The Effects of Mechanical Loading and ADAMTS5 Activity in a Tendinopathy Model

BY

REBECCA BELL
B.S. Trinity College, Hartford, CT, 2005

THESIS

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Defense Committee:

Dr. Vincent M. Wang, Advisor, Rush University
Dr. Anna Plaas, Co-advisor, Rush University
Dr. Thomas J. Royston, Chair
Dr. David Eddington
Dr. Karen Troy, Kinesiology

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Abstract

Tendinopathy is a chronic tendon disorder that accounts for ~50% of all sports-related "over-use" injuries and ~12% of work-related injuries (Bureau of Labor Statistics). Consistent findings in tendinopathic tissue are collagen fiber disorganization/rupture and mucoid degeneration (aggrecan rich deposits, or ARDs). Despite their prevalence, presently it is unknown whether ARDs are pathogenic. Aggrecan presence occurs at low levels in the tensile regions of tendon and undergoes rapid aggrecanase-mediated turnover in normal tendons. The lack of ADAMTS5 (aggrecanase-2) has been shown to lead to ARDs in collagen rich tissues (skin, cartilage). The objective of this dissertation was to test the hypothesis that accumulation of aggrecan is detrimental to normal and injured tendon function.

The first aim was to examine the effect of ADAMTS5 on the properties of flexor digitorum longus (FDL) and Achilles tendons in skeletally mature mice. FDL tendons of *adamts5*$^{-/-}$ (*ts5*$^{-/-}$) mice showed a 33% larger cross-sectional area, increased collagen fibril area fraction, and decreased material properties relative to those of age matched wild type mice. In Achilles tendons, cross-sectional area, stress relaxation, and structural properties were similar in *ts5*$^{-/-}$ and wild type mice; however, the *ts5*$^{-/-}$ tendons exhibited a higher tensile modulus and a weakened enthesis. In *ts5*$^{-/-}$ mice, aggrecan accumulated in the pericellular matrix of tendon fibroblasts within the tendon body as well as the insertion site. These results demonstrate that TS5 deficiency disturbs normal tendon collagen organization and alters biomechanical properties. Hence, the role of adamts5 in tendon is

to remove pericellular and interfibrillar aggrecan to maintain the molecular architecture responsible for normal tissue function.

The second aim was to develop an in vivo tendinopathy model characterized by ARDs and weakened biomechanical properties. TGFβ was used to create this model in wild type cage active mice by injecting the growth factor into the Achilles tendon body. At two weeks the cross-sectional area was increased, while the tensile stiffness and maximum stress were reduced relative to the respective properties of naïve (uninjured) wild type mice. The stiffness and maximum stress remained compromised compared to the naïve at 4 weeks. Histologic analysis demonstrated increased cellularity and aggrecan staining throughout the midportion of tendon near the injection site both two weeks and four weeks. Gene expression of aggrecan and MMP3 increased by greater than 100 fold relative to naïve at two weeks and remained elevated (> 75 fold increase) at 4 weeks, whereas gene expression for the collagen binding integrins (α1 and α2) was reduced throughout the 4-week period.

The third aim was to examine the role of mechanobiology in the development of tendinopathy using the murine model developed in Aim 2. Treadmill exercise following induction of the tendinopathy caused an increase of cross-sectional area and decrease of stiffness and maximum stress at 2 weeks but at 4 weeks, the stiffness and maximum stress returned to naïve levels. The increased presence of aggrecan deposits that was seen at 2 weeks was no longer present at 4 weeks with the treadmill exercising. Aggrecan and MMP3 gene expression at 4 weeks was also significantly reduced compared to the tendinopathy model. Gene expression for the collagen binding integrins α1 and α2 had also returned to levels seen in the naïve mice.

The fourth aim examined the *adamts5*$^{-/-}$ mice in the tendinopathy and mechanical stimulation models. Tendon cross-sectional area increased, while stiffness and maximum stress decreased at 2 weeks relative to the naïve *ts5*$^{-/-}$ mice. At 4 weeks, the maximum stress was still compromised. The histology showed increased cellularity and increased aggrecan staining at both 2 and 4 weeks. Aggrecan had reduced expression at 2 weeks compared to the wild type with mechanical stimulation but had a 4 fold increase in expression at 4 weeks. Type 1 and Type 3 collagens had significantly reduced expression at 4 weeks compared to the wild type with treatment. The integrin expression was considerably increased at both 2 weeks and 4 weeks. These results demonstrate the importance of both the ADAMTS5 enzyme and mechanical stimulation for restoration of tensile properties in this animal model of tendinopathy.

In all cases where there was histologic evidence of aggrecan accumulation in the tendon matrix, the tissue mechanical properties, maximum stress and stiffness, were reduced. Hence, collectively, these studies confirm our hypothesis that aggrecan rich deposits are detrimental to tendon mechanical function.

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Chapter 1

Significance

Chronic pain and functional impairment in tendons, (such as the rotator cuff, patellar and Achilles) represents a major health care problem. It is now widely held that tendon overuse (often leading to traumatic rupture) is the initiating factor for the majority of patients in the 30-50 year age group[1,2]. As early as 1951[3], de Quervain's tendinopathy was described to have "numerous cells lining the synovial layer were polyhedral rather than flattened, and these area of mucoid change were most obvious around the blood vessels. Further, the perisynovial fibrous tissue showed increased cellularity and collections of rounded cells with small areas of cartilage formation." In 1991[4], Kannus showed in ruptured tendons 20% of them had mucoid degeneration, which was the second most common degenerative change behind hypoxic degeneration. However, there is no animal model that was developed to study this type of tendinopathy.

Background

Tendon Structure and Function

Tendons are composed of dense connective tissues with longitudinal fibers made up of type I collagen (~80% of dry weight), several proteoglycans (decorin, fibromodulin and biglycan), and elastin. It is considered a hierarchical structure made up of fiber bundles, fascicles, and fibrils. Fibrils are visible in low power electron microscopy and fibers are bundles of fibrils that can be seen in light microscopy[5]. Fascicles are a collection of fiber bundles that are surrounded by endotenon. Fascicle is considered to be the smallest unit of the whole tendon which can be tested mechanically. In the rodent, a few tendons are single fascicle, including tail tendons and the flexor digitorum longus

(FDL) tendon of the mouse[5]. With multi-fascicle tendons, the surrounding sheath of the whole structure is called epitenon. A paratenon is a false tendon sheath that is separate from the tendon that consists of loose connective tissue and the tendon moves within that sheath[5]. Even though proteoglycans only make up less than 2% of tendons, they have a crucial role in assembly and regulation of collagen organization and differentiation status of tenocytes. In the tensile collagen fibril rich regions of the tendon, decorin is the most prevalent and has been proposed to be critical in organization and maintenance of collagen fibrillar structures, as well as regulating cell proliferation[6,7].

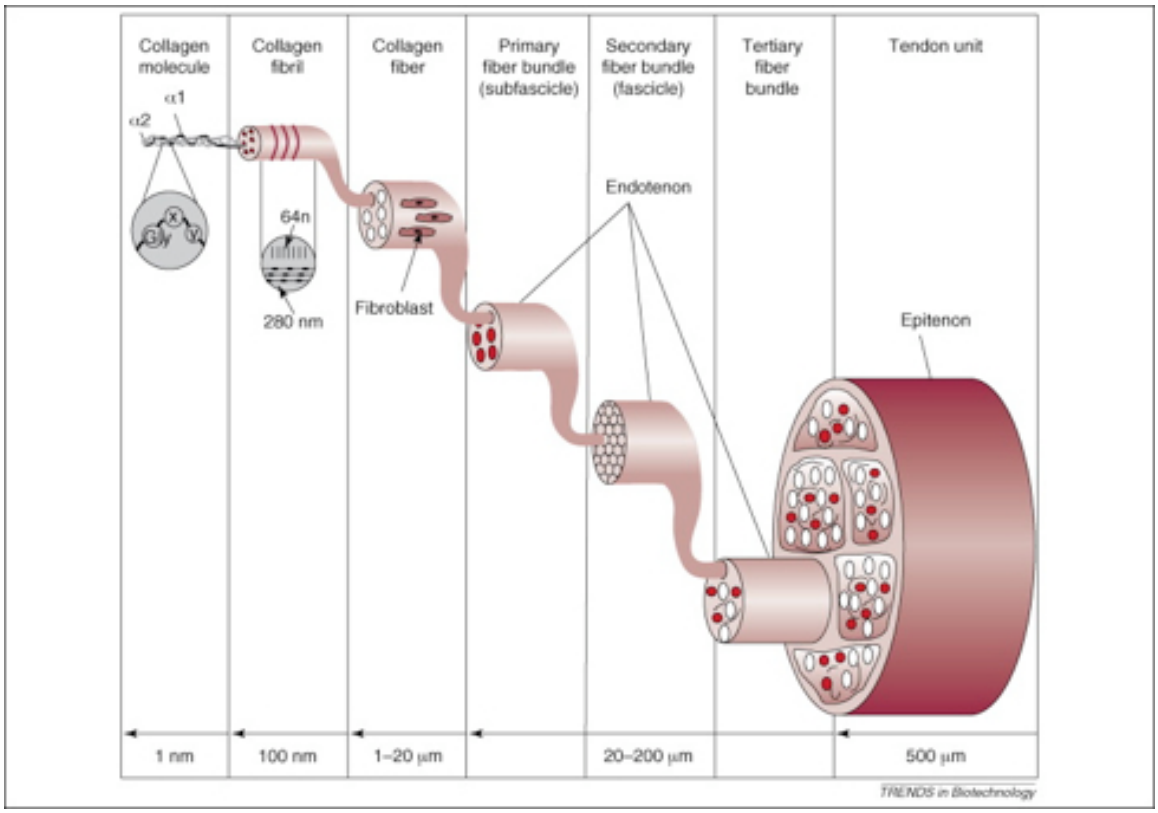

Figure 1.1-Diagram of the tendon[79].

Arranged in longitudinal rows separated by collagen fibers, the tendon cells are broad flat processes enclosing bundles of collagen fibers that extend lateral to cell bodies

to contact other adjacent cells. These cell bodies are able to communicate along this network[8].

Wrap-around tendons are defined as any tendon that bends around a bony pulley or threads through a fibrous one en route to its insertion. The locations of the wrap-around or compression area are frequently fibrocartilaginous and are an adaptation of the local compressive load the tendon experiences[15]. Proteoglycans can accumulate in the wrap-around area. The cells of fibrocartilage are large and oval and packed with intermediate filaments. These filaments might be involved in transduction of mechanical load via interactions between cytoskeleton, integrins and the extra cellular matrix (ECM)[5,9].

Other fibrocartilaginous regions of tendons occur at the insertion of the tendon into the bone. A common term of this region is "enthesis." There are two types of entheses, direct and indirect. Indirect insertions are made up of purely fibrous tissue and are seen in tendons like the FDL. Direct insertion goes through a transition zone from pure tendon to pure bone. The transition zone goes from pure tendon to uncalcified fibrocartilage to calcified fibrocartilage to bone[5,9].

Tendon Healing

Tendon injuries can be acute or chronic, with intrinsic or extrinsic factors contributing to those injuries. The extrinsic factors would be more considered a traumatic, acute injury where overuse injuries could be considered a combination of intrinsic and extrinsic factors. During the inflammatory phase (Table 1), a hematoma forms cells migrate from the peritendinous tissue such as the tendon sheath, epitenon and

endotenon. In the formative phase, the cells continue to proliferate and begin to differentiate[10].

Table 1.1- Tendon and ligament healing[10]

Time (Days)	Phase	Process
0	Immediately post-injury	Clot formation around the wound
0-1	Inflammatory	First battery of growth factors and inflammatory molecules produced by cells within the blood clot
1-2	Inflammatory	Invasion by extrinsic cells, phagocytosis
2-4	Proliferation	Further invasion by extrinsic cells, followed by a second battery of growth factors that stimulate fibroblast proliferation
4-7	Reparative	Collagen deposition; granulation tissue formation; revascularization
7-14	Reparative	Injury site becomes more organized; extracellular matrix is produced in large amounts
14-21	Remodeling	Decrease in cellular and vascular content; increases in collagen type 1
21+	Remodeling	Collagen continues to become more organized and cross-linked with healthy matrix outside the injury area. Collagen ratios, water content and cellularity begin to approach normal levels

Growth factors are essential to the healing response. In particular there are five growth factors, TGFβ, IGF-I, PDGF, VEGF and bFGF that are upregulated during healing[11]. In this thesis, TGFβ will be the main focus. TGFβ is active in both expression and in protein form in all stages of tendon healing and is responsible for stimulating cell migration, regulation of proteinases, fibronectin binding interactions, termination of cell proliferation via cyclin-dependent kinase inhibitors and stimulation of collagen production.

TGFβ

Transforming growth factor β (TGFβ) pathway is crucial in regulating cell physiology during development and adult life. It is critical and is linked to many human diseases ranging from cancers to fibrosis to neurological diseases [18,19]. There are three types of TGFβ isoforms in mammals (TGFβ-1, TGFβ-2, TGFβ-3), which are nearly identical[19]. TGFβ is active on all cell types. It stimulates and/or inhibits cell proliferation, differentiation, motility, adhesion or death depending on cell type and stage of the cell[19]. Another function for the growth factor is to regulate ECM proteins like collagens and fibronectin. It can also prevent ECM degradation by inhibiting MMPs by upregulating TIMPs[19]. It is considered a potent anabolic factor in deposition and repair of connective tissue[19]. Fenwick et al.[20] showed that the cells expressing TGFβ2 increases in chronic tendon pathology.

TGFβ helps drive fibrotic response in both in vivo and in vitro. Overexpressing of TGFβ can increase Smad2/3 phosphorylation and/or nuclear accumulation[19]. Velasco et al.[21] proposed that in an aggrecan rich environment, TGFβ binds to the TGFβRII/ALK-1 which leads to Smad 1/5/8 phosphorylation that causes chondrogenic response. With the removal of the aggrecan by ADAMTS5 allows TGFβ to bind to TGFβ1-TGFβRII/ALK5 that causes Smad2/3 phosphorylation that promotes fibroblastic response.

Robbins et al.[80] added TGFβ1 to tissue segments and increased the mRNA expression of aggrecan by 100%. In 2009, de Mos et al[81]. attempted to create a tendinopathy-like tissue explant. They accomplished this by exposing healthy tendons with TGFβ2 to the explant. This increased aggrecan expression by 2-fold. Both of these studies showed the presence of TGFβ created an increase of aggrecan.

Tendon Proteoglycans

Small leucine-rich proteoglycans (SLRPs) are the most common tendon PGs with decorin representing 80% of the total proteoglycan content in the tensional region (Figure 1.1)[12]. Biglycan, fibromodulin and lumican are other SLRPs that are located in the tensional region.

Corsi et al. [12,13] used biglycan knocked-out, decorin knocked-out mice and double knocked-out mice to determine the role of the SLRPs in collagen fibril assembly. Robinson et al.[14] examined the stress relaxation and pull to failure tests of three tendons. The tendon tail fascicles showed no difference in the tendon modulus, maximum stress and stress relaxation, however the flexor digitorum longus tendon and patellar tendon varied. The FDL had reduced maximum stress and modulus in the biglycan knockout compared to the control and decorin knockout, where the patellar tendon had an increases stress relaxation in the decorin knockout compared to the control. This study showed that there are tendon dependent changes and that decorin and biglycan are needed for normal tendon mechanics.

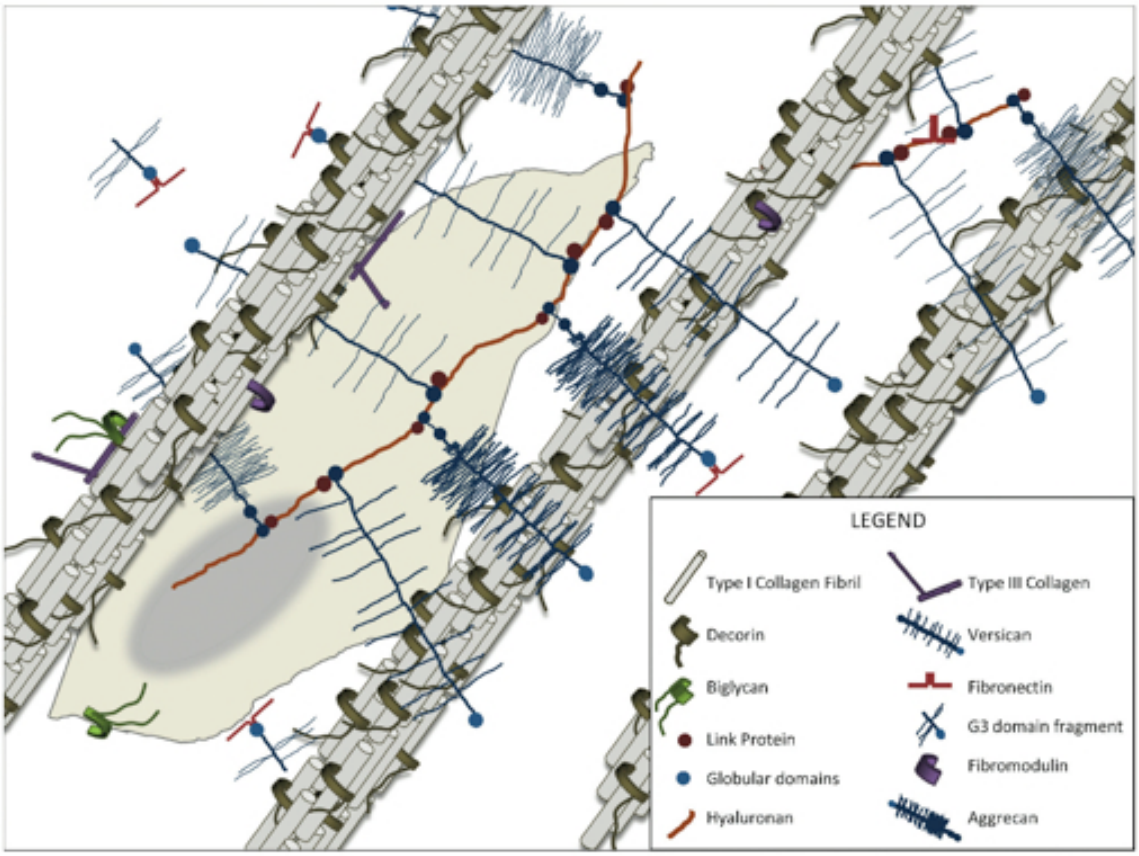

Figure 1.2- Diagram of interactions of the proteoglycans and other matrix macromolecules with the ECM in a normal tendon[12].

Aggrecan structure and function

The predominant large proteoglycans in tendon are aggrecan and versican. Aggrecan is composed of a core protein of about 220 kDa and is a monomer that consists of three globular domains (G1, G2 and G3). The G1 domain binds to hyaluronan forming large multimolecular aggregates. Versican contains G1 domain that binds to hyaluron and a carboxyl-terminal G3 domain but is missing the G2 domain. At the insertion site the proteoglycan content changes from mostly SLRPs to the larger proteoglycans especially aggrecan[15].

The tensional region of the tendon has degraded core proteins of aggrecan. These catabolic fragments contain the G3 domain indicating that the G3 domain may be

interacting with other components of ECM of the tendon. The fragments have been degraded by tissue proteinases which have high activity toward the interglobular domain and therefore generate G1-deficient, non-aggregating species[15,16].

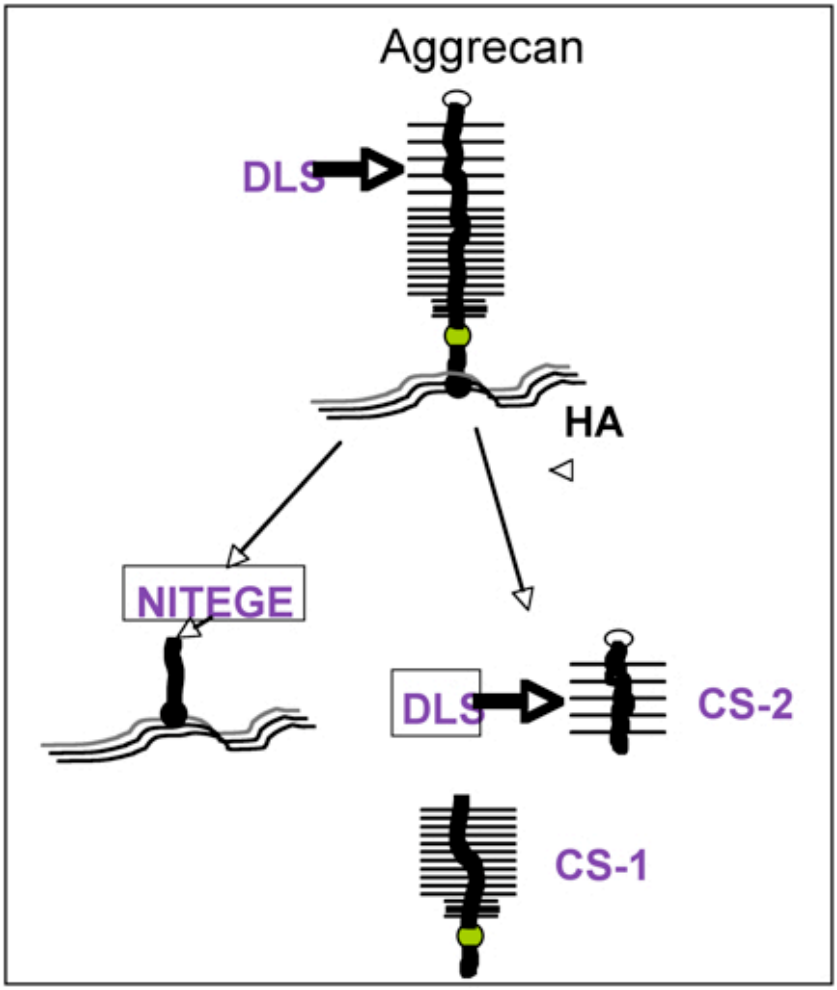

Figure 1.3- Diagram of full-length aggrecan, aggrecan cleaved at hylaronan (DLS) and aggrecanase-cleaved (NITEGE) aggrecan (Modified from Dr. John Sandy).

ADAMTS5

ADAMTS5 (A Disintegrin and Metalloproteinase with Thrombospondin motifs) is an aggrecanase that cleaves key sites of aggrecan core protein. ADAMTS5 has been frequently studied in the cartilage field. Glasson et al. in Nature studied ADAMTS5-/-

mice and proved they did not develop cartilage lesions in meniscus removal osteoarthritis model. Li et al.[48] also had the same results in a TGFβ injection and treadmill running oseteoarthritis model. Further research has been done with these mice in the dermal wound healing model[21].

Integrins

Integrins are transmembrane cell-signal receptors. Each integrin is made up with a α-subunit and a β-subunit and there are a total of 24 heterodimeric members (Figure 1.3). They mediate cell adhesion to the extracellular matrix and to other cells [22]. Integrins link extra cellular matrix and the cytoskeleton. Mechanical stimulation has been shown to increase integrin activity [22].

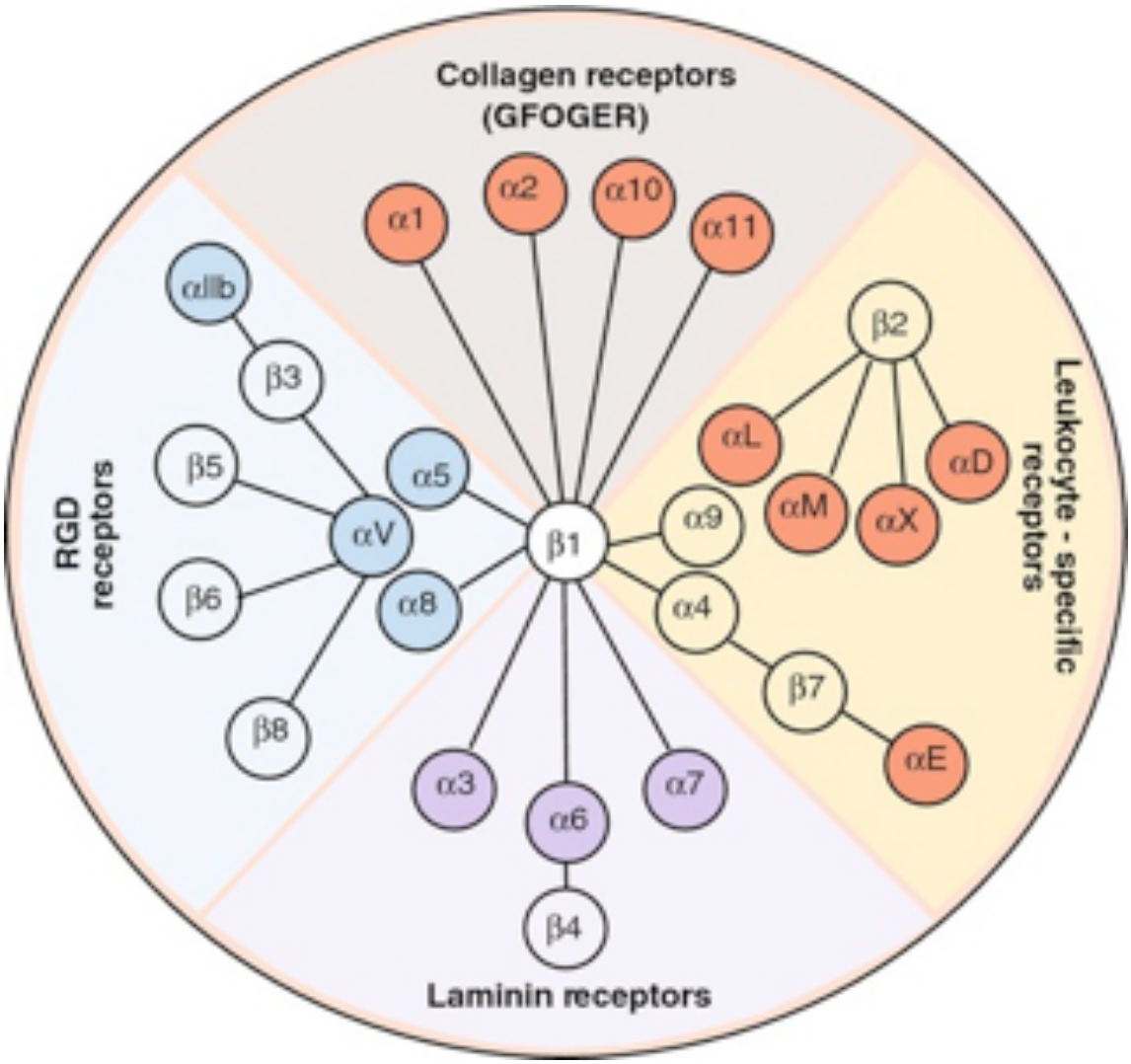

Figure 1.4- Representation of the integrin family. In vertebrates, the integrin family contains 24 heterodimers . The family can be split into collagen, RGD, laminin, and leukocyte receptors[22].

