us to study quality, functional adult healing vs. impaired natural healing in the wild type animal. I believe that findings related to these research areas will have a significant impact on progressing the field of tissue engineered repairs of musculoskeletal injuries.
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