In most cases with the presence of TGFβ (Table 1.2), integrin expressions are stimulated. There is a type of feedback loop since an increase of integrin expression can cause and increase of TGFβ [23].

Table 1.2- Overview of the regulation of integrin expression by TGF-β[23].

Integrin	*Main Ligand*	*Effect of TGFβ*	*Cell Type*	*Context*
α1β1	Collagens	Upregulation	Fibroblasts	Collagen remodeling and contraction, myofibroblast differentiation during wound healing and fibrosis
α2β1	Collagens	Upregulation. downregulation	Fibroblasts	Collagen remodeling and contraction, myofibroblast differentiation during wound healing and fibrosis, re-epithelialization during wound healing
α5β1	Fibronectin	Upregulation	Fibroblasts	Re-epithelialization during wound healing
αvβ3	RGD	Upregulation	Fibroblasts	Myofibroblast differentiation during wound healing and fibrosis
αvβ5	RGD	Upregulation	Fibroblasts	Myofibroblast differentiation during fibrosis, re-epithelialization during wound healing

Tendinopathy

Tendinopathy is a chronic tendon disorder that accounts for ~50% of all sports-related "over-use" injuries[24,25] and ~12% of work-related injuries (Bureau of Labor Statistics). The clinical symptoms include activity-related pain, tenderness, and swelling. Ruptured tendons have been reported to have signs of degeneration prior to the injury [4]. Histological examinations of tendinopathic tissue indicates a defective healing response with increase cellularity, vascularity, and collagen fibrillar matrix disorganization with increased proteoglycan accumulations; cell nuclei are often rounded and occasionally fatty cells[25,26]. Increased aggrecan (Figure 1.4) contents (often termed chondroid metaplasia in tendon), enhanced histological staining for interfibrillar GAGs along with collagenous matrix disruption, and adiposity are common features of human tendinopathy[27]. Clearly such remodeling causes the tendon to weaken, which predisposes to further damage and rupture. The pathogenesis of this disabling condition is poorly

understood, and scientific advancements to elucidate potential mechanisms may facilitate the development of more efficacious clinical treatment strategies.

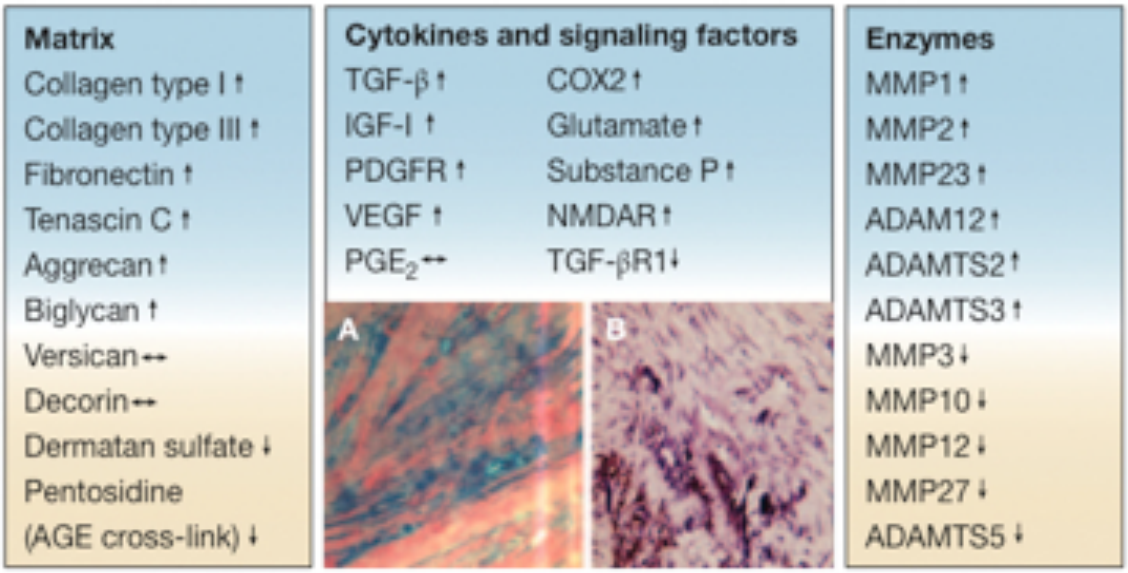

Figure 1.5- The changes of the matrix, signaling factors and enzymes in a tendinopathic tendon. (A) is an alcian blue/H&E stained section of supraspinatus tendon, the blue is showing the proteoglycans. (B) is an H&E stained Achilles tendinopathy showing increase cellularity and proliferation of blood vessels[28].

Treatments of Tendinopathy

There is no consensus in the best treatment for tendinopathy. The treatments can be separated into nonoperative management options and operative options. Nonoperative management options could range from exercise, shock therapy, to injectables. Operative options also range with how severe the problem is and how invasive the surgeon needs to be to remove the diseased area [29].

For nonoperative management options, the most common one is the prescribing of nonsteroidal anti-inflammatory drugs[29] even though chronic tendinopathy is a noninflammatory condition[29]. There has been no evidence of any benefits of this

treatment and patients use it to mask the early symptoms of the condition. This could cause more damage in the long run[29].

Another treatment is eccentric exercise. Eccentric exercise is the elongation of the muscle-tendon complex. This could be done in heel drops for the Achilles tendon. It is believed to promote collagen fiber cross-link formation, which creates tendon remodeling[30]. There are also theories that the decrease pain is caused by rapid strengthening of the calf muscle, stiffening and lengthening of the myotendinous unit and decreased neovascularization of the region[31]. Even with the different theories on how it works, it has been shown to reduce pain in 60-90% of the patients and has been shown the superior treatment in randomized trials[31,32]. This is consistent with athletic and sedentary patients[30].

Extracorporeal shock wave therapy is another nonoperative treatment. Its typical characteristics are high peak-pressure amplitudes (500 bar) with rise times of <10ns a short life cycle (10ms) and a frequency spectrum (16 to 20 MHz) ranging from audible to the far ultrasonic level, this rise is followed by periods of pressure dissipation and negative pressure before a gradual return to the ambient pressure[30]. It is believed the energy of the wave will create mechanical forces directly or indirectly on the tendon[30]. In animal studies, there was improved collagen fiber alignment in a rat Achilles, and a rabbit Achilles had neovascularization and an increase in the angiogenesis-related markers endothelial nitric oxide synthase as well as an increase in VEGF[29]. Zafar et al. [29] proposed the improvement in healing was due to an increase in growth factor levels, since there was elevated levels of TGFβ during the early stage and IGF-1 throughout[11].

Injectable substances have ranged from normal saline, corticosteroids, anesthetics, platelet-rich plasma, autologous blood injection, and polidocanol[30]. Platelet-rich plasma (PRP) has been proposed since the 1980s for wound healing and has been used in orthopedic surgery for bone grafting. It is a newer treatment for tendon wound healing. It releases TGFβ, PDGF, VEGF and cytokines[30]. Studies have shown very little improvement over injection of saline[30].

Surgical intervention is to excise fibrotic adhesions, remove or debride areas of failed healing, restore vascularity, and possibly stimulate viable cells to promote healing[30]. Radiofrequency microtenotomy, neovessel destruction and endoscopy-assisted treatment are three types of surgeries that are used. In all cases the operative treatments are more invasive and longer recovery time. The success rate of surgery is about 70% according to studies but that appears to be higher than actual clinical results[33].

According to most studies[30-33], treatments of tendinopathy should be kept conservative. Since eccentric exercise is low cost and low risk, it should be the first treatment to be tried.

Tendinopathy Models

There have been previous attempts to create animal models for tendinopathy, and they can be categorized into two groups: mechanical perturbation and chemical injury. Mechanical perturbation can be active stimulation and passive stimulation depending on the consciousness of the animal. In addition, [34,35] forced treadmill running to mimic the natural pathological injury has also been applied. The drawbacks of this approach are large variations in response between animals and the extended time frame of treadmill

running required. The passive mechanical stimulation has been done by loading the tendon with artificial stimulation or by direct repetitive tendon stretching via external loading device. Artificial stimulation has produced more repeatable and predictable outcomes but the outcomes do not resemble clinical tendinopathy [36]. The direct loading of the tendon using an external loading device requires surgery and is more of an acute injury model then a chronic one.

Chemical injury can be achieved by intra-tendon injection of collagenase, causes matrix damage, but its relevance to the human pathogenesis is unclear. Other chemical perturbation use injection of cytokines[37], PGE2, and PGE1[38] but none of them produced any clinically relevant responses in the treated tissues.

Specific Aims

Aim 1

To analyze morphologic properties, biomechanical properties, biochemical composition, and histological characteristics of tendons isolated from C57/Bl6 wild type mice (WT) and an ADAMTS-5 (aggrecanase) knockout mice, (TS5-KO).

Sub-Aim 1 – To study a single fascicle, no insertion tendon.

Sub-Aim 2 – To study a multi-fascicle with an insertion site tendon.

Aim 2

To develop a murine model of tendinopathy which results in accumulation of aggrecan rich deposits and has impairment of biomechanical properties.

Aim 3

To examine the effect of therapeutic interventions (treadmill exercise) on healing of tendinopathy.

Aim 4

To examine the effect of ADAMTS5 enzyme on healing of tendinopathy.

Chapter 2

Introduction

The failure of tendons to fully heal following traumatic injury or overuse implies an inadequate cellular reparative response to the disruption of the collagen-based matrix. The reparative task for cells, whether mature or progenitor, is primarily to re-establish the normal tensional properties of the tissue by the secretion and integration of newly synthesized collagen into the existing network, which is largely composed of heterotypic fibrils of collagens I and III along with minor amounts of type V. [39,40] This process also requires production and organization of a noncollagenous matrix which, among many other components, contains members of the collagen binding small leucine rich proteoglycans (SLRP) family and the large proteoglycans (PGs), versican, and aggrecan.[15,41]

To date, there has been no clearly defined mechanistic role for aggrecan (or versican) in tendon cell behavior, matrix homeostatic maintenance, tissue injury, and repair. This may be because of the inherent difficulties involved in analysis of the small amount of aggrecan present in all noncartilaginous tissues. Thus, studies directed toward determining the content and distribution of aggrecan in tendons have primarily used nonspecific measures based on glycosaminoglycan (GAG) analysis, such as hexuronic acid assay[42] and GAG reactivity with DMMB,[28] disaccharide compositional analysis [43,44] or histochemistry with cationic dyes.[45] Although GAG analysis alone does not determine what proportion of the chains are attached to aggrecan (compared to other CS/DS PG core proteins), purification steps such as gradient centrifugation and gel permeation chromatography, together with gel electrophoresis and protein sequence analysis, have

been used to provide definitive evidence for the presence of aggrecan core protein in extracts of tendon.[15,46] Further, Western analysis with antibodies specific to known aggrecan core epitopes and neoepitopes[47] has shown the presence of both intact aggrecan and ADAMTS-generated fragments in the same tendon and ligament samples.[46,47]

Aggrecan is most abundant in regions of tendon that experience mechanical compression and at tendon-bone insertion sites,[5] and proteolytically degraded aggrecan has also been identified by peptide analysis in tensional regions of normal adult tendons.[15] We have recently reported that diseased ligaments from horses with degenerative suspensory ligament desmitis (DSLD) contain highly elevated levels of intact aggrecan and fragments generated by ADAMTS- aggrecanase activity.[47] ADAMTS5 (TS5) was also abundant in the affected ligaments, but it was found to be complexed with hyaluronan around chondroid cellular clusters, and no active forms could be detected in these extracts. A causative role for this apparent deficiency of active TS5 in equine ligament degeneration, is supported by the finding that *ts5*$^{-/-}$ mice exhibit ineffective dermal repair. Indeed, this dermal defect, which is due to ablation of TS5, is characterized by the accumulation of aggrecan in the pericellular matrix of fibroblast progenitors and a concomitant failure to restore a functional dermal collagen matrix. [21]

On the basis of these equine ligament[47] and murine dermal[21] studies, we hypothesized that *ts5*$^{-/-}$ mice would also exhibit compromised tendon properties, and that this would be associated with aggrecan accumulation in the collagen matrix. Biomechanical and ECM properties were examined in the flexor digitorum longus (FDL) and Achilles tendons of wild type and *ts5*$^{-/-}$ mice. The FDL was chosen since it is a single fascicle tendon that lacks a prominent insertion site. The Achilles tendon is an example of

a multi-fascicle tendon containing a well-defined bone insertion.

Methods

Animal and Tissue Procurement

Twelve-week-old female mice were used for the study, which was approved by our Institutional Animal Care and Use Committee. *ts5*$^{-/-}$ mice (deletion of exon 2) were obtained from an in-house breeding colony[48] and C57BL/6 wild-type (WT) were either bred in house or purchased from The Jackson Laboratory, Bar Harbor, ME (see Table 1 legend). For biochemical analyses mice were euthanized, tissues immediately dissected into cold PBS with proteinase inhibitors and further processed for FACE and Western analyses. For biomechanical tests, euthanized mice were placed in sealed plastic bags and immediately frozen at 20^{o}C until the day of testing. Histologic analyses were performed on freshly dissected tendons.

Wet Weight/Dry Weight Measurements

FDL tendons, previously washed with proteinase inhibitors and stored at -20^{o}C, were thawed in PBS for 30 minutes then blotted dry. New centrifuge tubes were weighed and each tendon was placed into its own tube. The tendon and tube were weighed and then they were dried in a speed-vac for 1 hour. The tube and tendon were weighed again. The speed-vac and weighing process were repeated until the weights were consistent. The first weight and the last weight were used as the wet and dry weight, respectively.

Flexor Digitorum Longus Tendon Biomechanical Testing

Separate mice were used for the FDL testing and the Achilles testing. On the day of testing, the mice were thawed and the FDL tendons were dissected out. The tendon was cleaned off of any muscle by scraping gently (Figure 2.1). Five measurements along the width were taken with precision calipers and at the approximately the same location, the thickness was taken with a laser displacement sensor which has a 0.2 μm accuracy (Keyence model LK-G82). The tendon was assumed to be rectangular and the cross-sectional area was calculated as the product of the mean tissue width and thickness. A gauge length of 8 mm was set as the standard; this allowed 2-3 mm of tendon to be gripped with sand paper on both ends and prevented the need of super glue to hold the tendon without slipping.

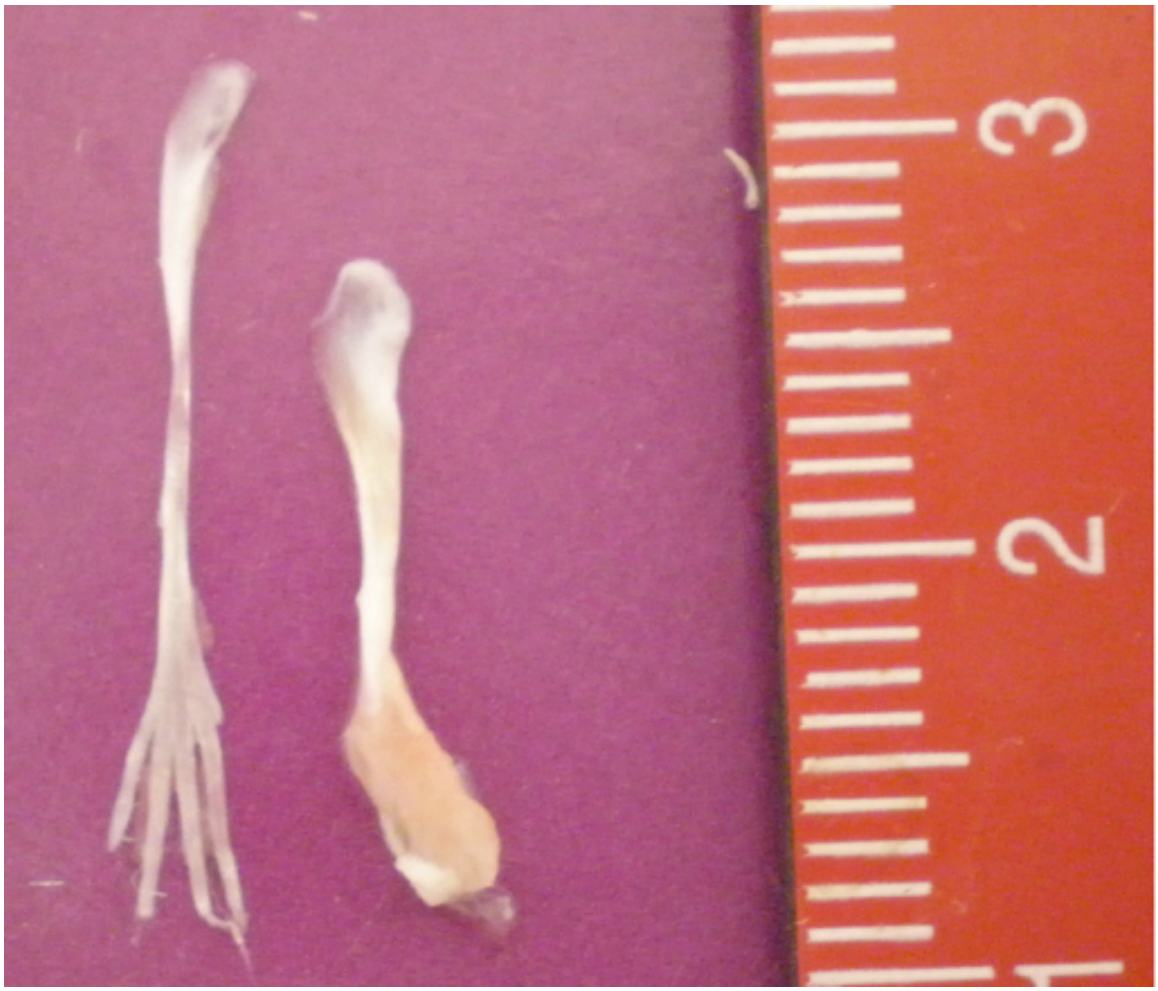

Figure 2.1- Full length of the FDL (on left) and Achilles (on right).

Once the tendon was in the grips, three marks were made evenly along the length of the tendon with a tissue marker. The tendon was then soaked for 15 minutes prior to testing in a 37^{o}C saline bath. While still in the bath, the tendon was preloaded at 0.05 N for 2 minutes; then it was preconditioned from 0.05 N to 0.15 N for 20 cycles at a rate of 0.10 N/sec. After five minutes of recovery time, the tendons were pulled to 5% strain at a rate of 2.5% strain/second and held for 600 seconds. The tendons were then pulled to failure at a rate of 0.05 mm/sec. During the failure test, a 1-megapixel digital video camera was used to record the test at 48 frames/second. This was used to determine the

strain between the outer two markers of the 3 marks on the tendon. Using the strain from the camera system, the force from the MTS and the cross-sectional area, optical tensile modulus was determined. The following tensile properties were computed: percent stress relaxation, maximum force, stiffness, maximum stress, and optically based elastic modulus. Stiffness and optical modulus were calculated as the steepest slope of the load-deformation curve (or stress-strain curve) spanning 40% of the data points collected between initiation of the load (or stress) and the maximum load (or stress) of the failure test.

Achilles Tendon Biomechanical Testing

The Achilles tendons were tested similarly to FDL but since the tendon has a more defined bony attachment (see Figure 2.1), adjustments were made. After the tendon was measured, the calcaneus was potted in a ~10 mm long brass tube with methyl methacrylate. The proximal end of the tendon was gripped with sand paper at a gauge length of 6 mm. Markings were made on the tendon but were not consistent in the amount and the location. The preload was again for two minutes at 0.05 N but the preconditioning was from 0.05 N to 0.55 N at 0.1 N per second. This was determined by pilot tests, which showed a higher failure load for the Achilles compared to the FDL. The rest of the testing was the same as FDL. Optical elastic modulus could not be calculated from the video due to the variations of the markings.

Transmission Electron Microscopy (TEM) of Collagen Fibril Diameters

A 1 mm long segment of tissue was harvested from the distal, central, and proximal regions of freshly dissected FDL tendons from WT (n=4) and *ts5*$^{-/-}$ (n=4) mice. Tendons were processed, embedded, and sectioned as previously described.[49] Using random sampling methods, three images per region were taken at 120,000-fold magnification. Individual fibrils within each image were outlined using ImageJ (NIH). The fibrils were assumed to be elliptical and the area and diameter were recorded for each fibril. Approximately 300 fibrils per tendon specimen were analyzed. The fibril area fraction was calculated by adding the area of all the fibrils in a given image then divided by the total area of the image. While area fraction demonstrated consistent results within a genotype, fibril diameter distribution and mean values varied considerably by anatomic region, and therefore the latter indices were not compared statistically by genotype.

Collagen Typing

Individual whole FDL tendons from the right hind limb of four WT and four *ts5*$^{-/-}$ mice were extracted by heat denaturation (100^{o}C) in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gel buffer[50]. The gel was run and qualitatively analyzed for any differences by an expert in the field (Dr. David Eyre).

Chondroitin Sulfate/Dermatan Sulfate (CS/DS) and Hyaluronan (HA) Contents

Glycoaminoglycans were solubilized and prepared for FACE[51,52]. Four FDLs were combined and digested at 55^{o}C with 10 ul proteinase K with a concentration of 10 mg/ml combined with 250 ul 0.1 M ammonium acetate overnight. The following day the digestion was stopped by heating to 100^{o}C for fifteen minutes then adding 1 ml of 1 mM

of AEBSF. The GAG's were then desalted with MicroCon3 filtration followed by digestion with chondroitinase ABC at 37^{o}C for overnight. Using the MicronCon3 filtration, the samples were rinsed of the chondrotinase ABC and dried overnight. Samples were then fluorotagged with 2-aminoacridone/sodium cyanoborohydride. After the samples were incubated at 37^{o}C overnight, 30 ul of 25% glycerol was added to each sample. The samples were then electrophoresis to have separation of disaccharide products. The gels were exposed at 1/100, 1/20, 1/10, ¼. ½, and 1 second. Gels were analyzed at the longest exposure time that does not cause saturation. A few of the gels had the standards analyzed at a faster time than the samples due to the intensity of the standards. Using ImageJ (NIH), the bands were quantified by comparing the intensity of the signal compared to the HA and CS standards. The HA standard lanes had 0.75 ug of HA and the CS standard lane had 0.6 ug of CS.

The recovery of GAGs by the above procedure was validated in a separate experiment. For this, 0.5, 1.0, and 5.0 ug of each, CS and HA was run as a standard gel, (seen in Figure 2.2) then the same amount was added to pooled FDLs and tissues solubilized with proteinase K or NaOH. Desalted GAGs were digested with chondroitinase ABC alone or S. Hyase followed by chondroitinase ABC. The recoveries for CS were 13% and for HA 14%, and these numbers were essentially identical for both proteinase K and NaOH solubilization methods. Data presented are corrected for these recovery values.

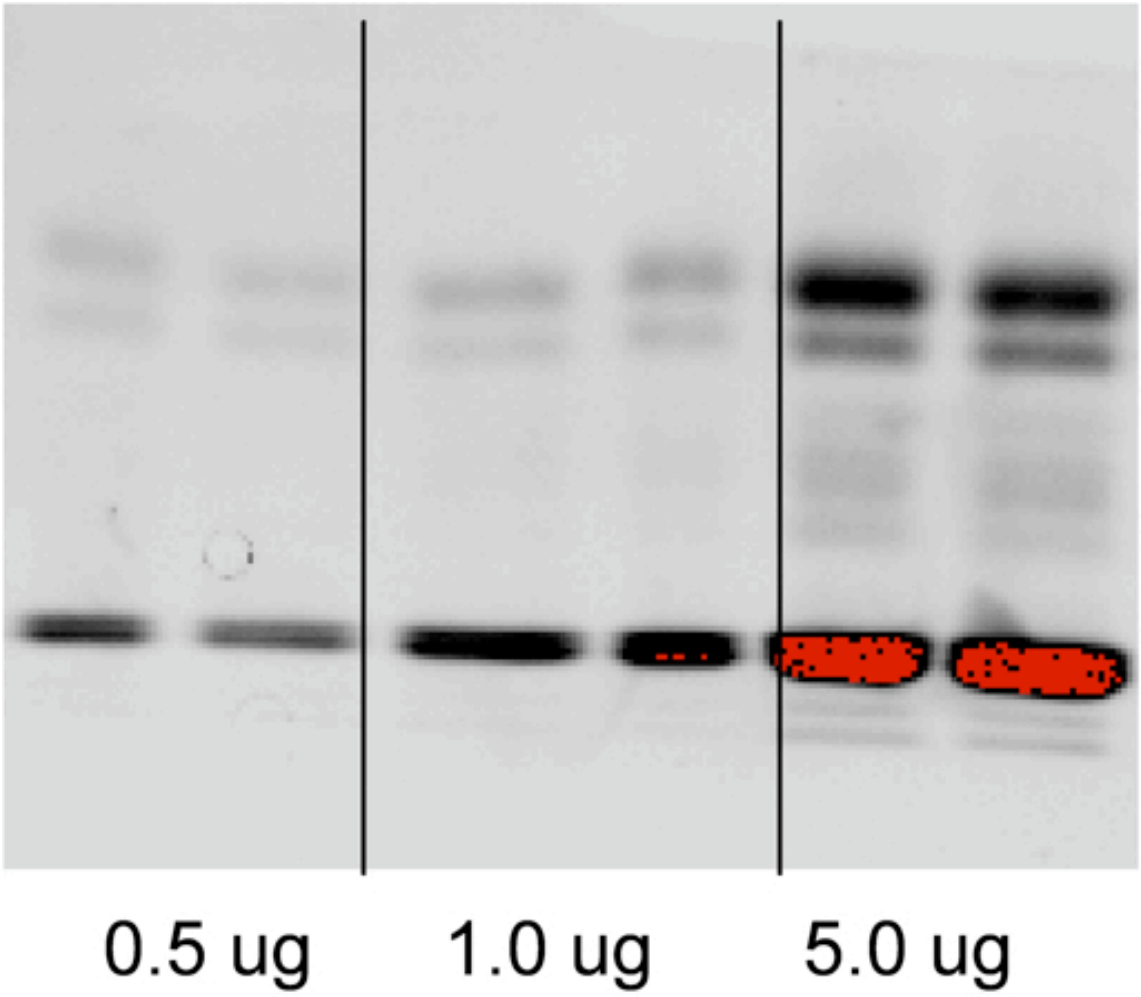

Figure 2.2- The standard gel with 0.5, 1.0, and 5.0 ug of CS and HA. Exposure time was 1 second for the gel. The red indicates saturation and 5.0 ug was analyzed at a faster exposure time.

Western Analysis of Aggrecan, Versican, and Decorin in FDLs

Following the methodology previously reported [47] FDL tendons (four each from WT and *ts5*$^{-/-}$ mice) were thawed, measured for wet weight and then deep-frozen in liquid nitrogen. The tendons were pulverized and incubated overnight in 4^{o}C in 500 ul of extraction buffer. Samples were then dialyzed against running water then run through DE52 cellulose columns. After the elution of the proteoglycans, the samples were

digested with chondroitinase ABCPF for one hour. Western blot analyses were performed with anti-bodies to aggrecan (a-DLS), aggrecanase-generated neoepitope aggrecan antibody (a-KEEE), versican (Ab1032/1033 (1:1 mix) from Millipore Inc., Billerica, MA) and decorin (Monoclonal 6D6, from Dr Paul Scott, U. Calgary).

Histology and Immunohistochemistry

FDL and Achilles tendons were processed for paraffin embedding as previously described.[53] Five-micron sections were stained with Toluidine blue or anti-aggrecan antibody (a-DLS) as previously described.[47] For the FDL, the number of cells per unit area (2,500 mm^2) was counted by three investigators blinded to the treatment group, as seen in Figure 2.3.

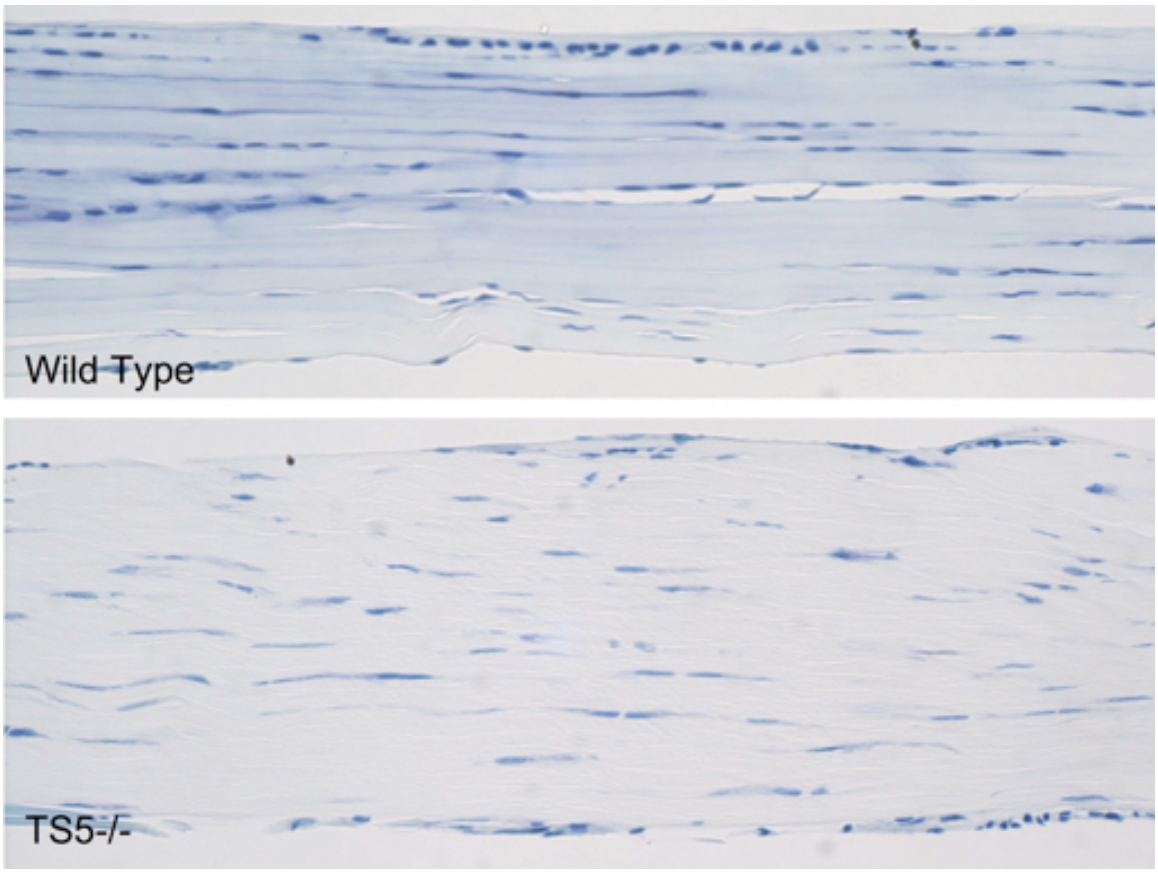

Figure 2.3- One of the five images that were measured for the amount of cells.

There were no differences in cell numbers between the genotypes (101± 25 for WT and 94± 40 for *ts5*$^{-/-}$), and no differences observed by tendon region.

Statistical Analysis

Ultrastructural, geometric, and biomechanical properties, as well as CS/DS/HA contents were compared between WT and *ts5*$^{-/-}$ tendons using unpaired t-tests.

Results

Biomechanical Properties of FDLs in WT and ts5$^{-/-}$ Mice

The cross-sectional area of the *ts5*$^{-/-}$ FDLs was 0.20±0.03 mm^2 compared to the significantly smaller (p=0.014) WT FDLs which was 0.15±0.04 mm^2. Even though the decrease in the area, the structural properties of peak load and stiffness showed no significant differences. There was no difference in the stress relaxation data either. However with the cross-sectional area taken into account in the material properties, the *ts5*$^{-/-}$ mice showed significantly lower maximum stress (p=0.019) and tensile modulus (p=0.032) than WT (Table 2.1). A typical comparative stress–strain optical curve is shown in Figure 2.4.

Table 2.1- Comparison of FDL Properties (mean ± std dev) between *ts5*$^{-/-}$(In-House Bred) and Wild Type (Purchased from Jackson Labs)[54]. NS indicates no significance.

FDL	Wild Type (n=7)	*ts5*$^{-/-}$ (n=7)	p-value
Cross-Sectional Area (mm^2)	0.15 ± 0.04	0.20 ± 0.03	0.014
% Relaxation	45.7 ± 6.5	52.3 ± 12.1	NS
Maximum Force (N)	3.6 ± 1.1	3.1 ± 1.1	NS
Stiffness (N/mm)	9.7 ± 1.3	7.9 ± 1.9	NS
Maximum Stress (MPa)	24.3 ± 6.6	15.6 ± 4.9	0.019
Modulus (MPa)	1138.8 ± 766.8	336.6 ± 179.3	0.032

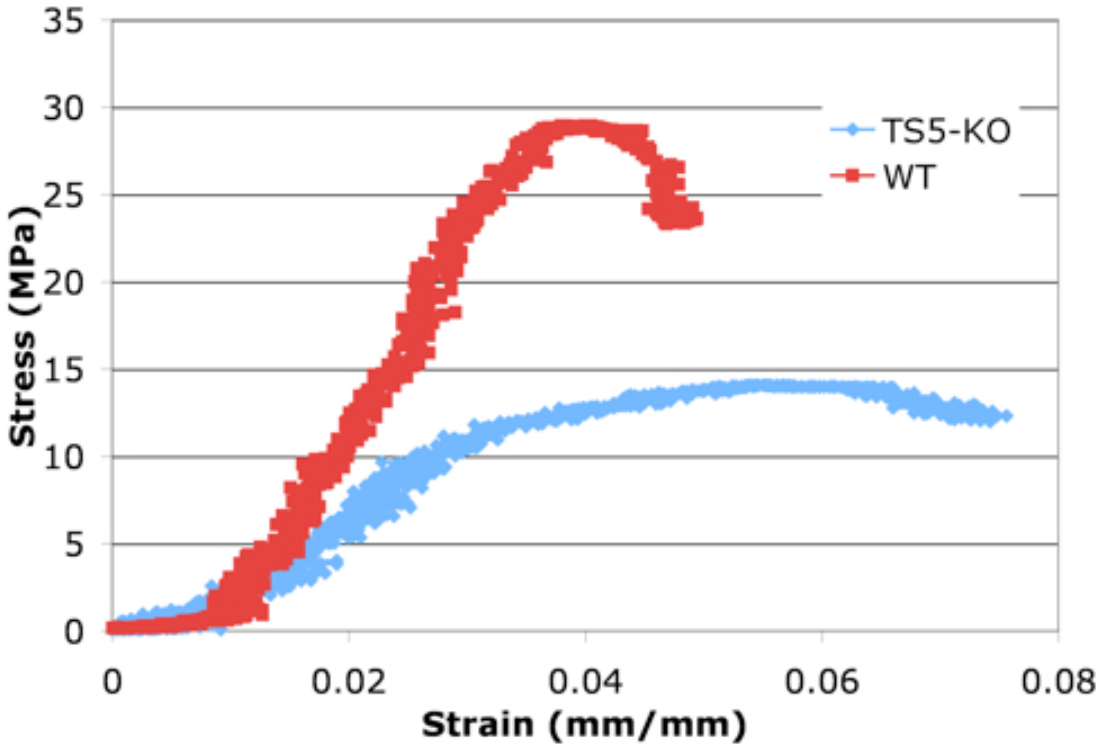

Figure 2.4- A typical stress- optical strain curve for the FDL[54].

Collagen Structure of FDLs in WT and ts5$^{-/-}$ Mice

Since the mean cross-sectional area of *ts5*$^{-/-}$ tendons was approximately 33% greater than that of WT (p=0.014), the collagen structure of the tendons were further analyzed. TEM analyses (Figure 2.5A,B) indicated that the fibril area fraction was increased (p=0.027) in *ts5*$^{-/-}$ mice when compared to WT, and greater heterogeneity of fibril diameters was observed for the former (Figure 2.6). Individual fibril borders were generally more consistent and well defined in wild type compared to *ts5*$^{-/-}$ tendons (black arrowheads in Figure 2.5B).

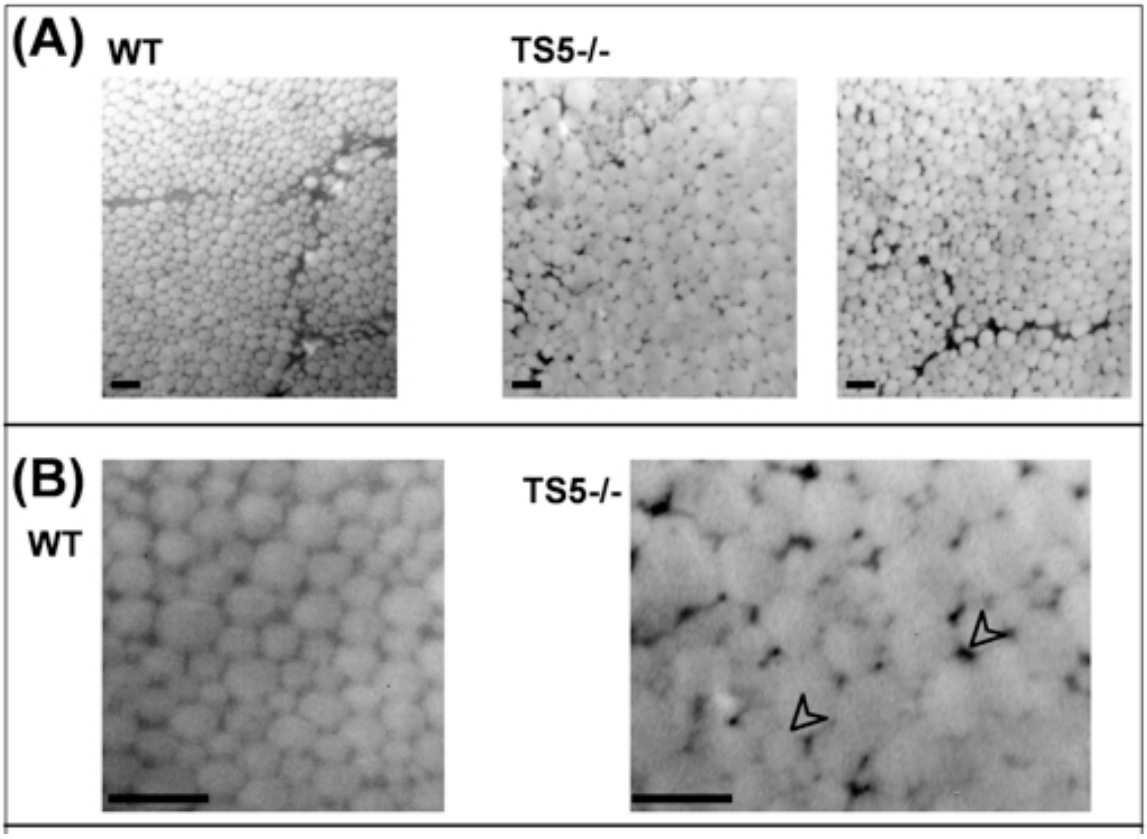

Figure 2.5- TEM images (scale bar =200 nm) of collagen fibrils from FDL tendons of wild type and *ts5*$^{-/-}$ mice at 60,000 (A) and 120,000 magnification (B)[54].

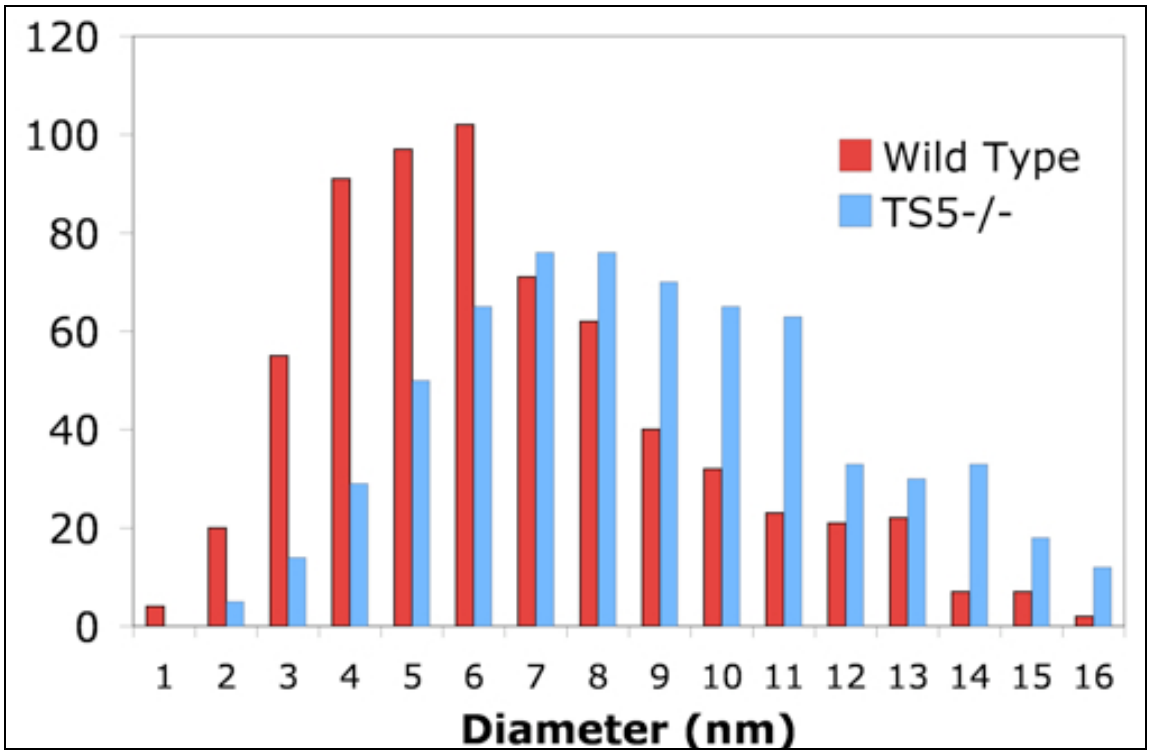

Figure 2.6- Histogram of the fibril diameters of WT and *ts5*$^{-/-}$ FDL tendons.

Despite the fibrillar changes, the total and relative abundance of collagen type I a-chains (and cross-linked forms) was not appreciably different for the two genotypes (Figure 2.7).

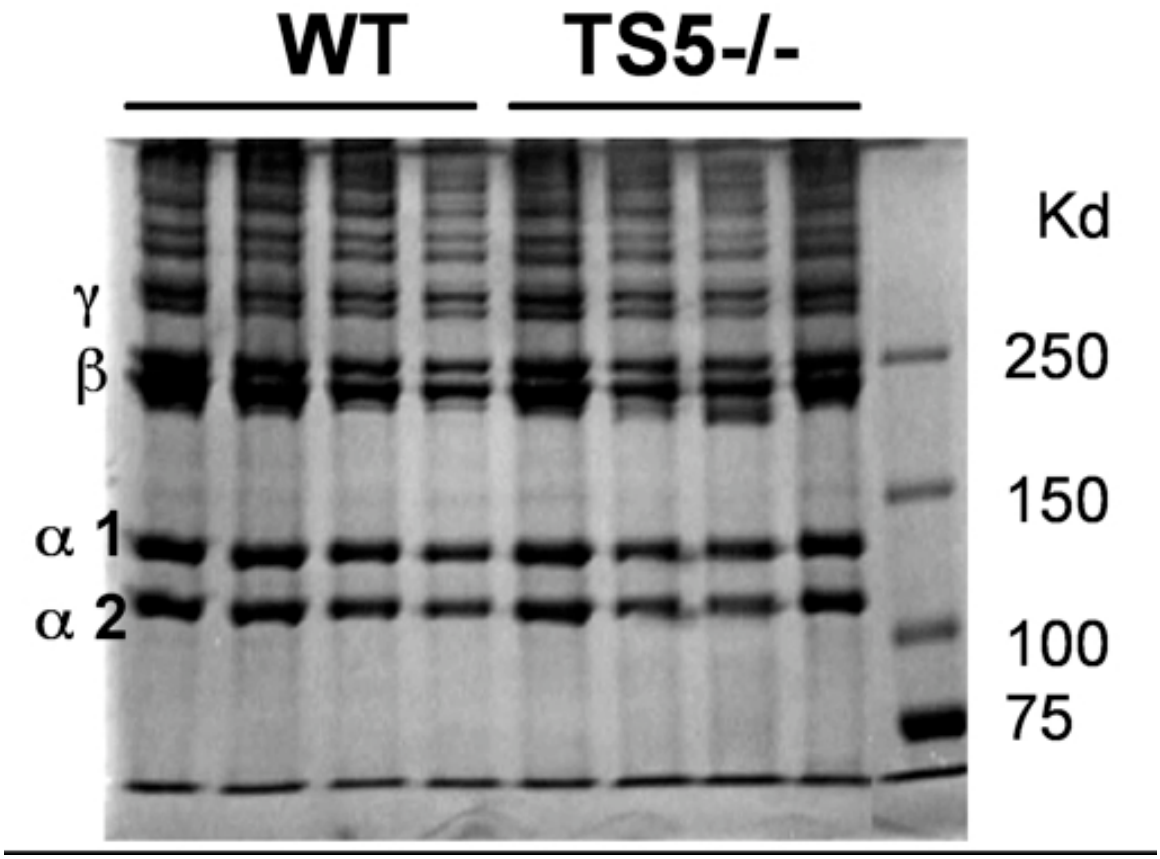

Figure 2.7- For collagen analysis, for electrophoresis, the loads (dry weight tissue per lane) were the same, and the gel was stained with Coomassie blue. No obvious quantitative or qualitative difference was detectable in the patterns of α1, α2, β, γ, or higher polymeric forms of collagen in the two sets of tendon samples[52].

Quantitative Assessment of GAGs Isolated from FDLs of WT and ts5$^{-/-}$ Mice

FACE analysis of FDLs (Figure 2.8) showed an average CS/DS content of 250 ng/tendon and was similar for both genotypes. HA content in WT FDLs was 1.8 mg/tendon and was fourfold higher than in the *ts5*$^{-/-}$ FDLs ($p < 0.0001$). It was also noted that in wild type tendons the HA content (based on disaccharide analysis) was about eightfold higher than the combined CS/DS content.

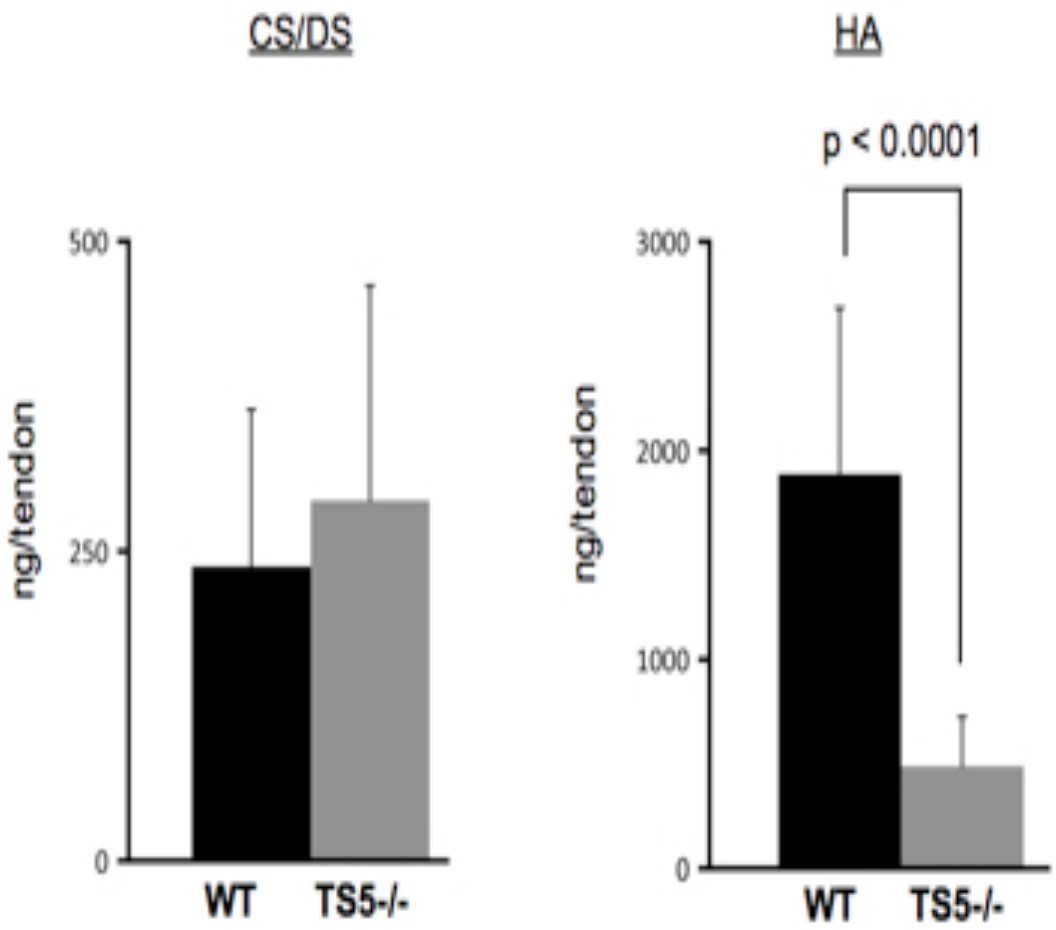

Figure 2.8- FACE analyses of FDL tendons showed that the total CS/DS content (left panel) was similar for each genotype whereas the hyaluronan content (right panel) was markedly higher ($p < 0.0001$) in WT tendons[54].

Quantitative and Qualitative Assessment of PGs Isolated from FDLs of WT and ts5$^{-/-}$ Mice

Western analyses for aggrecan (Figure 2.9) showed that both genotypes contained a major core protein species at 250 kDa, with minor species at 160 kDa and 300–400 kDa. Western analysis of the same samples with a-KEEE (specific for aggrecanase-generated fragments) showed the presence of an immunoreactive species at about 250 kDa (data not shown). Densitometric analysis of the blots showed that the mean

abundance of aggrecan in FDL extracts was approximately threefold higher (p=0.013) in the *ts5*$^{-/-}$ (n=14) than WT (n=14) FDLs. Versican analysis with a mix of antibodies to the murine a-GAG and b-GAG regions, showed two major core species at about 110 and 80 kDa in both genotypes. Decorin analysis with monoclonal Ab 6D6, which specifically detects the tetrapeptide sequence 241HLRE244, showed a single major core at about 48 kDa in both genotypes. A minor 52 kDa species (which might, for example, result from propeptide retention) was evident in some samples. Densitometric analysis (Figure 2.9B) of Westerns for both versican (n=14 for each genotype) and decorin (n=5 for each genotype) showed no differences in abundance for these PGs between genotypes.

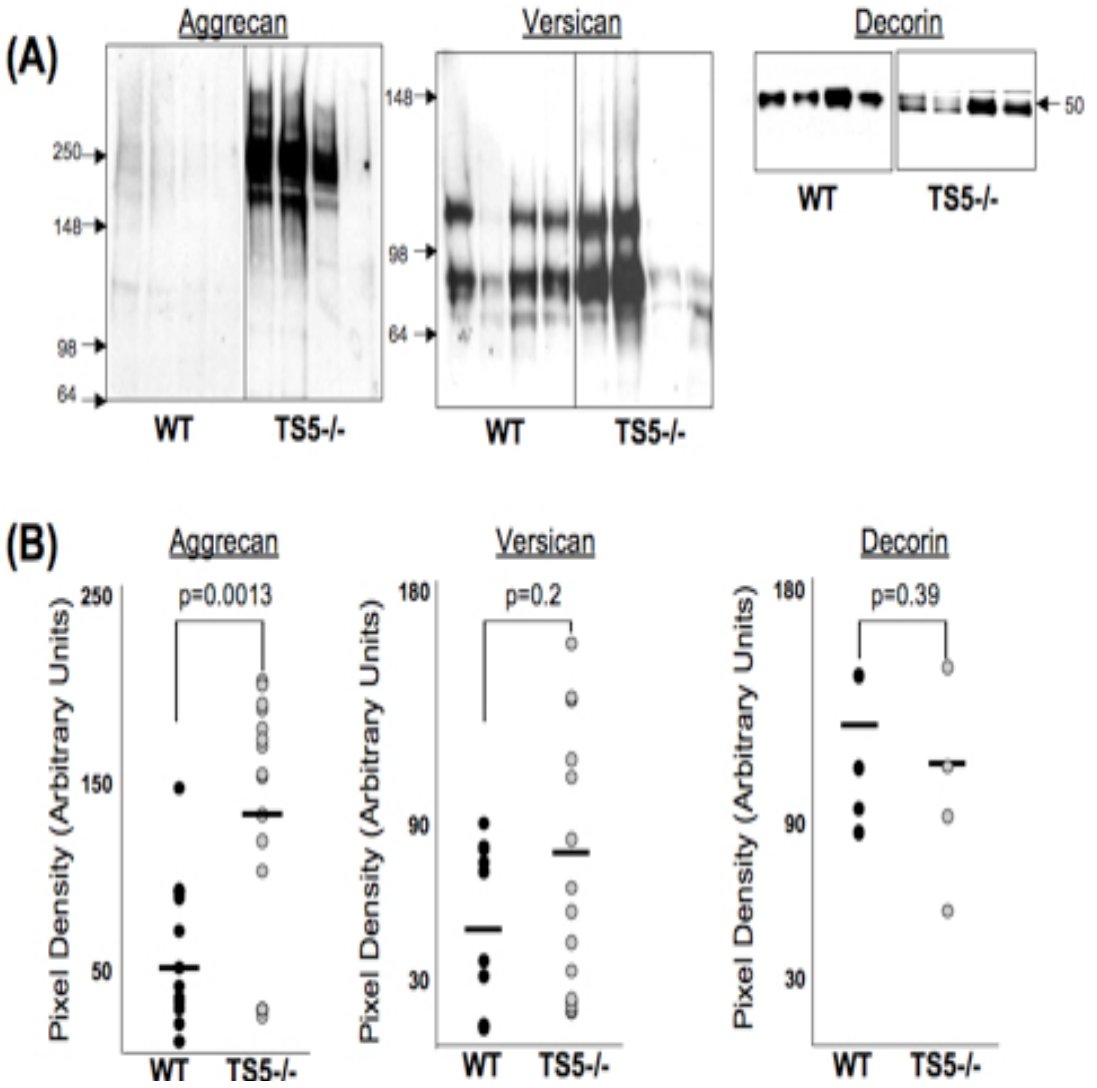

Figure 2.9- (A) Typical Western blots of PGs purified from FDL tendons of four WT and four *ts5*$^{-/-}$ mice. (B) Densitometric analysis of Western blots for aggrecan (WT, n=14; *ts5*$^{-/-}$, n=14), versican (WT, n=14; *ts5*$^{-/-}$, n=14), and decorin (WT, n=9; *ts5*$^{-/-}$, n=13)[54].

Histology and Aggrecan IHC of FDL Tendons from WT and ts5$^{-/-}$ *Mice*

WT FDLs contained typically elongated flattened tenocytes whose nuclei appeared closely aligned with the long-axis of the collagen bundles (Figure 2.10). By comparison, *ts5*$^{-/-}$ FDLs also contained the flattened aligned consistent with low abundance of aggrecan in WT FDL tendons, shown by Western analyses of tissue extracts (Figure 2.9). However, in *ts5*$^{-/-}$ FDLs (Figure 2.10), the cells with more rounded

nuclei stained strongly for aggrecan. Most notably, the immunoreactivity was largely cell-associated, suggesting a preferential localization of aggrecan to the pericellular space in the absence of TS5.

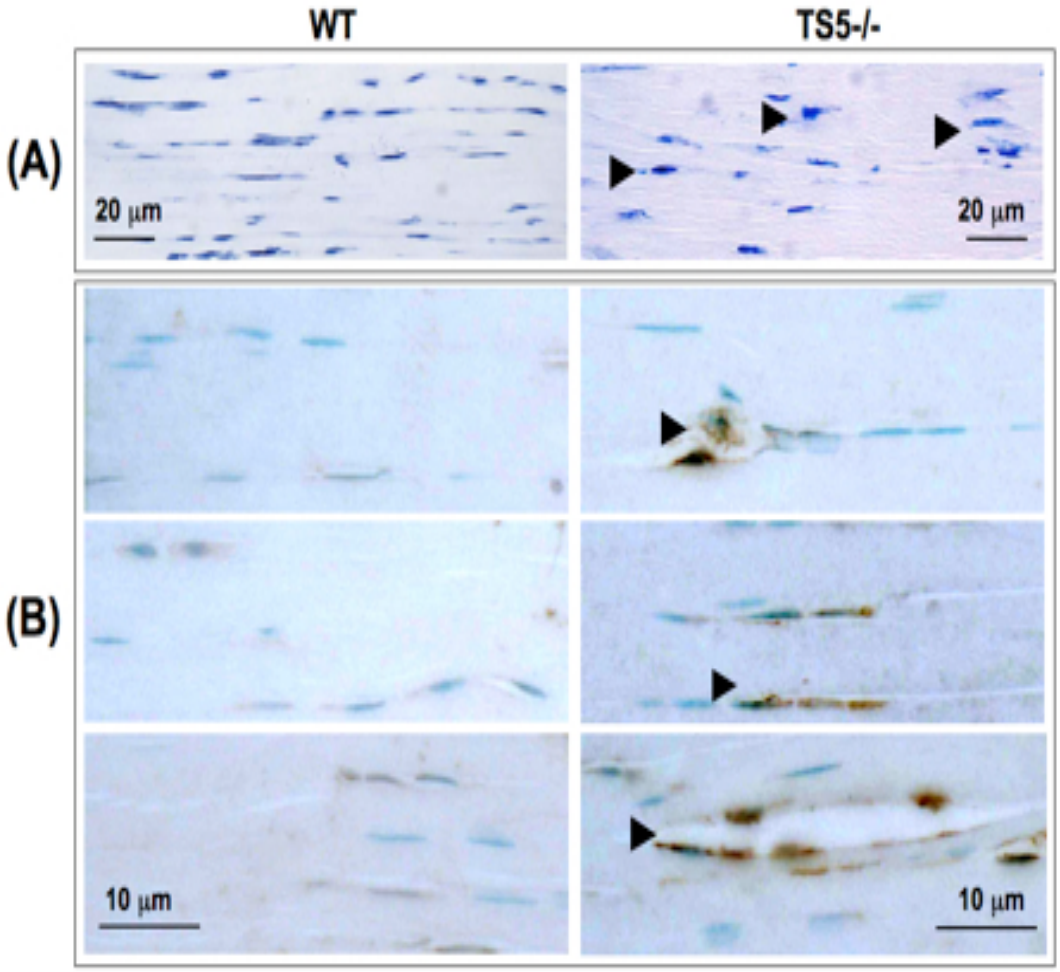

Figure 2.10- (A) Typical images of Toluidine Blue staining from the midportion of WT and *ts5*$^{-/-}$ FDLs taken at 20x magnification. (B) Typical images of immunohistochemical localization of aggrecan with (a-DLS) in midportion of WT and *ts5*$^{-/-}$ FDLs taken at 40x illustrate pericellular aggrecan deposition in *ts5*$^{-/-}$ tendons (black arrowheads)[54].

Geometric and Biomechanical Properties of Achilles Tendons from WT and ts5$^{-/-}$ Mice

The mean cross-sectional area of Achilles tendons was similar ($p > 0.7$) among the two genotypes. While the tensile modulus of *ts5*$^{-/-}$ Achilles tendons was higher than that of the WT ($p=0.047$), the maximum stress, maximum force, stiffness, and the percent stress relaxation were not significantly different between the two groups (Table 2.2). Notably, the TS5-/ mice showed abnormally poor mechanical integration of the Achilles tendon into its bony insertion, as 5 of 8 *ts5*$^{-/-}$ tendons failed within the insertion site, in contrast to the WT group, in which no insertional failures were observed (Table 2.2). In *ts5*$^{-/-}$ tendons, the failure location appeared macroscopically at the interface between unmineralized and mineralized fibrocartilage regions. For WT tendons as well as *ts5*$^{-/-}$ tendons which did not fail within the insertion, rupture occurred within the proximal tendon approximately 1–2 mm below the upper tissue grip.

Table 2.2- Comparison of Achilles Tendon Geometric and Mechanical Properties (mean ± std dev) between *ts5*$^{-/-}$ and Wild Type Mice. NS= no significance[54].

Achilles	Wild Type (n=7)	*ts5*$^{-/-}$ (n=8)	p-value
Cross-Sectional Area (mm^2)	0.50 ± 0.02	0.49 ± 0.09	NS
% Relaxation	38.9 ± 3.2	40.0 ± 3.2	NS
Maximum Force (N)	6.6 ± 1.7	7.4 ± 1.1	NS
Stiffness (N/mm)	8.2 ± 1.4	8.5 ± 1.5	NS
Maximum Stress (MPa)	13.4 ± 3.7	15.7 ± 2.8	NS
Modulus (MPa)	86.8 ± 15.5	106.6 ± 19.4	0.047
% Failures at insertion	0	62.5	0.004

Histology and Immunohistochemistry of Achilles Insertion Sites from WT and ts5$^{-/-}$ Mice

Representative images of GAG-enriched bony insertion sites are depicted in Figure 2.11. In all specimens examined, the nonmineralized fibrocartilaginous region of *ts5*$^{-/-}$ tendon insertions was more intensely stained with Toluidine blue and had a higher number of rounded, chondrocyte-like cells (Figure 2.11) and IHC for aggrecan (Figure 2.12) confirmed Toluidine blue staining results. Higher magnification images of sections showed a greater abundance of aggrecan-positive cells throughout the *ts5*$^{-/-}$ Achilles tendon, which was localized to both pericellular regions and throughout the collagen bundles (Figure 2.11).

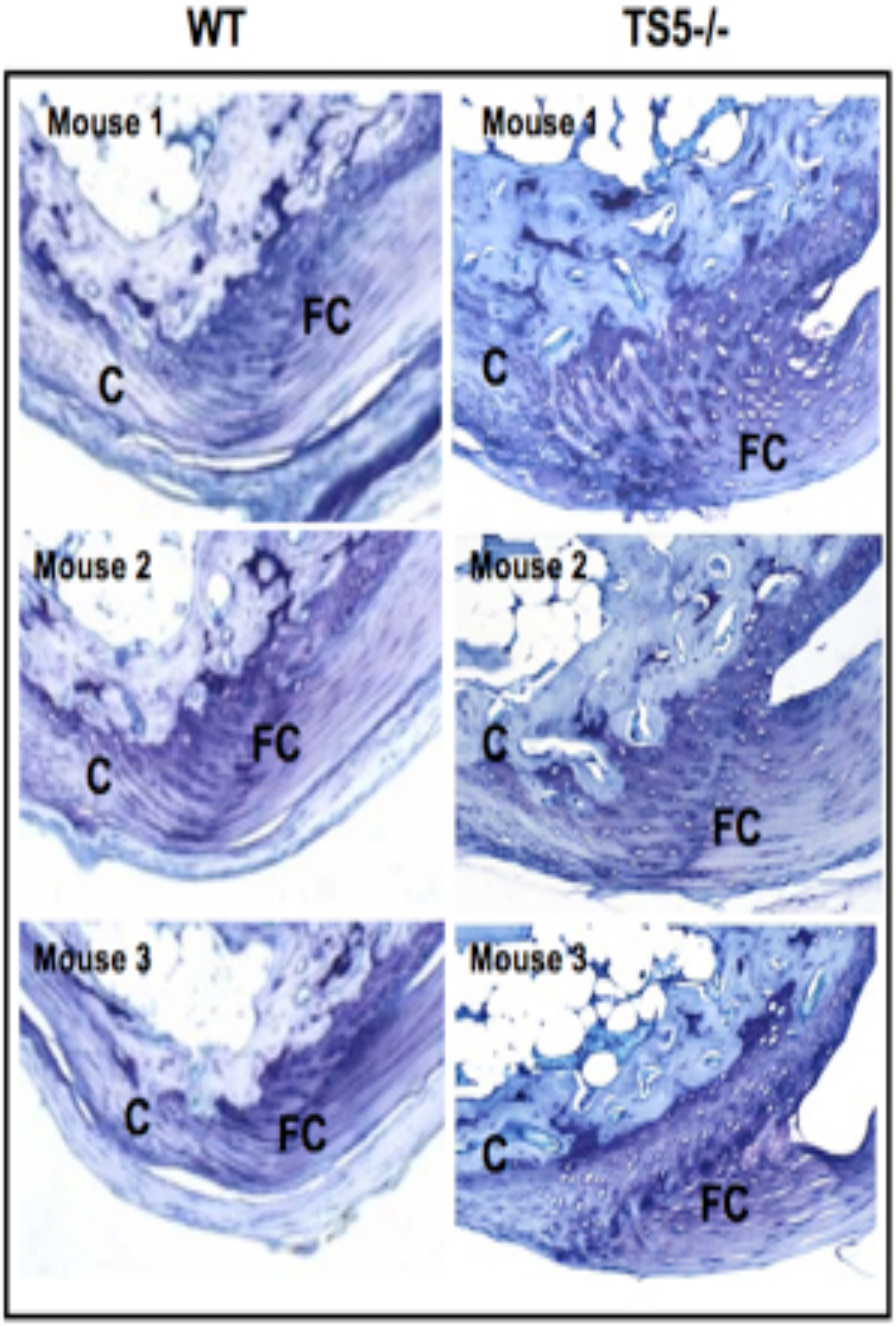

Figure 2.11- WT mice on left side, *ts5*$^{-/-}$ mice on right. Stained in Tol Blue. C-Cartilage, FC-Fibrocartilage. *ts5*$^{-/-}$ had a higher number of chondrocyte-like cells[54].

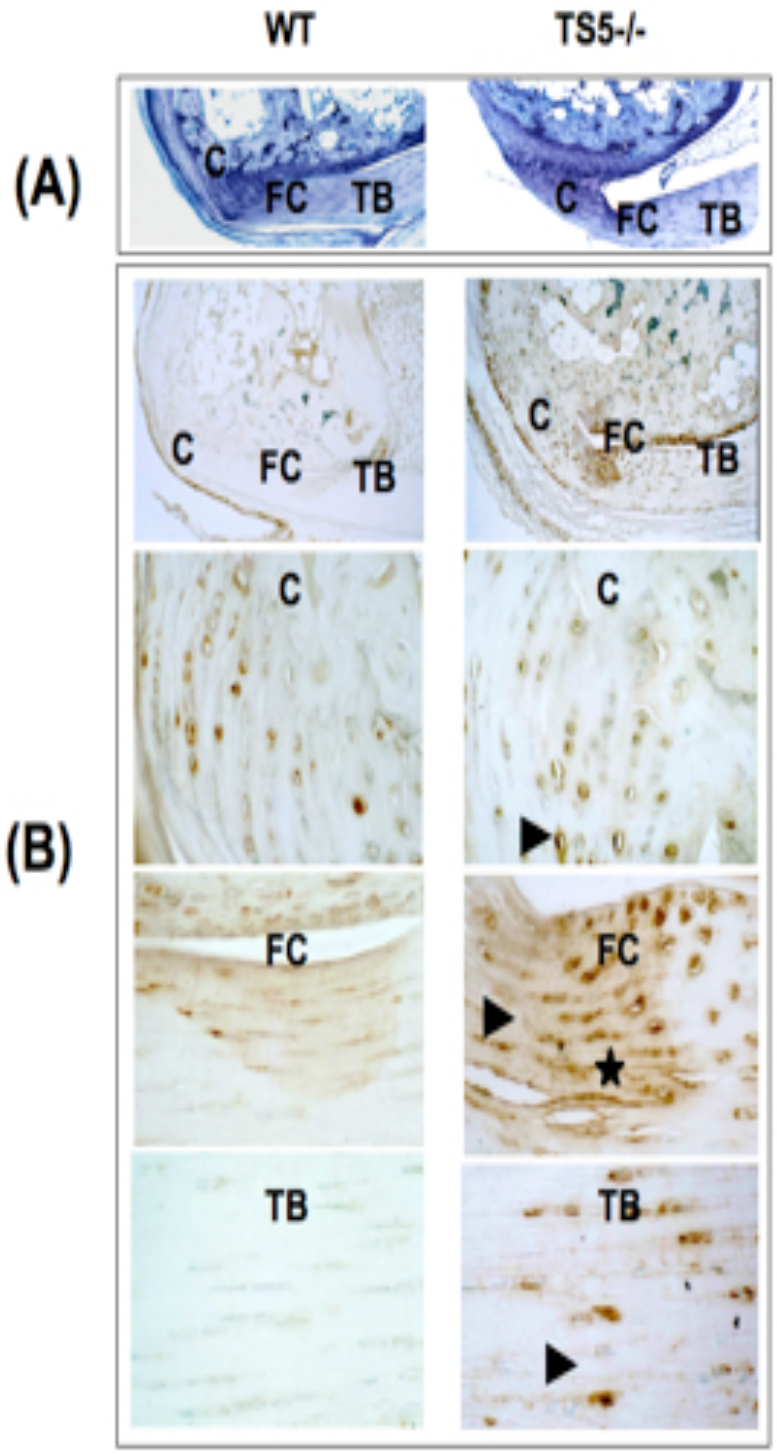

Figure 2.12-(A) Typical images of Toluidine blue stained Achilles tendons from WT and *ts5*$^{-/-}$ mice (10x). (B) 40x images of immunoreactive aggrecan (a-DLS) in Achilles tendons of WT and *ts5*$^{-/-}$ mice. Intense staining was found in pericellular regions (black arrowheads) and throughout collagen bundles (asterisk) primarily in *ts5*$^{-/-}$ tendons. (C) C, calcified fibrocartilage, FC, unmineralized fibrocartilage; T, tendon body[54].

Discussion

The earliest studies with *ts5*$^{-/-}$ mice reported that they are protected from cartilage erosion in experimental OA[55]and it was widely assumed that this was due to the absence of aggrecanase activity from the cartilage itself. However, recently we found[48]that the mechanism of cartilage protection is a blockade of meniscal, synovial, and periarticular fibrosis, along with deposition of aggrecan within the cartilage surface layers. Consistent with these findings, we show here that the same *ts5*$^{-/-}$ mice have tendons (FDL and Achilles) with altered biomechanical properties associated with changes in collagen fascicle (FDL) organization, along with excessive deposition of aggrecan around chondroid cell clusters within the tissue. In our ongoing, unpublished studies of male, 16-week old mice, cage control (untreated) mice exhibit very similar genotypic comparisons to those reported in the present study; hence, it is unlikely that the current results reflect a gender or age bias. It therefore appears that inhibition of TS5 can both promote chondrogenic cartilage repair, and also result in the chondroid deposits which are a hallmark of the tendinopathies. We have proposed [47-48] that both of these outcomes can be explained by a single cell response mechanism, in which deletion of TS5 results in the conversion of progenitors from a fibrogenic to a chondrogenic phenotype. In tendinopathies, the affected regions are typically characterized by focal hypercellularity, collagen disorganization with fibrotic lesions, granulation tissue, neovascularization, increased GAG contents and fibrocartilaginous and bony metaplasia.[4]

Our recently published studies[47,21]have suggested that the production, deposition and turnover of aggrecan by cells in all fibrous tissues, represents a critical process in the reparative response. Thus, aggrecan is produced at early stages of fibroblast

differentiation and is organized at cell surfaces by binding to hyaluronan which is itself immobilized by binding to the cell-surface receptor CD44. Most importantly, in order for fibroblastic differentiation, collagen deposition and wound contracture to occur, this pericellular aggrecan must first be eliminated via a TS5-dependent process.

In this regard it is interesting to note that knockout of CD44 results in enhanced healing of tendon defects in mice, [56] a result which may be primarily explained by elimination of the normal process of post-injury CD44- dependent aggrecan accumulation. Thus, as we have found in dermal studies, the elimination of aggrecan by knockout of CD44, reverses the poor repair seen in *ts5*$^{-/-}$ mice. [21]

The increase in tendon pericellular aggrecan seen in *ts5*$^{-/-}$ mice was shown in the current study to compromise the material (e.g., tensile modulus, an intrinsic mechanical property) but not structural properties (e.g., stiffness and maximum load) of the FDL tendon.

The reduction in tensile modulus and maximum stress was attributable to the 33% larger cross-sectional area of tendons from *ts5*$^{-/-}$ mice relative to wild type controls. In comparison to WT, *ts5*$^{-/-}$ mice exhibited significantly increased collagen fibril area density as well as greater heterogeneity of fibril diameters (Fig. 1). Histologic and biochemical results, however, did not reveal gross differences in collagen I content or organization. Variability of aggrecan and versican content was noted among mice within a genotype, possibly attributable to differences in the patterns, and perhaps magnitudes, of mechanical loading of their FDLs. Since a similar decorin content was noted for FDLs of *ts5*$^{-/-}$ and WT mice, there is presently no obvious explanation for the change in fibril geometry. It will therefore be important to determine whether the accumulation of

fibroblast pericellular aggrecan in the *ts5*$^{-/-}$ mice alters the turnover of other SLRPs (e.g., fibromodulin or biglycan) or minor collagens, which are involved in fibrillogenesis. [57]

In contrast to the FDL findings, a higher tensile modulus (computed using grip-to-grip strain) for *ts5*$^{-/-}$ Achilles tendons was observed relative to wild type, while maximum stress and cross-sectional area were similar. However, as seen in the FDL, the absence of TS5 did not alter the stress relaxation or structural properties of Achilles tendons. Importantly, among the eight *ts5*$^{-/-}$ Achilles tendons tested biomechanically, five ruptured within the insertion site. By contrast, the failure mode for all of the WT tendons was the tissue midsubstance. Further analysis revealed that the nominal biomechanical values were similar when comparing the *ts5*$^{-/-}$ specimens by failure mode (i.e., insertion vs. midsubstance, data not shown). Future studies assessing optical strain will examine whether regional surface strains differed between the more proximal and distal tissue; this, in turn, will enable direct comparison of tensile modulus results derived from grip-to-grip motion versus tissue-based strain measurements. A potential explanation for the predisposition for failure within the insertion site of Achilles tendons from *ts5*$^{-/-}$ mice is the abundance of aggrecan in the pericellular matrix of cells within the fibrocartilaginous interface between tendon and bone, as well as in the adjacent tendon body. Since the architecture and composition of the native insertion site is optimized to reduce stress concentrations, [58] we speculate that the accumulation of aggrecan disrupts the normal mechanism of load transmission from tendon to bone, thereby increasing the likelihood of failure.

In summary, we describe here a novel and apparently nodal role of TS5-mediated aggrecan turnover in tendon structure and function. Accumulation of aggrecan in the

pericellular matrix of fibroblasts can be expected to have several effects on both homeostatic and post-injury response of resident cells. For example, aggrecan is known to impair TGFb1, and likely TGFb2/TGFb3, signaling through the ALK5/SMAD3 pathway. [21,59] This is important, since it offers a reasonable explanation for why SMAD3$^{-/-}$ mice exhibit poor repair of the FDL. [60]Also, pericellular aggrecan might sterically hinder normal cell matrix binding through integrins, interactions which play a critical role in maintaining the differentiated state of the tenocyte and its appropriate response to mechanical stimuli. Finally, we propose that therapies targeted to stimulate the TS5-mediated aggrecanolytic pathway or decrease aggrecan production and its pericellular deposition around tenocytes, could minimize or even abolish overuse or injury-induced tendinopathies.

Chapter 3

Introduction

Chronic pain and functional impairment in tendons (such as the rotator cuff, patellar and Achilles) represents a major health care problem. It is now widely held that tendon overuse (often leading to traumatic rupture) is the initiating disease factor for the majority of patients in the vulnerable 30-50 year age group[1].

The major *pathognomonic* feature of tendinopathies is the deposition within the site of injury of a glycosaminoglycan-rich (proteoglycan-rich, mucoid, or chondroid) matrix materials, which we will refer to as aggrecan rich deposits. Samiric et al.[61] found fragmentation of large proteoglycans of aggrecan and versican in patellar tendinopathy. Corps et al.[62] found increase of aggrecan mRNA expression in painful tendinopathy. In 1991, Kannus and Jozsa[4] looked at 891 ruptured tendons and 445 control tendons, 20% and 9% respectively had mucoid degeneration. Recent reviews[38,63,64] concluded that tendinopathy in general is caused by a mucoid degeneration which results in collagen fibers that are thinner than normal. Our previous work[47] showed increase of aggrecan accumulation in diseased equine suspensory ligaments. While widely acknowledged that mucoid deposits are pathognomonic for tendinopathies, it has been unclear, until now, whether they are also *pathogenic.*

The objective of this aim was to develop a murine model of tendinopathy which result in accumulation of aggrecan rich deposits and impairs biomechanical properties.

Using our previous work[21] as a guideline (Figure 3.1) we hypothesized that using TGFβ we would be able to create a chondrogenic or pSmad 1/5/8 response in the tendon

which would result in aggrecan rich deposits thereby impairing and impeding the resulting remodeling response.

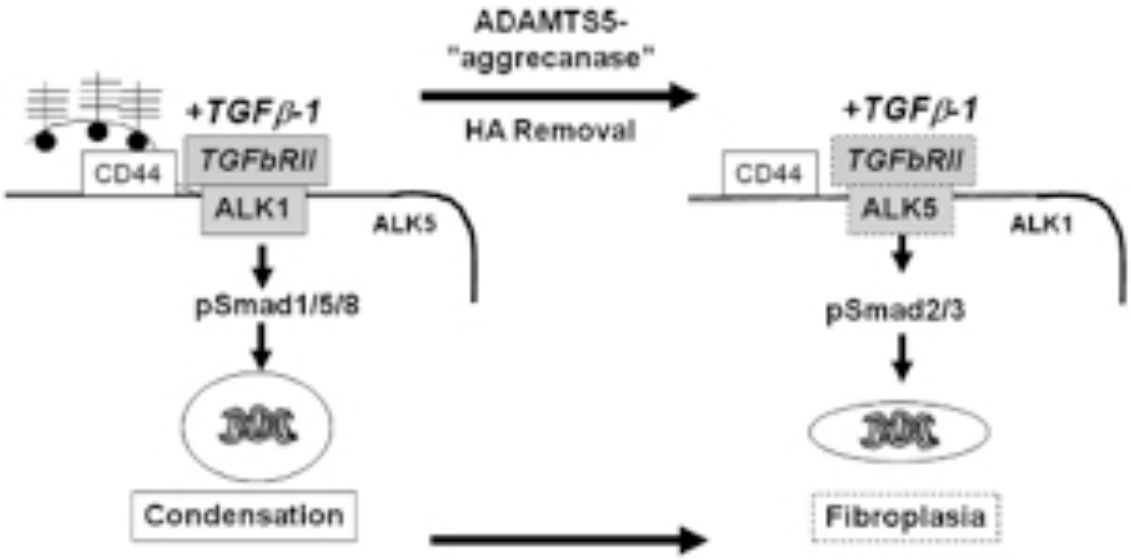

Figure 3.1- Model for regulation of TGFβ1 signaling[21].

Methods

Animal Studies

All in vivo studies were carried out under approved protocols by RUMC IACUC (#10-037). Mice were twelve-week old male C57Bl6 and were bred in-house. Each mouse was weighed prior to injection and weighed each week until sacrifice. The average weight for the mice at the time of injection was 29.0 ± 2.9 grams and there was no significant differences in weights between any of the assays.

Induction of Tendinopathy

Male mice at 12 weeks of age were anesthetized 100 ng hrTGFβ1 (Active Form, PrepoTech Inc) in 6 ul of sterile saline containing 0.1% ultrapure BSA (Sigma Aldrich) was injected into the mid-portion of the right Achilles tendon using a Kendall Monoject U-100 Insulin syringe (28G needle). The left tendon remained intact. Mice were then maintained at cage activity with free access to food and water and sacrificed at 48 hours, 12 days (2 weeks) or 26 days (4 weeks) post-injection for histological, biochemical and biomechanical properties of injected tendons, as described below.

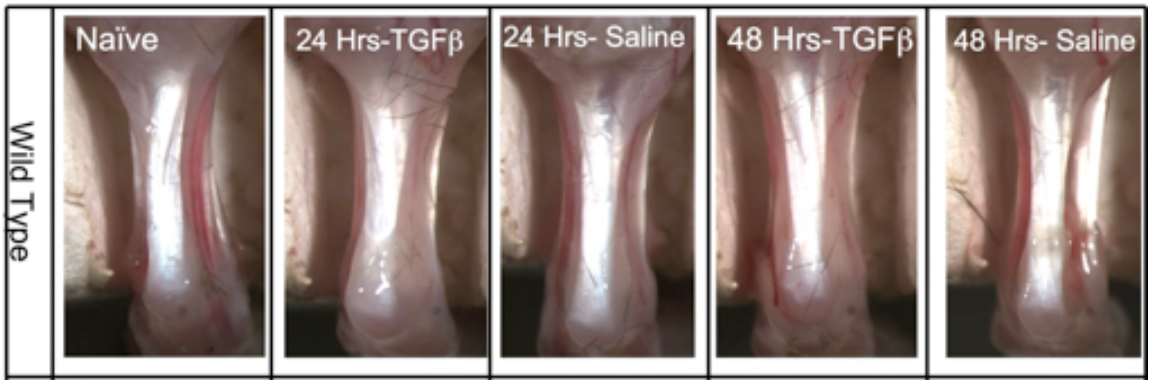

Figure 3.2- Macroscopic images at 24 hours and 48 hours post injection of TGFβ or saline alone.

Biomechanics

Mechanical testing was performed as described by Wang et al.[54] and in Chapter 2. Briefly, tendons were dissected and prior to loading the specimen into the MTS apparatus, five width and thickness measurements were taken along the tendon length with precision caliper and laser displacement sensor (Keyence model LK-G82), and the cross-sectional area was determined. Mechanical testing was conducted with the specimen in a 37°C saline bath. A 1-megapixel digital video camera for the first eight of the studies[65] and the remaining 10 tests with a 4-megapixel camera was used to determine

modulus of the failure test optically. A preload of 0.05N was applied to determine the initial length of the tendon, then preconditioning between 0.05 N and 0.55 N at a rate of 0.1 N/s for 20 cycles. After five minutes of recovery time in an unloaded state, a stress relaxation test was performed by straining the tissue to 5% grip-to-grip strain at a rate of 2.5%/s then held for 600 seconds. Specimens were then pulled to failure at a rate of 0.5%/s. One-way ANOVA for within genotype and condition comparing the effect of time was used. Significance was set to $p<0.05$.

Quantitative Real Time PCR Analysis

Tendons were removed above the calcaneus to exclude the fibrocartilagenous junction and below the muscle-tendon junction and stored at -20^{o}C in RNA later. At time of analysis, a total of 20 tendons were combined placed in liquid nitrogen, and pulverized in a Bessman Tissue pulverizer. The tissue samples were placed in 1 ml of Trizol and vortexed at high speed for 60 secs. Chloroform was added and the samples were again vortexed for 2 minutes. The samples were then centrifuged for 15 minutes. After centrifuge, the samples were separated into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The aqueous phase was removed and added to isopropyl alcohol. After incubating at room temperature for 5 minutes, the samples were once again centrifuged for 15 minutes. 75% ethanol was used to wash the samples, then samples were dried. RNAase free water was added to the samples then the RNA was purified using the RNeasy Mini Kit (Qiagen). Using a nanodroper, the concentration of the samples were measured. Yields of RNA were approximately 16 ng per tendon. cDNA synthesis was performed with SuperScript First-Strand Synthesis

System for RT-PCR (Invitrogen) with 1 ug of RNA. All following primers used were mouse-specific and purchased from Applied Biosystems, Inc.: *aggrecan* (Mm00545794_m1); *adamts5* (Mm01344180_m1); *gapdh* (Mm99999915_g1); *collagen1a* (Mm00801666_g1); *collagen2a* (Mm01309565); *collagen3a* (Mm00802331_m1); *MMP3* (Mm00440295_m1); *MMP13* (Mm00439491_m1); *fibronectin* (Mm01256744_m1); *α1* (Mm01306375_m1); *α2* (Mm00434371_m1); *α5* (Mm00439797_m1); *αV* (Mm00434506_m1); *β1* (Mm01253230_m1); *β3* (Mm00443980_m1); and *β5* (Mm00439825_m1). Amplification reactions were performed in at least triplicate using Applied Biosystems 7300 Real-Time PCR System (Life Technologies Corp., Carlsbad, CA) following cycling conditions: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds, 60°C for one minute and were repeated 39 times. The delta Ct data (relative to *gapdh*) for each gene at each time point in each genotype were determined. A fold change is calculated by the delta Ct of *gapdh* subtracted by the naïve's delta Ct of *gapdh*. Student's t-test was used to compare 48 hours, 2 weeks, and 4 weeks to naïve.

Histology and Immunohistochemistry

Mice were euthanized and the right and left legs below the knee were skinned. They were then photographed as seen in Figure 3.2. The leg was detached at the knee and placed in biopsy bags at an anatomical flexion (~45°) and fixed with 10% neutral buffered formalin for a minimum of 3 days then decalcified in 5% EDTA/PBS for 3 weeks. After processing, the specimens were paraffin embedded and twelve six-micron sections were taken across the entire joint from medial to lateral in the sagittal plane.

After removal of paraffin and dehydration in ethanol, sections 1 AND 12 were stained with Safranin O and the remaining sections were taken for immunohistochemistry, with at least two adjacent sections being stained with each antibody. Immunohistochemisty was performed as described previously[47]. Briefly, deparaffinized sections were incubated in primary antibodies overnight at 4 degrees at 10ug/ml. Commercial antibodies were from Novus USA (Littleton, CO; anti-integrin-α1), Bioss USA (Woburn, MA; anti-integrin-α2), Lifespan Bioscience (Seattle, WA; anti-integrin-α5), Abcam (Cambridge, MA; anti-integrin-αV, anti-integrin-β1, anti-integrin-β5). Aggrecan and ADAMTS5 were detected using anti-DLS and anti-KNG as described[47]. Sections were stained with anti-KNG. Following primary antibody exposure, sections were incubated with biotinylated goat anti-rabbit IgG and horseradish peroxidase labeled avidin-biotin complex and bound complexes were visualized with 3, 3'-diaminobenzidine substrate. Nuclei and cartilage matrix were counter-stained with methyl green.

Cells were counted in four Safranin o stained images at 20x at each time point and treatment using ImageJ (NIH) by two investigators blinded to the group

Results

In the tendinopathy model, the anterior region had similar features to the posterior region (Figure 3.4) with an increase of aggrecan. The aggrecan was seen to be intrafibrillar, especially at 2 weeks (Figure 3.4). The peritenon had an increase of cellularity at 2 weeks. At 4 weeks (Figure 3.4) the cells were still more rounded compare to naïve and located in between the fibers of the tendon. The cell count showed the naïve had 177 ± 1 cells, the 48 hour acute had 194 ± 13 cells, the 2 week group had 283 ± 28

cells, and the 4 week group had 444 ± 25 cells. Even though the n was too small to run comprehensive statistics, the differences in the 4-week group compared to the naïve was substantial.

The tendinopathy model had an increase ($p<0.01$) of cross-sectional area at 2 weeks post injection (0.89 ± 0.26 mm^2) compared to naïve (0.64 ± 0.20 mm^2), as seen in Table 3.1. Stress relaxation and maximum load had no significant difference ($p=0.18$ and $p=0.08$, respectively) across the time points. Stiffness was higher ($p=0.01$) in the naïve (8.56 ± 2.72 N/mm) compared to 48 hours (4.85 ± 2.04 N/mm), 2 weeks (4.87 ± 2.59 N/mm), and 4 weeks (4.97 ± 1.52 N/mm) post-injection. As seen in Table 3.1, maximum stress significantly decreased ($p=0.0001$) at 48 hours (4.15 ± 2.51 MPa), 2 weeks (3.12 ± 1.29 MPa) and 4 weeks (3.54 ± 1.06 MPa) compared to the naïve (9.05 ± 3.19 MPa).

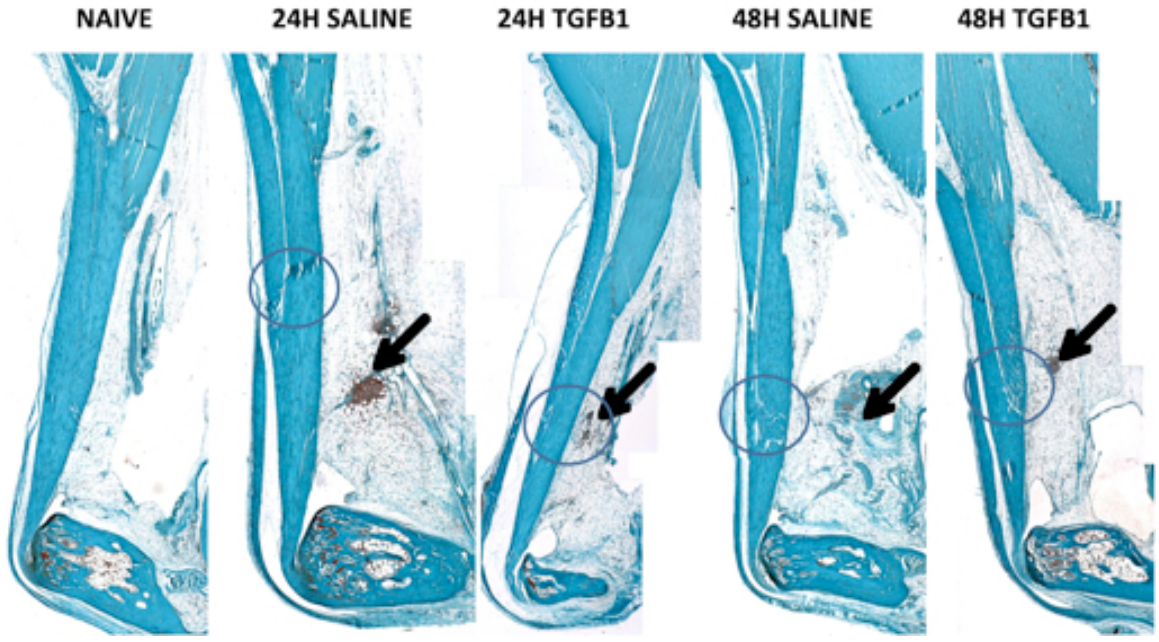

Figure 3.3- Full length of tendon at naïve, 24 hours post saline injection, 24 hours post TGFβ injection, 48 hours post saline injection and 48 hours post TGFβ injection. The circle indicates possible injection location and arrows are showing cellular response.

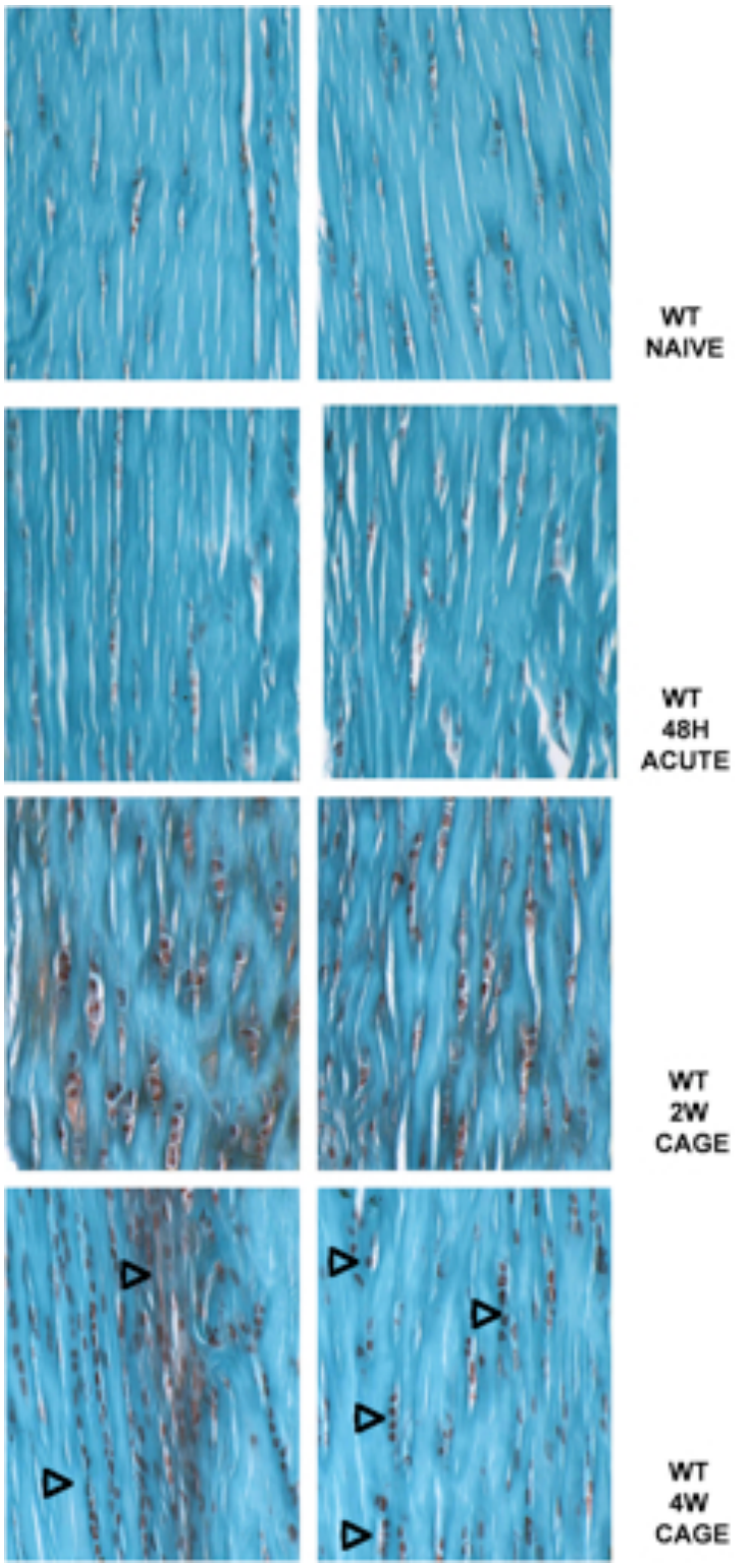

Figure 3.4- 40x of the midportion of the Achilles tendon. Anterior region on the left, posterior region on the right.

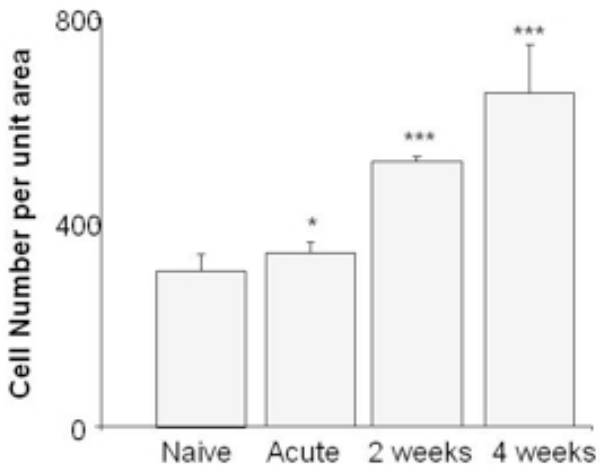

Figure 3.5- Cell number count of tendinopathy model. * is $p<0.05$, *** is $p<0.001$.

Table 3.1- Mechanical data of the tendinopathy model mean, with SD in parentheses?. Like symbols means $p<0.05$.

Property	Naïve	48 Hours Cage	2 Weeks Cage	4 Weeks Cage
CSA (mm^2)	0.64^{b} (0.20)	0.89 (0.26)	1.19^{b} (0.31)	0.94 (0.25)
Stress Relaxation (%)	45.7 (8.1)	51.1 (12.0)	53.1 (15.1)	59.1 (7.3)
Maximum Load (N)	5.50 (1.97)	3.43 (1.47)	3.64 (1.91)	3.40 (1.39)
Stiffness (N/mm)	$8.56^{a,b,c}$ (2.72)	4.85^{a} (2.04)	4.87^{b} (2.59)	4.97^{c} (1.52)
Maximum Stress (MPa)	$9.05^{a,b,c}$ (3.19)	4.15^{a} (2.51)	3.12^{b} (1.29)	3.54^{c} (1.06)

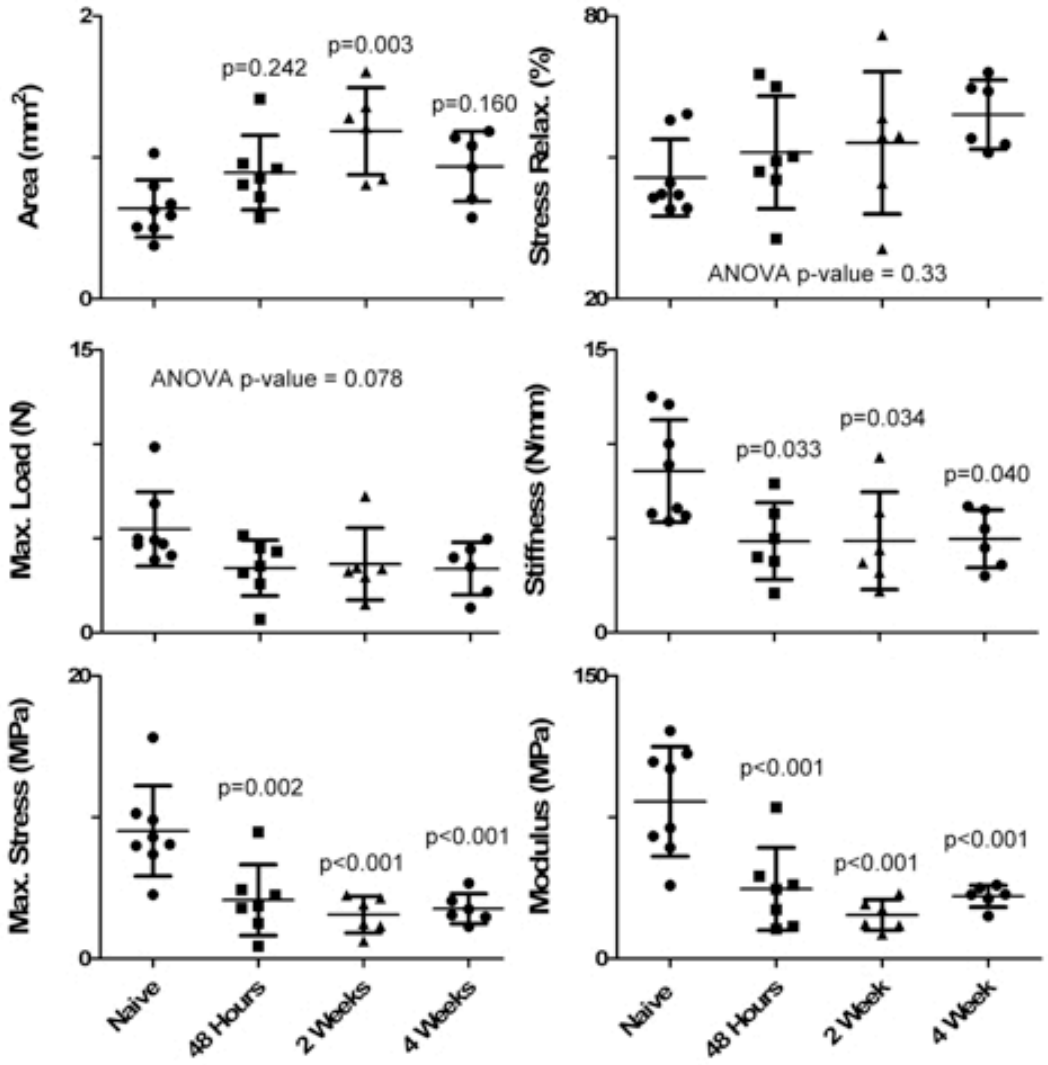

Figure 3.6- Mechanical properties for each time point. Area is the cross-sectional area and modulus is the tensile grip-to-grip modulus.

Gene expression data (Table 3.2) had significant changes at all time points. At 48 hours, there was substantial increase of expression for aggrecan, type 1 and type 3 collagens, fibronectin, and MMP3, however there was a decrease in ADAMTS5 expression. The increase in gene expression for aggrecan, type 1 collagen, fibronectin, and MMP 3 continued throughout all of the time points, where the type 3 collagen

returned to naïve levels at 4 weeks. ADAMTS5 was back to naïve levels at 2 weeks but was lower expressed at 4 weeks.

Table 3.2- Gene expression of tendinopathy model. ΔCT is the change from GAPDH and standard deviations in parentheses. Fold values are from Naïve Wild Type. Student's T-test was run comparing time point to naïve. Significance values: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, NS= no significance.

Wild Type	Naïve	48 Hour Cage		2 Weeks Cage		4 Weeks Cage	
Gene	**ΔCT**	**ΔCT**	**Fold**	**ΔCT**	**Fold**	**ΔCT**	**Fold**
Col 1	-0.14 (0.16)	-1.61* (0.38)	2.82	-5.13*** (0.11)	31.9	-3.52* (0.51)	10.7
Col 2	16.65 (0.39)	16.95 (0.64)	NS	12.28*** (0.15)	20.66	10.90*** (0.38)	54.79
Col 3	4.04 (0.07)	2.08* (0.19)	3.93	-0.32 ** (0.42)	21.2	3.99^{NS} (0.05)	1.04
Col 5	7.11 (0.13)	12.19*** (0.58)	0.03	10.25** (0.34)	0.12	13.66*** (0.21)	0.01
Fibronectin	1.38 (0.03)	0.35** (0.11)	2.04	-0.43 * (0.43)	3.60	0.36** (0.16)	2.04
Aggrecan	15.91 (0.61)	10.56** (0.08)	40.84	7.92*** (0.37)	258	9.78*** (0.45)	72.1
ADAMTS5	7.26 (0.07)	8.44* (0.14)	0.44	7.27 ^{NS} (0.16)	1.00	7.83** (0.03)	0.68
MMP3	11.35 (0.20)	6.58*** (0.14)	27.19	4.70 *** (0.31)	101.5	4.50*** (0.31)	117.3
MMP13	16.76 (0.77)	ND		2.50** (0.55)	20634	3.40** (0.24)	10634

In the tendinopathy model, the central integrin of β1 was down regulated at 48 hours and 4 weeks but it was at naïve level at 2 weeks. The TGD integrins had a varied response with β3 not changing in 48 hours but was more expressed at 2 weeks and 4 weeks. β5 had no significant changes throughout the four weeks. α5 had higher expression at 48 hours and 2 weeks but then was less expressed at 4 weeks compared to the naïve. αV was less expressed at 48 hours and 4 weeks but no changes at 2 weeks. The collagen integrins of α1 and α2 had extreme decrease levels of expression at all time points.

Table 3.3- Integrin expression for tendinopathy model. ΔCT is the change from GAPDH and standard deviations in parentheses. Fold values are from Naïve Wild Type. Student's T- test was run comparing time point to naïve. Significance values: * p<0.05, **p<0.01, ***p<0.001, NS= no significance.

	Wild Type Gene	Naïve	48 Hour Cage		2 Weeks Cage		4 Weeks Cage	
		ΔCT	ΔCT	Fold	ΔCT	Fold	ΔCT	Fold
Central	**β1**	1.69 (0.15)	3.25** (0.04)	0.34	1.36 (0.03)	NS	3.26* (0.42)	0.35
RGD	**β3**	11.17 (0.21)	10.90 (0.57)	NS	8.78*** (0.14)	5.27	10.65* (0.14)	1.44
	β5	11.47 (0.13)	12.73 (0.67)	NS	9.97 (0.63)	NS	9.26 (0.53)	NS
	α5	7.44 (0.14)	6.19** (0.06)	2.38	5.65*** (0.11)	3.48	9.18** (0.14)	0.30
	αV	5.28 (0.15)	6.62*** (0.19)	0.40	4.77 (0.37)	NS	7.92** (0.32)	0.16
Collagen	**α1**	8.42 (0.15)	8.75* (0.12)	0.80	10.09** (0.32)	0.32	9.88** (0.32)	0.37
	α2	9.48 (0.08)	12.33** (0.26)	0.14	9.99** (0.11)	0.70	11.55*** (0.06)	0.24

Discussion

By injecting TGFβ directly into the tendon, a novel tendinopathy murine model was created. This model had an increase amount of aggrecan and deposits in the intrafibrillar region similar to the human tissues from epicondylitis biopsies. The model also fit the criteria suggested by Lui et. al [25] for a successful tendinopathy animal model of having proteoglycan accumulation, changes in gene expression, defective long-term healing, increased cross-sectional area and decreased mechanical properties.

We hypothesized that using TGFβ we would be able to create a chondrogenic or pSmad 1/5/8 response in the tendon, which would need both signaling from mechanical

stimulation and ADAMTS5 in order to change pathways into a fibrogenic or pSmad 2/3 response. The initial response of a tendon wound is assumed to be like any wound repair setting. It will go through the initial inflammatory phase, a longer-term proliferative and matrix synthesis phase to an ongoing homeostatic control phase. TGFβ is most active in the inflammatory phase and helps regulates cell migration, proteinase expression, fibronectin binding interactions, termination of cell proliferation and stimulation of collagen production [8].

Our new model of tendinopathy presents an improvement on previously described chemical models in that it uses a single dosage injection of TGFβ1, which has been demonstrated to be the critical biological factor translating mechanical overuse injury of tendon cells into a biological response[66]. The other strengths of the model is the relatively quick induction of an injury (2 weeks). At 4 weeks the injury is still present proving this is a long term/chronic injury. Also the labor time is relatively short and the consistency is relatively high, especially at the 2 week timepoint.

Chapter 4

Introduction

Treatments of tendinopathy have varied from non-invasive of passive stretching to eccentric exercise to injecting drugs to invasive surgery, however there is not a clear consensus of which is the best mode of treatment[30]. Killian et al.[67] reviewed articles looking at the effect of overloading, controlled loading and complete unloading on tendon healing. They concluded the correct amount of controlled loading can be beneficial tendon, however it is tendon to tendon dependent and type of tendinopathy dependent. Both the overloading and the complete unloading were detrimental to the tendon. This shows the mechanical stimulation is key to proper tendon repair and matrix turnover.

Verrall et al.[68] used a modified eccentric heel-drop program in chronic Achilles tendinopathy. 86% of the patients had a satisfaction rating and the mean time to return to normal activity was ten weeks. Silbernagel et al.[69] followed out patients with Achilles tendinopathy for five years. They concluded majority recovered with exercise alone. They also concluded that 12 weeks and even 6 months is to early of time to determine if the patient would recover and would recommend following the treatment up to a year. They would recommend not using more invasive treatment until it is clear the patient would net recover fully with exercise alone.

Methods

Animal Studies

All in vivo studies were carried out under approved protocols by RUMC IACUC (#10-037). Mice were C57Bl6 and were bred in-house. If there were pups, they stayed in the cage until three weeks of age and then were weaned. The activity of the mice in the cage varied based on personality of the mouse, the amount of mice in cage, amount of pups in cage, and the age of the pups. Each mouse was weighed prior to injection and weighed each week until sacrifice. The average weight for the mice at the time of injection was 27.3 ± 2.0 grams and there was no significant differences in weights between any of the assays.

Induction of Tendinopathy

Male mice at 12 weeks of age were anesthetized 100 ng hrTGFβ1 (Active Form, PrepoTech Inc) in 6 ul of sterile saline containing 0.1% ultrapure BSA (Sigma Aldrich) was injected into the mid-portion of the right Achilles tendon using a Kendall Monoject U-100 Insulin syringe (28G needle). Mice were then maintained at cage activity with free access to food and water and sacrificed at 48 hours, 12 days (2 weeks) or 26 days (4 weeks) post-injection for histological, biochemical and biomechanical properties of injected tendons, as described below.

Mechanical Stimulation

The mice were subjected to uphill (17^{o}) running on a Stoelting/Panlab treadmill at 32 cm/s for 20 min/day for 5 days/week, starting one-day post injection. All of the mice

were able to run the first day. Nearly (95%) all of the mice were able to complete the running to designated sacrifice time.

Biomechanics

Mechanical testing was performed as described by Wang et al.[54], Chapter 2 and Chapter 3. Briefly, tendons were dissected and prior to loading the specimen into the MTS apparatus, five width and thickness measurements were taken along the tendon length with precision caliper and laser displacement sensor (Keyence model LK-G82), and the cross-sectional area was determined. Mechanical testing was conducted with the specimen in a 37^{o}C saline bath. A 1-megapixel digital video camera was used to determine modulus of the failure test optically. A preload of 0.05N was applied to determine the initial length of the tendon, then preconditioning between 0.05 N and 0.55 N at a rate of 0.1 N/s for 20 cycles. After five minutes of recovery time in an unloaded state, a stress relaxation test was performed by straining the tissue to 5% grip-to-grip strain at a rate of 2.5%/s then held for 600 seconds. Specimens were then pulled to failure at a rate of 0.5%/s. One-way ANOVA for within genotype and condition comparing the effect of time was used. Significance was set to $p<0.05$.

Quantitative Real Time PCR Analysis

Tendons were removed above the calcaneus to not include the fibrocartilagenous junction and below the muscle-tendon junction and stored at -20^{o}C in RNA later. At time of analysis, a total of 20 tendons were combined placed in liquid nitrogen, and pulverized

in a Bessman Tissue pulverizer. The tissue samples were placed in 1 ml of Trizol and vortexed at high speed for 60 secs. RNA was purified using the RNeasy Mini Kit (Qiagen) and yields of RNA were approximately 16 ng per tendon. cDNA synthesis was performed with SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) with 1 ug of RNA. All following primers used were mouse-specific and purchased from Applied Biosystems, Inc.: *aggrecan* (Mm00545794_m1); *adamts5* (Mm01344180_m1); *gapdh* (Mm99999915_g1); *collagen1a* (Mm00801666_g1); *collagen2a* (Mm01309565); *collagen3a* (Mm00802331_m1); *MMP3* (Mm00440295_m1); *MMP13* (Mm00439491_m1); *fibronectin* (Mm01256744_m1); *α1* (Mm01306375_m1); *α2* (Mm00434371_m1); *α5* (Mm00439797_m1); *αV* (Mm00434506_m1); *β1* (Mm01253230_m1); *β3* (Mm00443980_m1); and *β5* (Mm00439825_m1). Amplification reactions were performed in at least triplicate using Applied Biosystems 7300 Real-Time PCR System (Life Technologies Corp., Carlsbad, CA) following cycling conditions: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds, 60°C for one minute and were repeated 39 times. The delta Ct data (relative to *gapdh*) for each gene at each time point in each genotype were determined. A Student's t-test was also utilized to compare genotypes (WT vs $ts5^{-/-}$) within a time point and conditions (treadmill vs no treadmill).

Histology and Immunohistochemistry

Mice were euthanized and the right and left legs below the knee were skinned. The leg was detached at the knee and placed in biopsy bags at an anatomical flexion (~45°) and fixed with 10% neutral buffered formalin for a minimum of 3 days then decalcified in 5% EDTA/PBS for 3 weeks. After processing, the specimens were

paraffin embedded and twelve six-micron sections were taken across the entire joint from medial to lateral in the sagittal plane. After removal of paraffin and dehydration in ethanol, sections 1 AND 12 were stained with Safranin O and the remaining sections were taken for immunohistochemistry, with at least two adjacent sections being stained with each antibody. Immunohistochemisty was performed as described previously (PMID 21418601). Briefly, deparaffinized sections were incubated in primary antibodies overnight at 4 degrees at 10ug/ml. Commercial antibodies were from Novus USA (Littleton, CO; anti-integrin-α1), Bioss USA (Woburn, MA; anti-integrin-α2), Lifespan Bioscience (Seattle, WA; anti-integrin-α5), Abcam (Cambridge, MA; anti-integrin-αV, anti-integrin-β1, anti-integrin-β5). Aggrecan and ADAMTS5 were detected using anti-DLS and anti-KNG as described [47]. Sections stained with anti-KNG. Following primary antibody exposure, sections were incubated with biotinylated goat anti-rabbit IgG and horseradish peroxidase labeled avidin-biotin complex and bound complexes were visualized with 3, 3'-diaminobenzidine substrate. Nuclei and cartilage matrix were counter-stained with methyl green.

Cells were counted in four safranin o stained images at 20x at each time point and treatment using ImageJ (NIH) by two investigators blinded to the group.

Results

With the treatment of mechanical loading, the histology still had some clusters of aggrecan (Figure 4.1) but there was no adhesion of the tendon to the tissue above the calcaneus bone. At 40x (Figure 4.2), there were more cells at 2 weeks and 4 weeks compared to the naïve but the cell types were more back to naïve type at 4 weeks unlike

the tendinopathy model. The cell count showed the naïve had 177 ± 1 cells, the 2 week group had 253 ± 56 cells, and the 4 week group had 288 ± 48 cells. Again the n was small to make conclusive statements, the 2 week and 4 week group had more cells than the naïve group but not as extreme as the tendinopathy model.

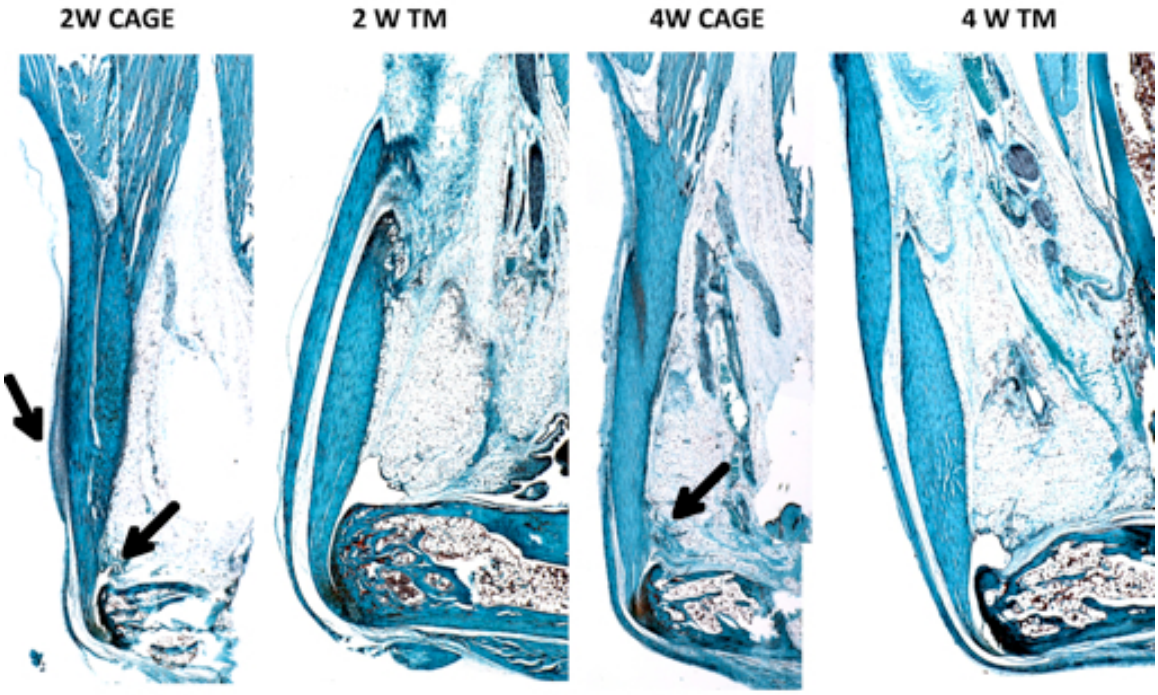

Figure 4.1- 4x of the full Achilles tendon. Arrows point to adhesion points and increased cellularity of the cage active mice compared to the treadmill mice.

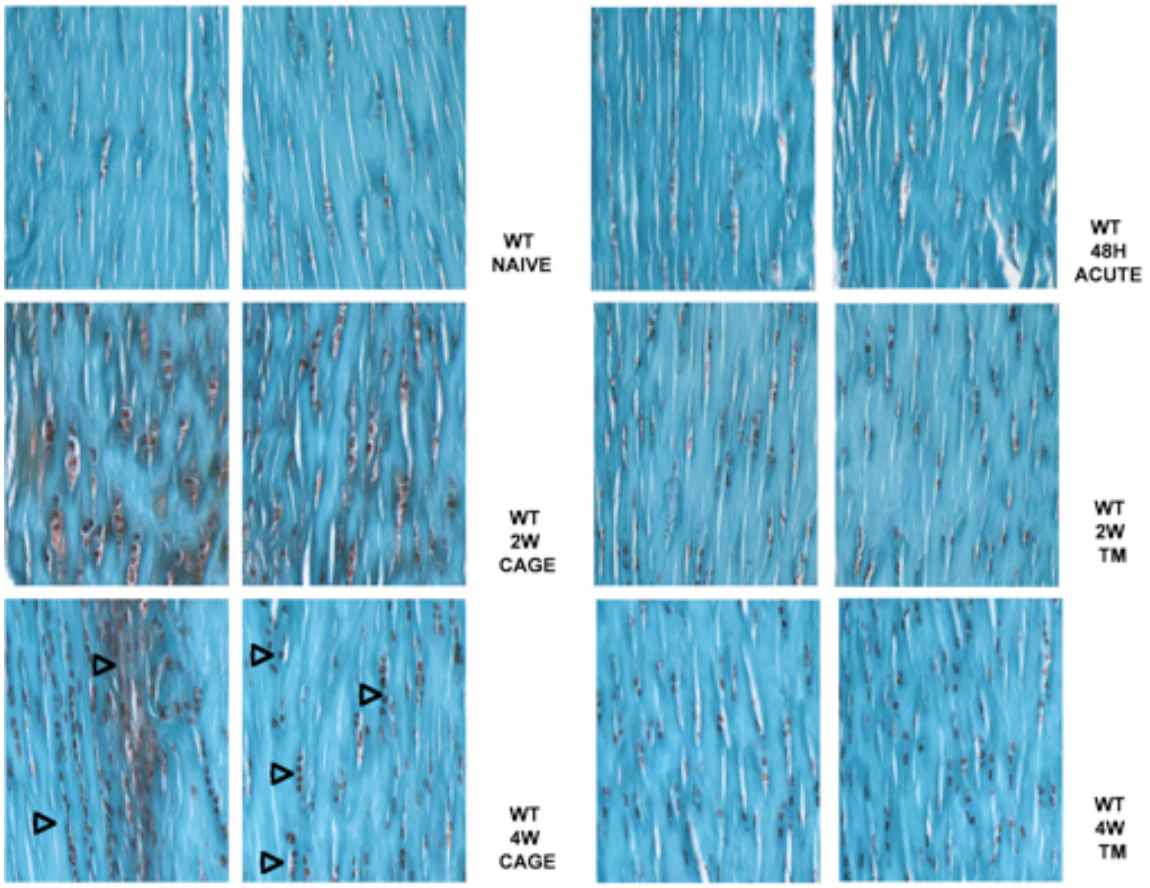

Figure 4.2- 40x of the midportion of the Achilles. Cage active mice on left, treadmill run mice on the right. Arrow heads pointing at chondrocyte like cells which is lacking in the treadmill run group.

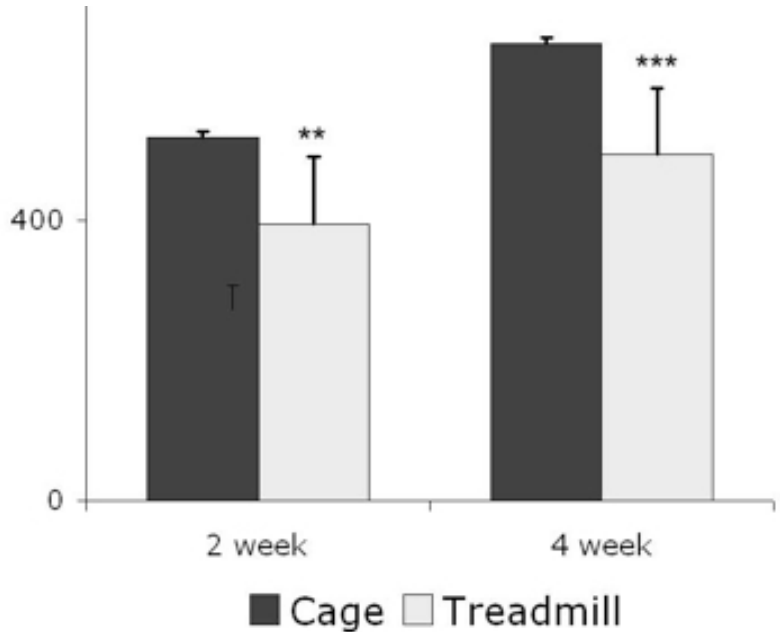

Figure 4.3- Cell counting of treadmill mice compared to the cage active mice. ** is $p<0.01$ and *** is $p<0.001$.

With the mechanical data (Table 4.1), the 2 weeks mice had significantly larger cross-sectional area and had lower stiffness than the naïve mice. Figure 4.3 shows a typical sample at naïve, 2 weeks and 4 weeks. The 2 weeks had lower stiffness, lower maximum load and higher extension but those are returned at 4 weeks. The 4 weeks specimens had all the properties returned to naïve levels. In addition to the return to naïve levels, both the naïve and 4 weeks had significantly higher and maximum load ($p=0.0024$) and maximum stress ($p=0.006$, seen in Figure 4.4) compared to the 2 weeks.

Table 4.1- Table of mechanical data from the wild type mice receiving mechanical stimulation. The same symbols indicated significance of $p<0.05$.

Property	Naïve	2 Weeks Treadmill	4 Weeks Treadmill
CSA (mm^2)	0.64 [a] (0.20)	0.95 [a] (0.21)	0.82 (0.14)
Stress Relaxation (%)	45.7 (8.1)	53.5 (8.1)	48.7 (10.0)
Maximum Load (N)	5.50 [a] (1.97)	2.51 [a,b] (1.31)	6.95 [b] (1.39)
Stiffness (N/mm)	8.56 [a] (2.72)	4.56 [a] (1.44)	8.18 (2.49)
Maximum Stress (MPa)	9.05 [a] (3.19)	2.53 [a,b] (0.77)	8.52 [b] (1.75)

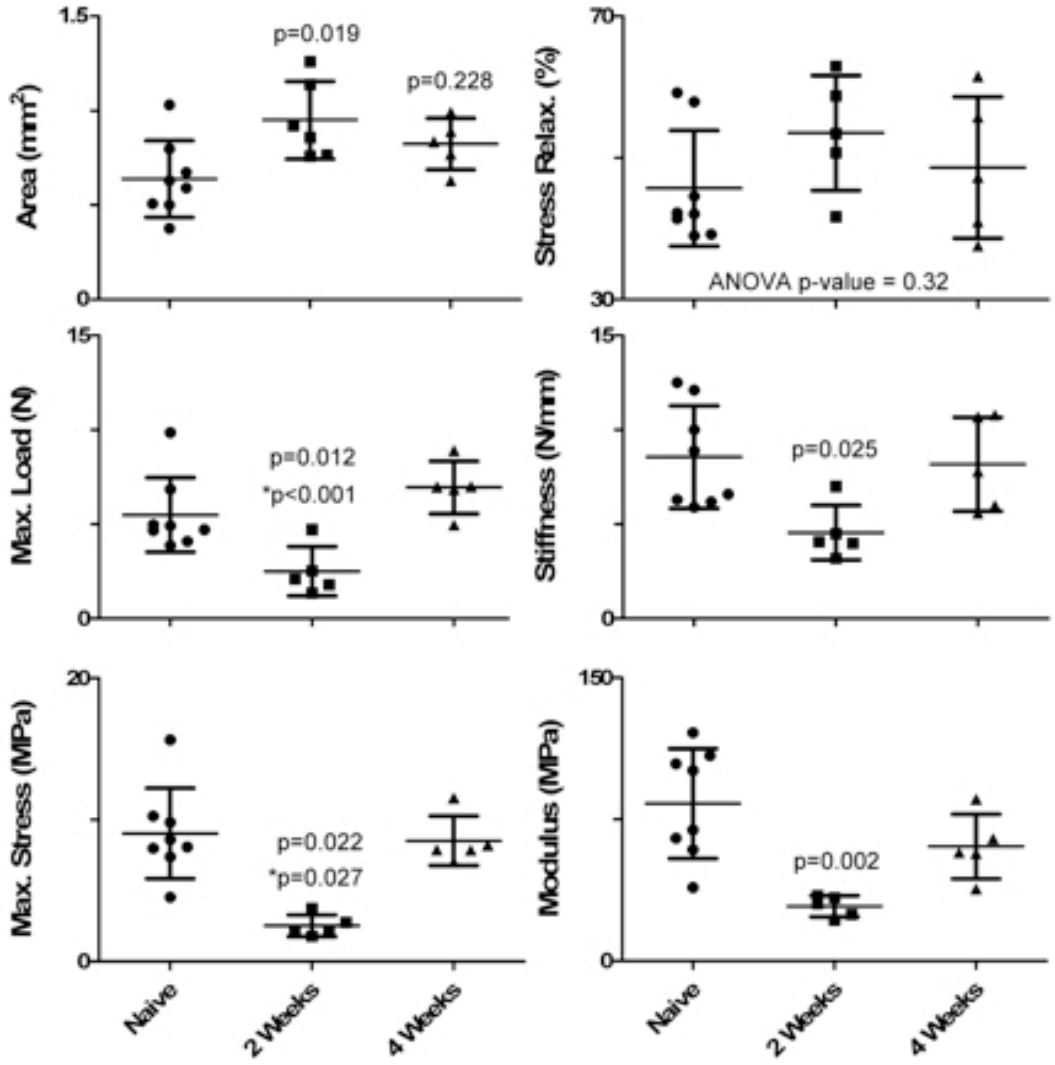

Figure 4.4- Mechanical properties at naïve, 2 weeks and 4 weeks. P-values indicate posthoc tests compared to naïve. *p-values indicated posthoc tests compared to the 4 week time point.

When comparing the ΔCT values (Table 4.2), Type 1 (Figure 4.5) and 3 (Figure 4.7) collagens had significantly lower expression at both 2 weeks and 4 weeks when comparing to the tendinopathy model at the respective time points. Type 2 collagen (Figure 4.6) had a 5 fold higher expression at 2 weeks then a reduced expression at 4 weeks compared to the tendinopathy model. Type 5 collagen (Figure 4.8) had higher

expression than the tendinopathy model at both time points but had the same expression as naïve at 2 weeks and lower expression than naïve at 4 weeks. On the other hand, fibronectin (Figure 4.9) had higher expression at both time points when the mice had mechanical stimulation. The aggrecan (Figure 4.10) significantly had lower (p=0.0005) expression at 4 weeks compared to the tendinopathy model. This was also consistent with the ADAMTS5 (Figure 4.11) and MMP3 (Figure 4.12) expression. MMP13 had the same high expression at 2 weeks but at 4 weeks the expression drops considerably compared to the tendinopathy model.

Table 4.2- Exercise intervention- Wild type Treadmill activity. ΔCT is the change from GAPDH and standard deviations in parentheses. Fold values are from Cage Wild Type at the same time point. Student's T- test was run comparing the same time point of cage activity to treadmill treated. Significance values: * p<0.05, **p<0.01, ***p<0.001, NS= no significance.

Wild Type	**2 Weeks Cage**	**2 Weeks Treadmill**		**4 Weeks Cage**	**4 Weeks Treadmill**	
Gene	**ΔCT**	**ΔCT**	**Fold**	**ΔCT**	**ΔCT**	**Fold**
Col 1	-5.13 (0.11)	-4.47** (0.10)	0.63	-3.52 (0.51)	0.11* (0.15)	0.08
Col 2	12.28 (0.15)	9.96** (0.02)	5.02	10.90 (0.38)	15.66*** (0.18)	0.04
Col 3	-0.32 (0.42)	0.94* (0.08)	0.42	3.99 (0.05)	8.96*** (0.04)	0.03
Col 5	10.25 (0.34)	6.12** (0.57)	18.39	13.66 (0.21)	12.71** (0.18)	1.94
Fibronectin	-0.43 (0.43)	-3.19** (0.23)	6.84	0.36 (0.16)	-1.04* (0.47)	2.71
Aggrecan	7.92 (0.37)	7.33 (0.29)	NS	9.78 (0.45)	13.44*** (0.42)	0.07
ADAMTS5	7.27 (0.16)	6.95 (0.24)	NS	7.83 (0.03)	9.96** (0.22)	0.24
MMP3	4.70 (0.31)	4.97 (0.02)	NS	4.50 (0.31)	8.85*** (0.14)	0.05
MMP13	2.50 (0.55)	1.63 (0.20)	NS	3.40 (0.12)	10.36*** (0.12)	0.01

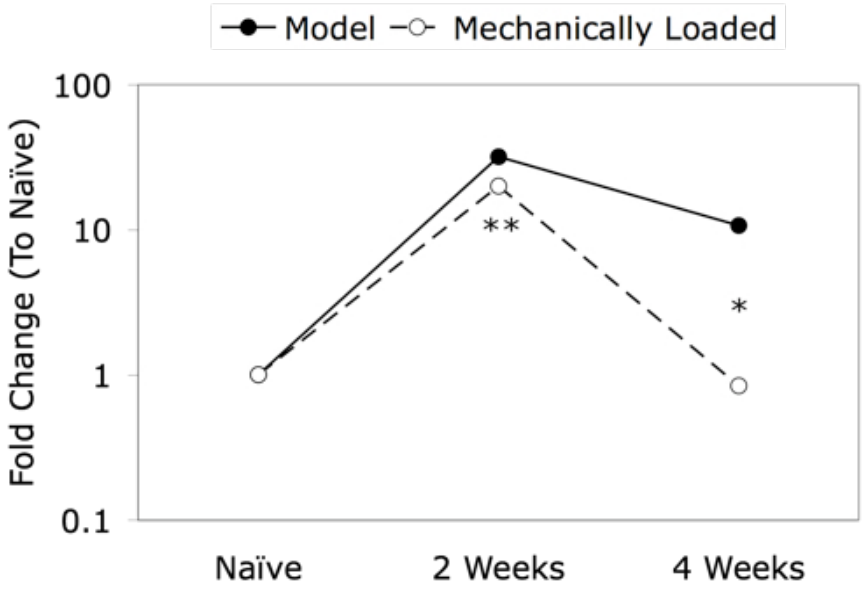

Figure 4.5- Type 1 collagen expression data. Fold values are from Naïve Wild Type using the ΔCT values. Significance values: * p<0.05, **p<0.01, ***p<0.001.

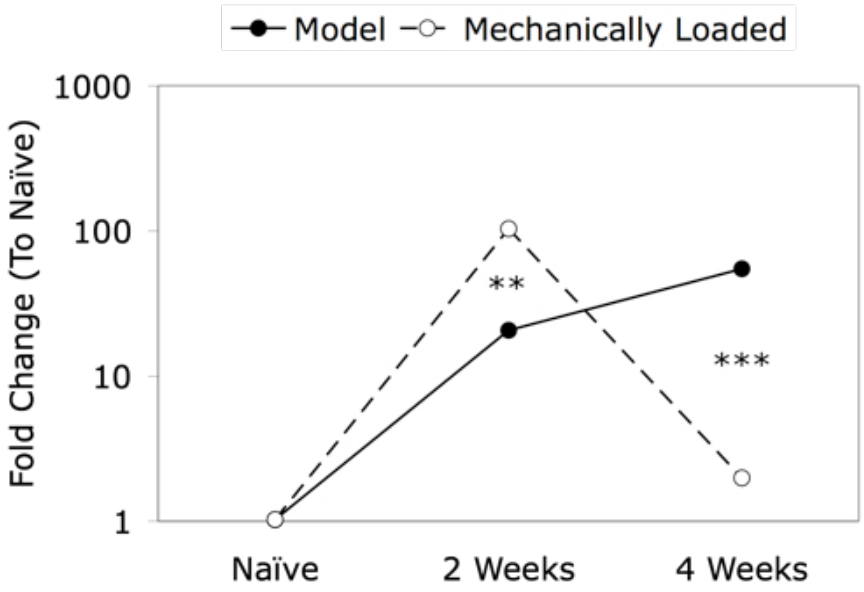

Figure 4.6- Type 2 collagen expression data. Fold values are from Naïve Wild Type using the ΔCT values. Significance values: * p<0.05, **p<0.01, ***p<0.001.

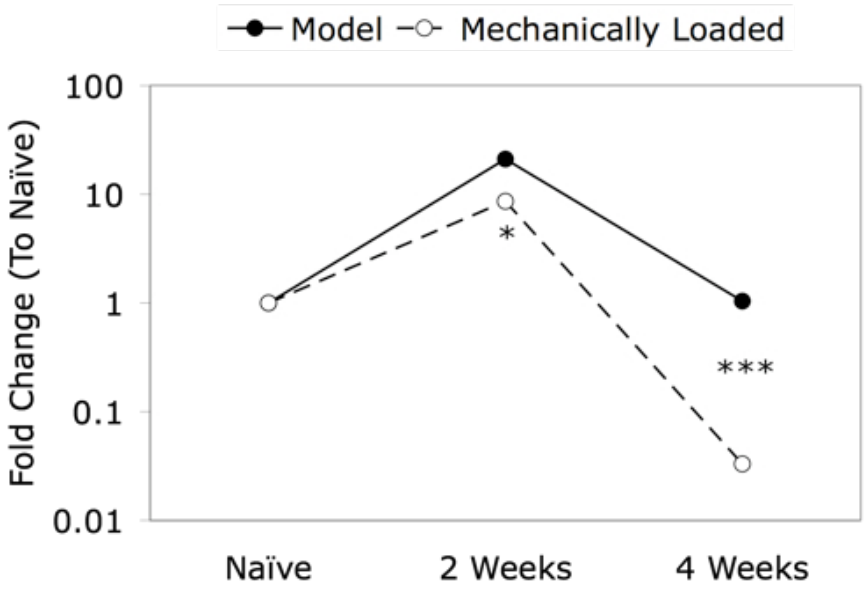

Figure 4.7- Type 3 collagen expression data. Fold values are from Naïve Wild Type using the ΔCT values. Significance values: * p<0.05, **p<0.01, ***p<0.001.

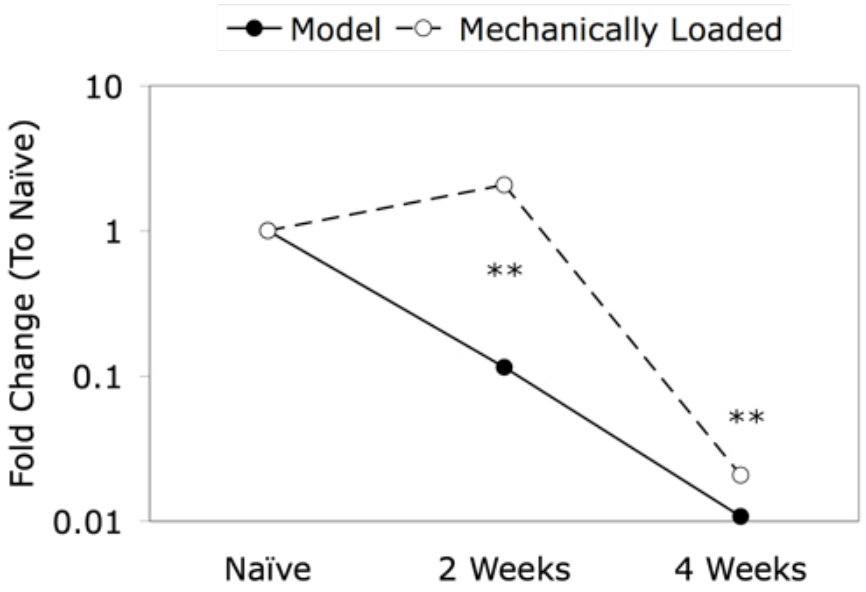

Figure 4.8- Type 5 collagen expression data. Fold values are from Naïve Wild Type using the ΔCT values. Significance values: * p<0.05, **p<0.01, ***p<0.001.

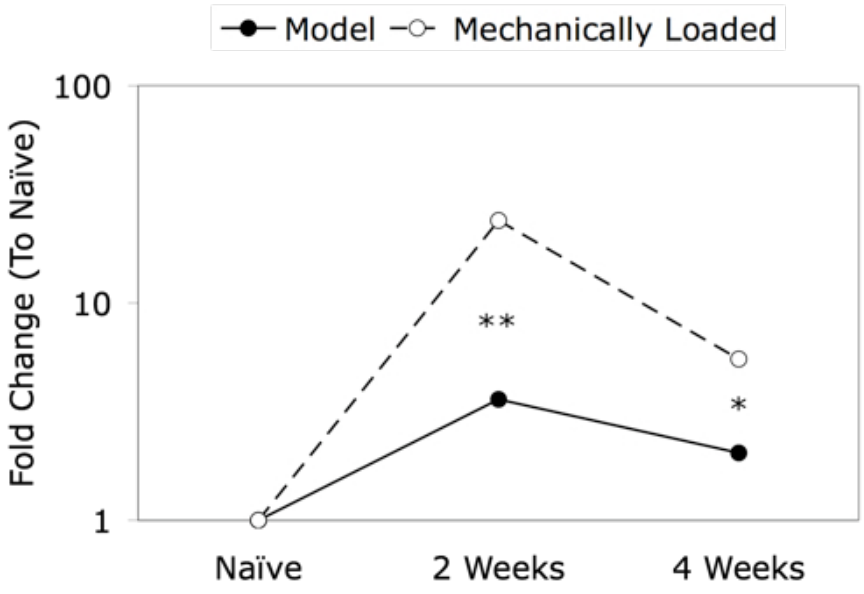

Figure 4.9- Fibronectin expression data. Fold values are from Naïve Wild Type using the ΔCT values. Significance values: * p<0.05, **p<0.01, ***p<0.001.

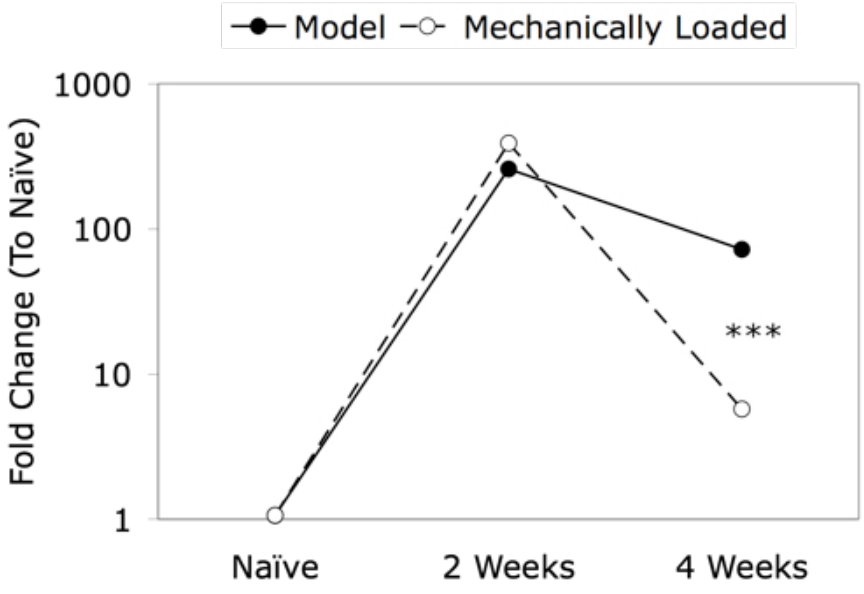

Figure 4.10- Aggrecan expression data. Fold values are from Naïve Wild Type using the ΔCT values. Significance values: * p<0.05, **p<0.01, ***p<0.001.

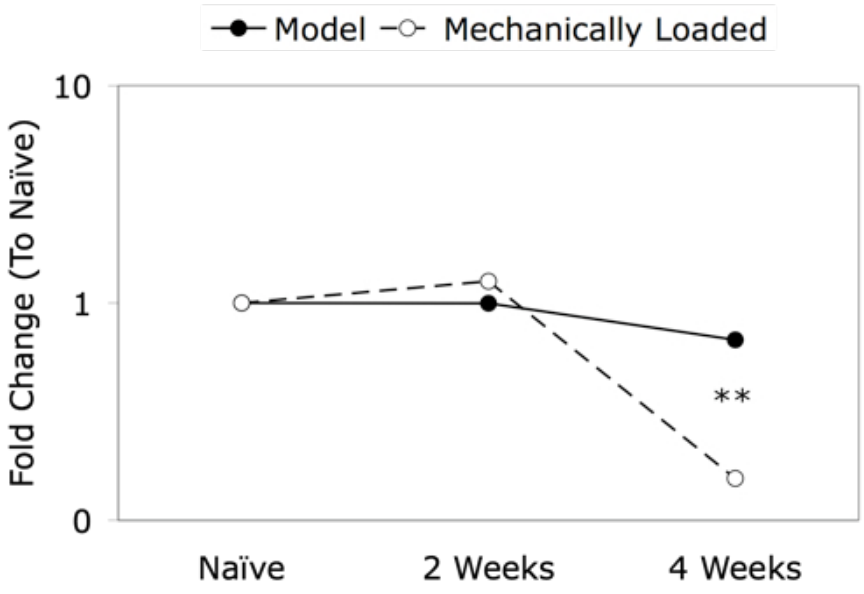

Figure 4.11- ADAMTS5 expression data. Fold values are from Naïve Wild Type using the ΔCT values. Significance values: * p<0.05, **p<0.01, ***p<0.001.

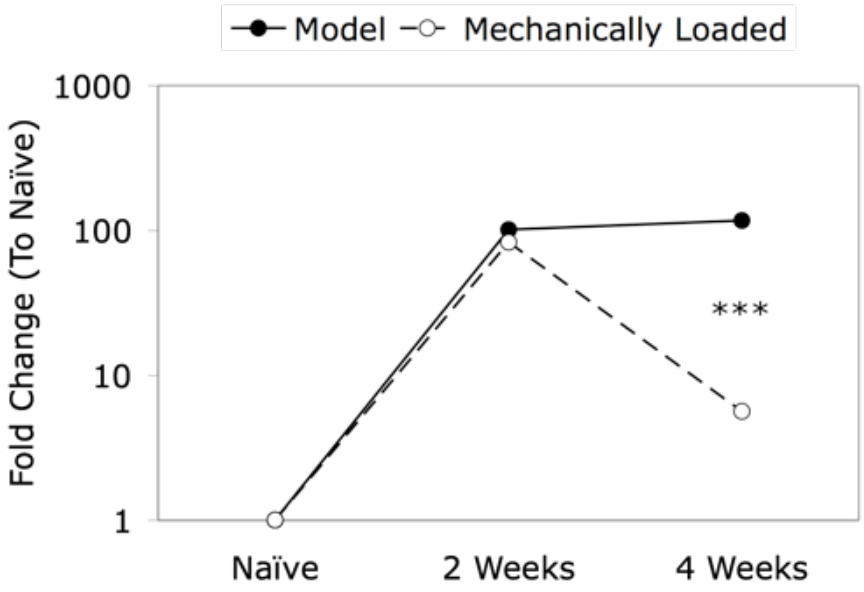

Figure 4.12- MMP3 expression data. Fold values are from Naïve Wild Type using the ΔCT values. Significance values: * p<0.05, **p<0.01, ***p<0.001.

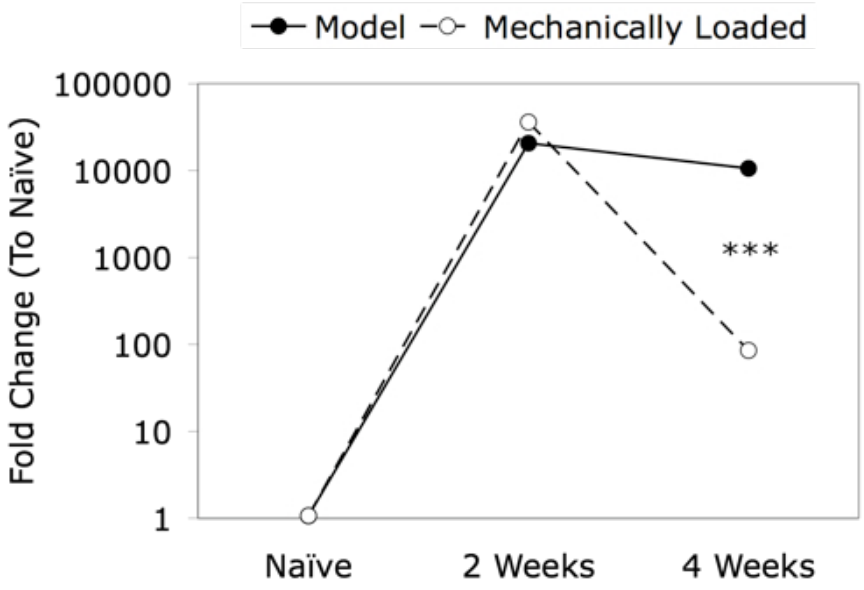

Figure 4.13- MMP13 expression data. Fold values are from Naïve Wild Type using the ΔCT values. Significance values: * p<0.05, **p<0.01, ***p<0.001.

When the wild type mice go through the treadmill treatment, the integrin expression stayed at a more naïve levels than the non-treated at 2 weeks and 4 weeks. There were no significant differences of the integrin expression at 2 weeks and 4 weeks for both β1 (Figure 4.14) and β5 (Figure 4.16). β3 (Figure 4.15) had a reduction of expression at 4 weeks compared to the model. α2 (Figure 4.20) and αV (Figure 4.18) had no changes at 2 weeks but at 4 weeks there was an increase of expression compared to the cage active mice. α5 (Figure 4.17) and α1 (Figure 4.19) had a slight decrease at 2 weeks than a slight increase at 4 weeks. This was especially the case in the collagen integrins as well as the RGD integrins of β3, α5 and αV.

Table 4.3- Exercise intervention-Wild type Treadmill activity. Significance values: * p<0.05, **p<0.01, ***p<0.001, NS= no significance.

	Wild Type Gene	2 Weeks Cage ΔCT	2 Weeks Treadmill ΔCT	2 Weeks Treadmill Fold	4 Weeks Cage ΔCT	4 Weeks Treadmill ΔCT	4 Weeks Treadmill Fold
Central	**β1**	1.36 (0.03)	1.19 (0.28)	NS	3.26 (0.42)	2.58 (0.08)	NS
RGD	**β3**	8.78 (0.14)	8.90 (0.17)	NS	10.65 (0.14)	12.06*** (0.17)	0.38
	β5	9.97 (0.63)	10.40 (0.56)	NS	9.26 (0.53)	9.52 (0.41)	NS
	α5	5.65 (0.11)	6.50* (0.13)	0.55	9.18 (0.14)	8.76* (0.06)	1.34
	αV	4.77 (0.37)	5.27 (0.26)	NS	7.92 (0.32)	6.34* (0.69)	3.24
Collagen	**α1**	10.09 (0.32)	8.75** (0.23)	2.55	9.88 (0.32)	8.87* (0.12)	2.01
	α2	9.99 (0.11)	9.68 (0.27)	NS	11.55 (0.06)	10.43* (0.24)	2.20

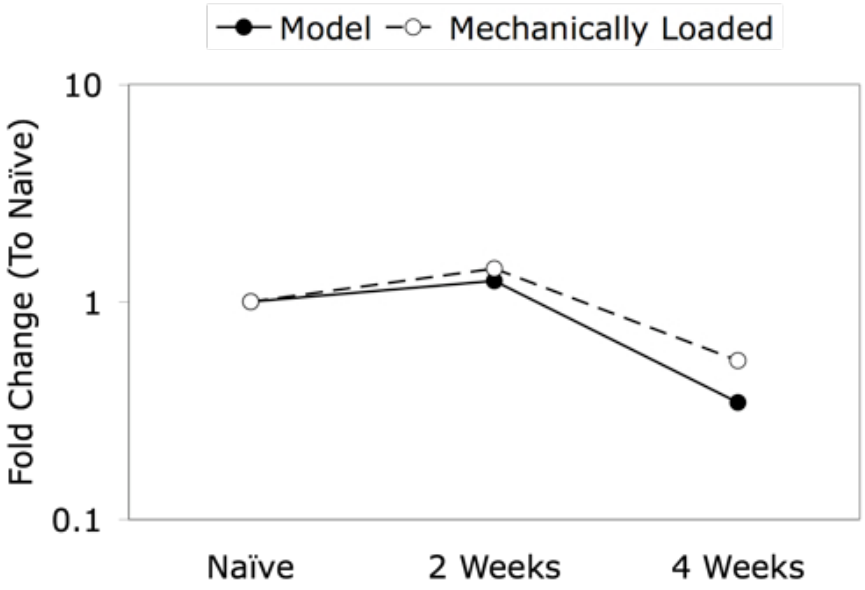

Figure 4.14- β1 expression data. Fold values are from Naïve Wild Type using the ΔCT values. Significance values: * p<0.05, **p<0.01, ***p<0.001.

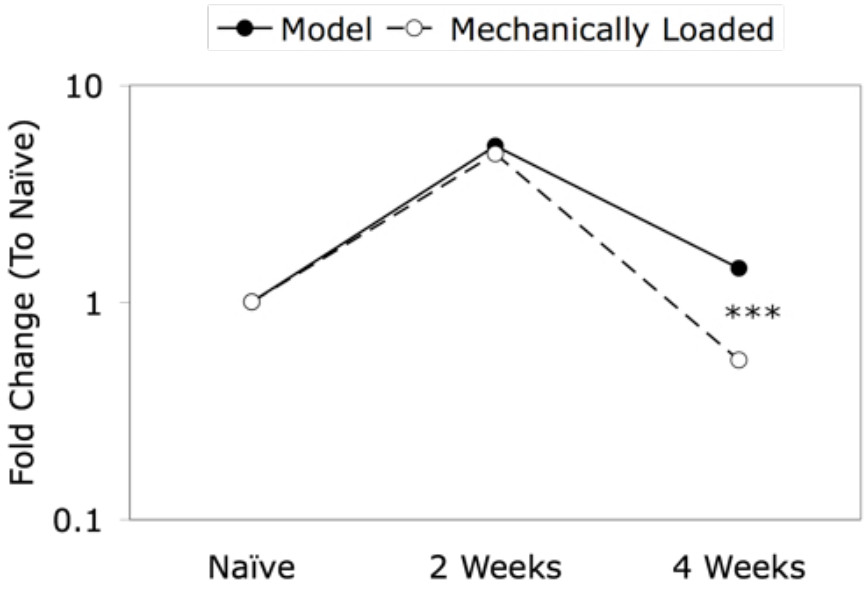

Figure 4.15- β3 expression data. Fold values are from Naïve Wild Type using the ΔCT values. Significance values: * p<0.05, **p<0.01, ***p<0.001.

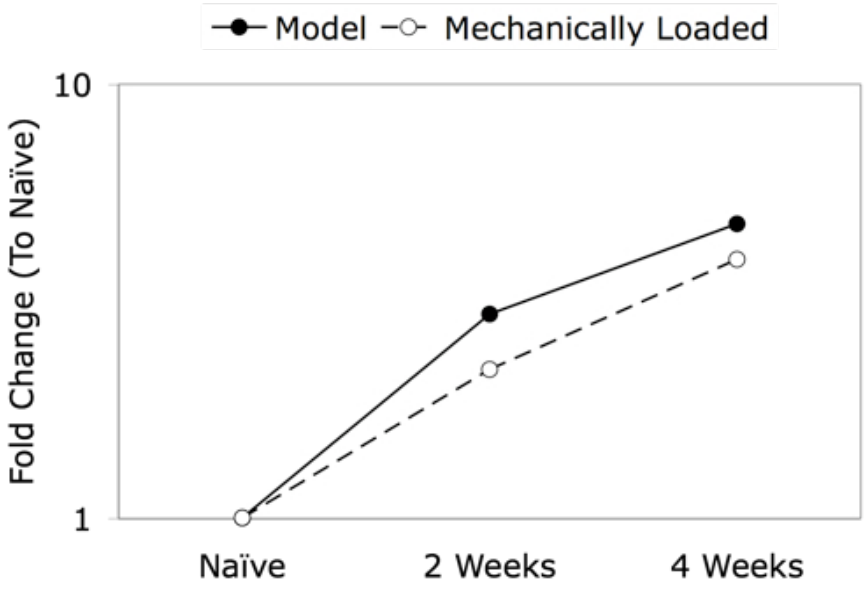

Figure 4.16- β5 expression data. Fold values are from Naïve Wild Type using the ΔCT values. Significance values: * p<0.05, **p<0.01, ***p<0.001.

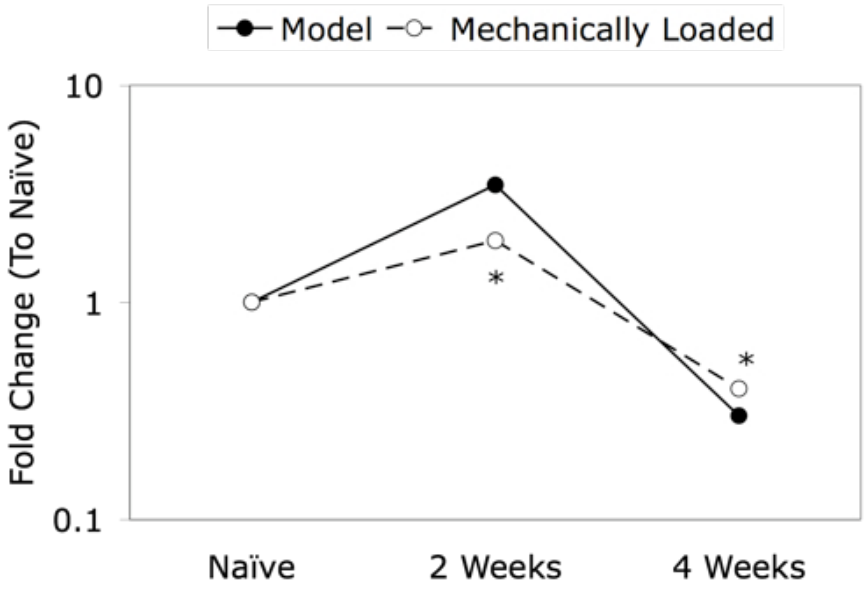

Figure 4.17- α5 expression data. Fold values are from Naïve Wild Type using the ΔCT values. Significance values: * p<0.05, **p<0.01, ***p<0.001.

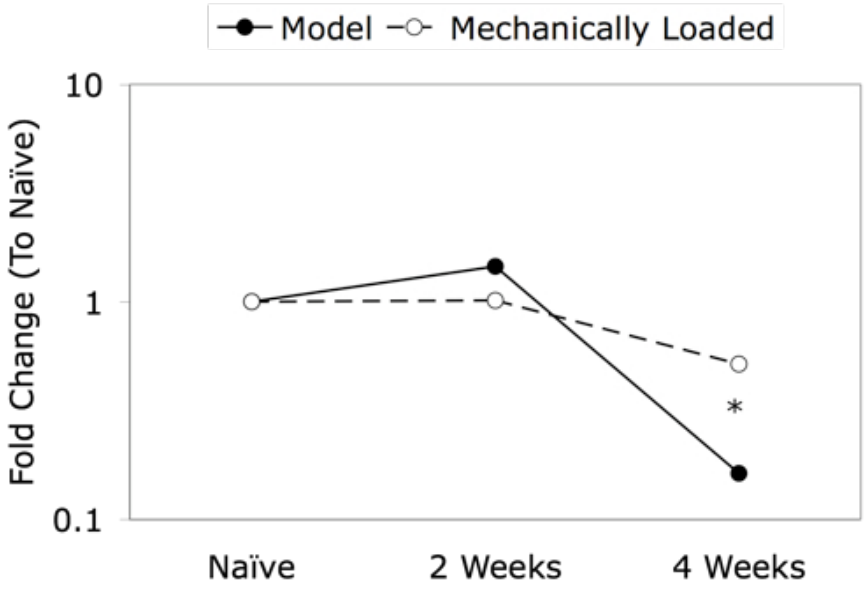

Figure 4.18- αV expression data. Fold values are from Naïve Wild Type using the ΔCT values. Significance values: * p<0.05, **p<0.01, ***p<0.001.

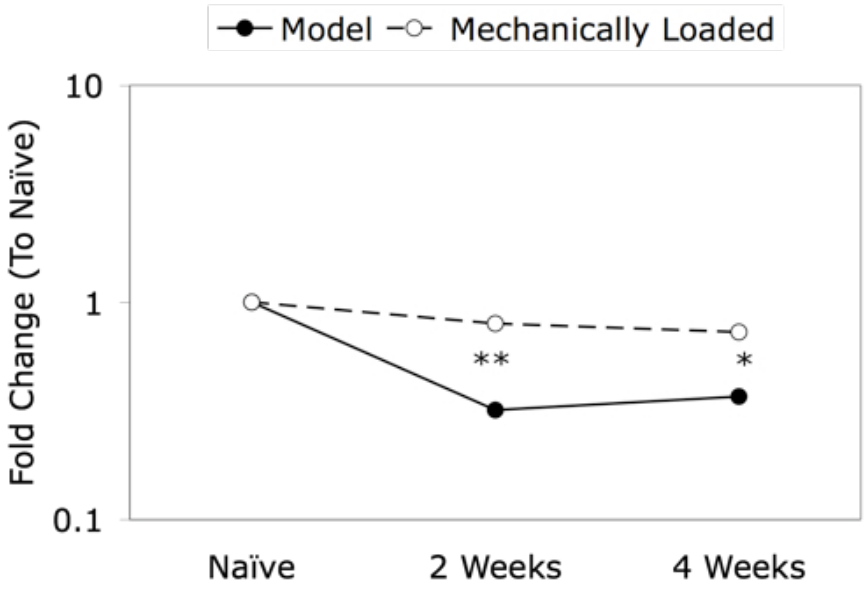

Figure 4.19- α1 expression data. Fold values are from Naïve Wild Type using the ΔCT values. Significance values: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

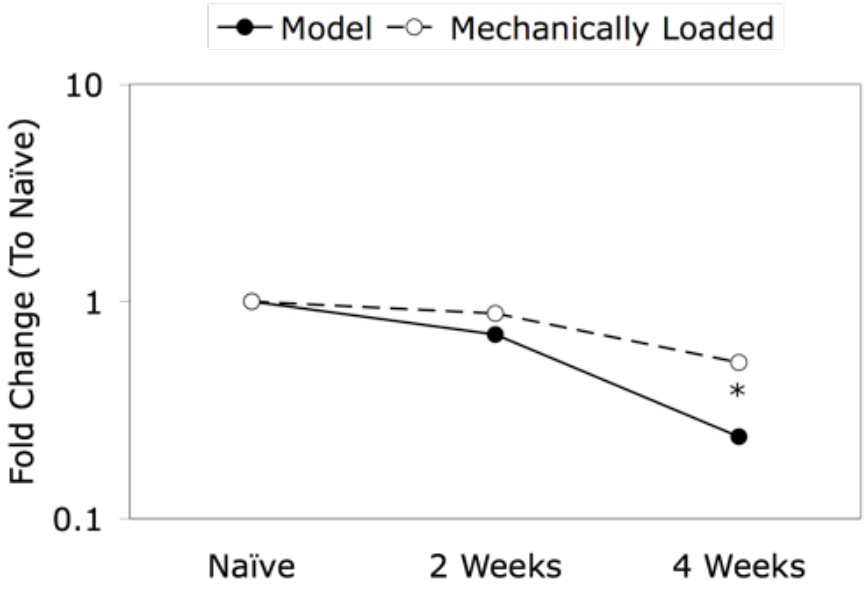

Figure 4.20- α2 expression data. Fold values are from Naïve Wild Type using the ΔCT values. Significance values: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Discussion

By adding mechanical stimulation to the tendinopathy model, the mice were able to recover from the initial injury of the injection. Mechanical stimulation was used to treat the tendinopathy and at two weeks the histology already showed improvement with fewer chondrocyte-like cells. At 4 weeks, the tendon appeared to be back to having naïve characteristics. This was consistent with the mechanical properties. At 2 weeks, the tendons were similar to the model tendons but by 4 weeks the tendons were able to recover back to their naïve properties. Other studies have shown similar beneficial results in humans with mechanical treatment[68,69].

Adhesions have been shown [70,71] to be created by TGFβ and mechanical stimulation can prevent adhesions. This is consistent with the histology of the wild type cage compared to the wild type with treadmill. It has also been shown the need for integrin activity to absorb TGFβ to prevent adhesions. α1 and α2 had the highest fold change in the integrins and those are need for collagen remodeling and myofibroblast differentiation during wound healing and fibrosis [23,71].

By using the treadmill to act as an eccentric exercise treatment, we showed that the tendinopathy model is a model that can be healed like the majority of patients who are healed with exercise alone.

Chapter 5

Introduction

Even with the high incidences of mucoid deposits in human tendinopathy, no animal model focused on this type of pathology. Other models have had a loss of material properties and had the pathology of an induction of chondrogenic genes and evidence of mucoid accumulations. For example, collagenase injection into horse[72] or rat [73,74,75] tendons produced an inflammatory response with tendon collagen bundle disarray, mucoid deposits and at the molecular level, expression of type II collagen and sox9 by fibroblast-like cells and expression of BMP-4/7 by chondrocytic cells, suggesting that aberrant differentiation of healing tendon fibroblasts may account for failed healing. Further, when a mixture of cytokines (IL-1 etc.) and growth factors (TGFß1, FGF2) was injected into rabbit tendons[37] both fibrosis and mucoid accumulation resulted.

ADAMTS5 is an aggrecanase that cleaves aggrecan in normal healthy tendons[76] and cartilage[48]. In knockout mice, it has been studied frequently in osteoarthritis models [48,55,77]. Using the knockout mice in a dermal wound healing model, Velasco et al.[21] proposed the importance of ADAMTS5 in the TGFβ pathway. It is needed to cleave aggrecan to change from the chondrogenic pathway of pSmad 1/5/8 to the fibrogenic pathway of pSmad 2/3. In Wang et al.[54], we saw the increase of aggrecan at the insertion site of the Achilles tendon of the *Adamts5*$^{-/-}$ mice caused the failure location to move from the tendon to the insertion site.

Methods

Animal Studies

All in vivo studies were carried out under approved protocols by RUMC IACUC (#10-037). Mice were of the C57Bl6 background and were bred in-house. The a*damts5*$^{-/-}$ line was established by deletion of exon 2, as previously described Plaas et al. and Wang et al.[54] Each mouse was weighed prior to injection and weighed each week until sacrifice. The average weight for the mice at the time of injection was 28.9 ± 2.7 grams and there was no significant differences in weights between any of the assays.

Induction of Tendinopathy

Male mice at 12 weeks of age were anesthetized 100 ng hrTGFβ1 (Active Form, PrepoTech Inc) in 6 ul of sterile saline containing 0.1% ultrapure BSA (Sigma Aldrich) was injected into the mid-portion of the right Achilles tendon using a Kendall Monoject U-100 Insulin syringe (28G needle). Mice were then maintained at cage activity with free access to food and water and sacrificed at 48 hours, 12 days (2 weeks) or 26 days (4 weeks) post-injection for histological, biochemical and biomechanical properties of injected tendons, as described below.

Mechanical Stimulation

The mice were subjected to uphill (17^{o}) running on a Stoelting/Panlab treadmill at 32 cm/s for 20 min/day for 5 days/week, starting one-day post injection. All of the mice were able to run the first day. By three weeks, the mice began to struggle to run consistently at the front of the treadmill. Most mice would run to the front of the

treadmill then stop running until it was at the back of the treadmill then it would jump/run to the front and repeat the cycle. Of the *ts5*$^{-/-}$ mice, 78% were able to complete the full 4 week running protocol.

Biomechanics

Mechanical testing was performed as described by Wang et al.[54], Chapter 2, Chapter 3, and Chapter 4. Briefly, tendons were dissected and prior to loading the specimen into the MTS apparatus, five width and thickness measurements were taken along the tendon length with precision caliper and laser displacement sensor (Keyence model LK-G82), and the cross-sectional area was determined. Mechanical testing was conducted with the specimen in a 37^{o}C saline bath. A 1-megapixel digital video camera was used to determine modulus of the failure test optically. A preload of 0.05N was applied to determine the initial length of the tendon, then preconditioning between 0.05 N and 0.55 N at a rate of 0.1 N/s for 20 cycles. After five minutes of recovery time in an unloaded state, a stress relaxation test was performed by straining the tissue to 5% grip-to-grip strain at a rate of 2.5%/s then held for 600 seconds. Specimens were then pulled to failure at a rate of 0.5%/s. One-way ANOVA for within genotype and condition comparing the effect of time was used. Significance was set to $p<0.05$.

Quantitative Real Time PCR Analysis

Tendons were removed above the calcaneus to not include the fibrocartilagenous junction and below the muscle-tendon junction and stored at -20^{o}C in RNA later. At time of analysis, a total of 20 tendons were combined placed in liquid nitrogen, and pulverized

in a Bessman Tissue pulverizer. The tissue samples were placed in 1 ml of Trizol and vortexed at high speed for 60 secs. RNA was purified using the RNeasy Mini Kit (Qiagen) and yields of RNA were approximately 16 ng per tendon. cDNA synthesis was performed with SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) with 1 ug of RNA. All following primers used were mouse-specific and purchased from Applied Biosystems, Inc.: *aggrecan* (Mm00545794_m1); *adamts5* (Mm01344180_m1); *gapdh* (Mm99999915_g1); *collagen1a* (Mm00801666_g1); *collagen2a* (Mm01309565)*; collagen3a* (Mm00802331_m1); *MMP3* (Mm00440295_m1); *MMP13* (Mm00439491_m1)*; fibronectin* (Mm01256744_m1); *α1* (Mm01306375_m1); *α2* (Mm00434371_m1); *α5* (Mm00439797_m1); *αV* (Mm00434506_m1); *β1* (Mm01253230_m1); *β3* (Mm00443980_m1); and *β5* (Mm00439825_m1). Amplification reactions were performed in at least triplicate using Applied Biosystems 7300 Real-Time PCR System (Life Technologies Corp., Carlsbad, CA) following cycling conditions: 50^{o}C for 2 minutes, 95^{o}C for 10 minutes, 95^{o}C for 15 seconds, 60^{o}C for one minute and were repeated 39 times. The delta Ct data (relative to *gapdh*) for each gene at each time point in each genotype were determined. A Student's t-test was also utilized to compare genotypes (WT vs *ts5*$^{-/-}$) within a time point and conditions (treadmill vs no treadmill).

Histology and Immunohistochemistry

Mice were euthanized and the right and left legs below the knee were skinned. The leg was detached at the knee and placed in biopsy bags at an anatomical flexion (~45^{o}) and fixed with 10% neutral buffered formalin for a minimum of 3 days then

decalcified in 5% EDTA/PBS for 3 weeks. After processing, the specimens were paraffin embedded and twelve six-micron sections were taken across the entire joint from medial to lateral in the sagittal plane. After removal of paraffin and dehydration in ethanol, sections 1 AND 12 were stained with Safranin O and the remaining sections were taken for immunohistochemistry, with at least two adjacent sections being stained with each antibody. Immunohistochemisty was performed as described previously (PMID 21418601). Briefly, deparaffinized sections were incubated in primary antibodies overnight at 4 degrees at 10ug/ml. Commercial antibodies were from Novus USA (Littleton, CO; anti-integrin-α1), Bioss USA (Woburn, MA; anti-integrin-α2), Lifespan Bioscience (Seattle, WA; anti-integrin-α5), Abcam (Cambridge, MA; anti-integrin-αV, anti-integrin-β1, anti-integrin-β5). Aggrecan and ADAMTS5 were detected using anti-DLS and anti-KNG as described [47]. Sections stained with anti-KNG. Following primary antibody exposure, sections were incubated with biotinylated goat anti-rabbit IgG and horseradish peroxidase labeled avidin-biotin complex and bound complexes were visualized with 3, 3'-diaminobenzidine substrate. Nuclei and cartilage matrix were counter-stained with methyl green.

Cells were counted in four safranin o stained images at 20x at each time point and treatment using ImageJ (NIH) by two investigators blinded to the group.

Results

The histology at 2 weeks had increased aggrecan staining throughout the length of the tendon (Figure 5.1). The whole tendon showed the injection site as well as the cell infiltration from the blood vessel. Figure 5.2 shows more adhesions compared to the wild

type mice with treadmill. In Figure 5.3, the cells were round and intrafibrillar. At 4 weeks, there were still regions of aggrecan rich deposits and increased cellularity compared to the treated wild type mice. The cell count showed the naïve had 160 ± 16 cells, the 48 hour acute group had 196 ± 48 cells, the 2 week group had 378 ± 27 cells, and the 4 week group had 369 ± 4 cells. Again the n was small to make conclusive statements, the 2 week and 4 week group had more cells than the naïve group and the 48 hour group.

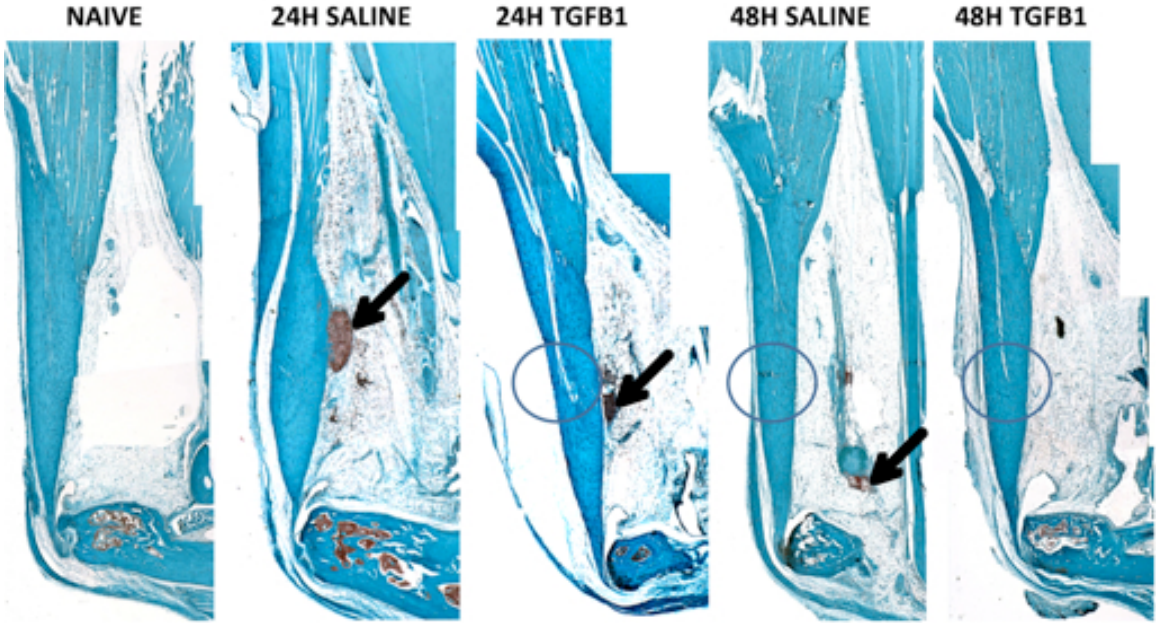

Figure 5.1- Full length of tendon at naïve, 24 hours post saline injection, 24 hours post TGFβ injection, 48 hours post saline injection and 48 hours post TGFβ injection. The circle indicates possible injection location and arrows are showing cellular response.

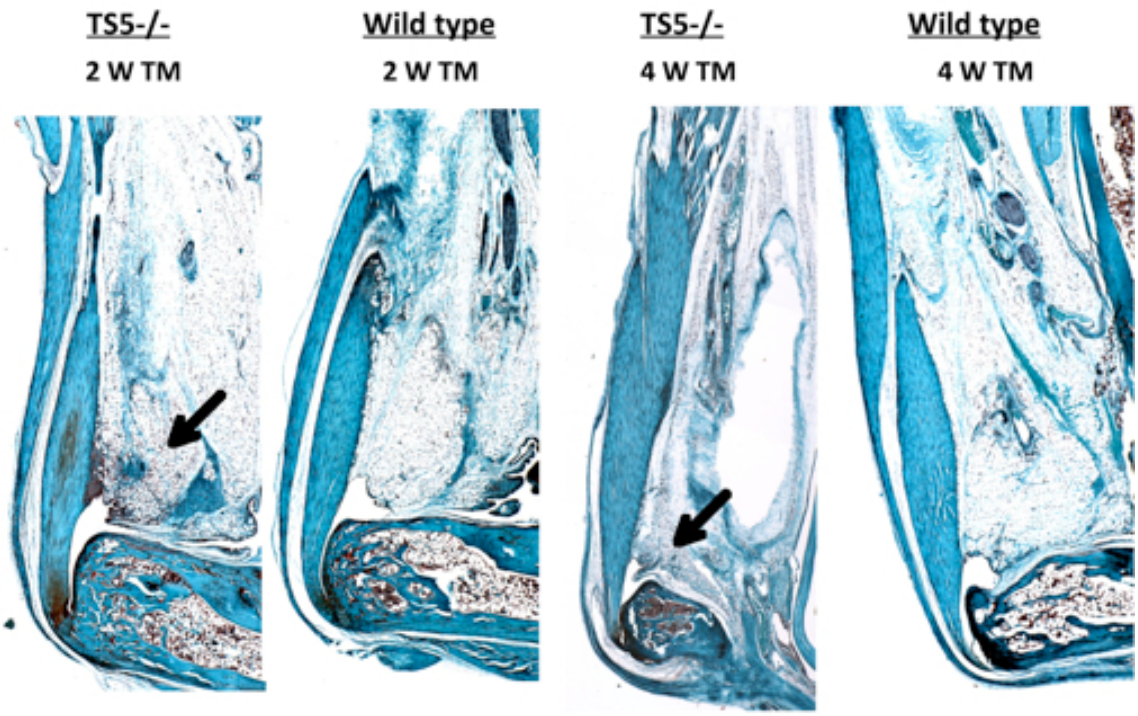

Figure 5.2- 4x of the full Achilles tendon. Arrows point to adhesion points and increased cellularity of the *ts5*$^{-/-}$ mice compared to the wild type treadmill mice.

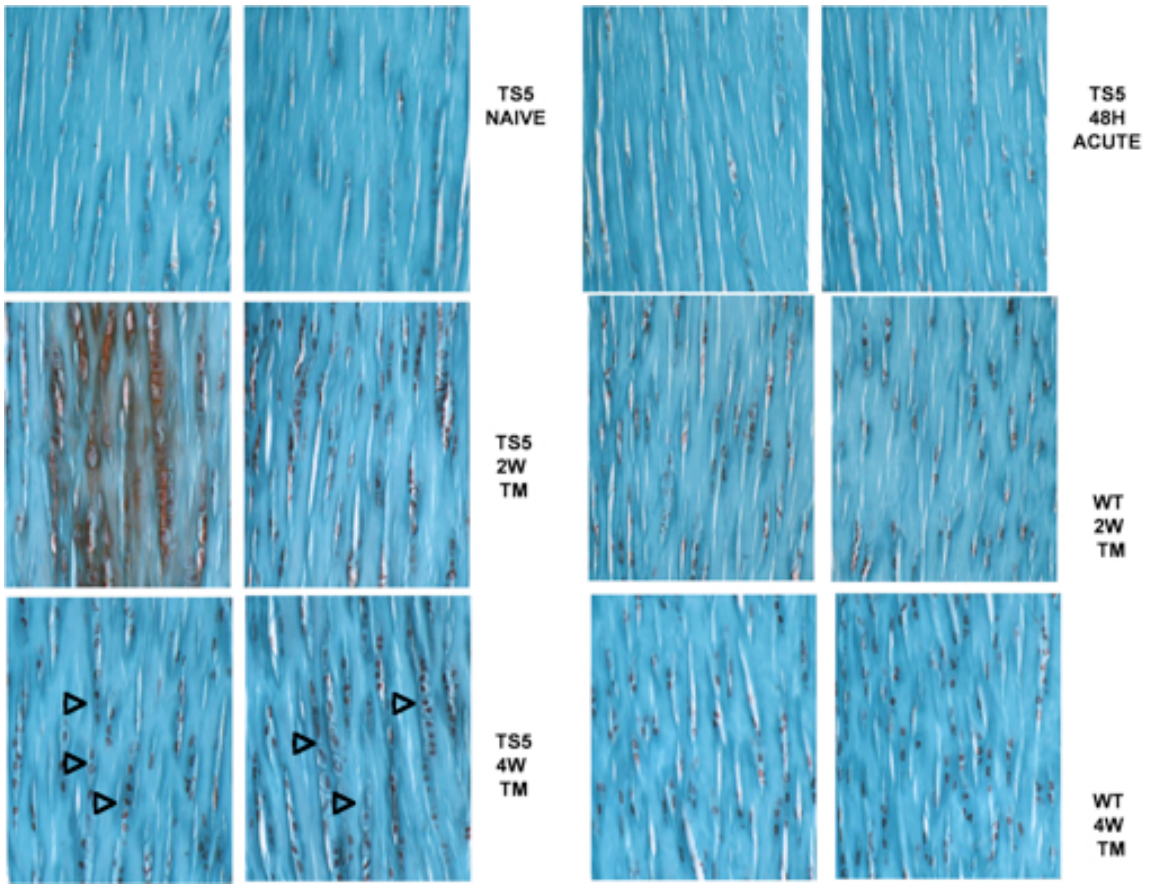

Figure 5.3- 40x of the midportion of the Achilles. Cage active mice on left, treadmill run mice on the right. Arrow heads pointing at chondrocyte like cells which is lacking in the treadmill run group.

The cross-sectional area of the tendon was larger in the 2 weeks group compared to all the other groups, Table 5.1. There was a larger amount of stress relaxation in the 48 hours group compared to the naïve. Stiffness was significantly higher in the naïves then the 2 weeks group, Figure 5.4. As seen in Figure 5.5, the maximum stress was higher in the naïve compared to all other time points.

Table 5.1- Table of mechanical data from the wild type mice receiving mechanical stimulation. The same symbols indicated significance of $p<0.05$.

Property	Naïve	48 Hours	2 Weeks Treadmill	4 Weeks Treadmill
CSA (mm^2)	0.72 [a] (0.10)	0.84 [b] (0.18)	1.13 [a,b,c] (0.14)	0.85 [c] (0.12)
Stress Relaxation (%)	45.0 [a] (5.6)	55.3 [a] (6.8)	59.7 (10.5)	48.6 (7.7)
Maximum Load (N)	6.51 (3.94)	3.50 (2.06)	4.02 (0.73)	3.51 (1.84)
Stiffness (N/mm)	9.37 [a] (3.95)	5.93 (2.70)	5.14 [a] (1.31)	6.48 (1.67)
Maximum Stress (MPa)	8.86 [a,b,c] (4.95)	4.44 [a] (2.64)	3.58 [b] (0.60)	4.19 [c] (2.06)

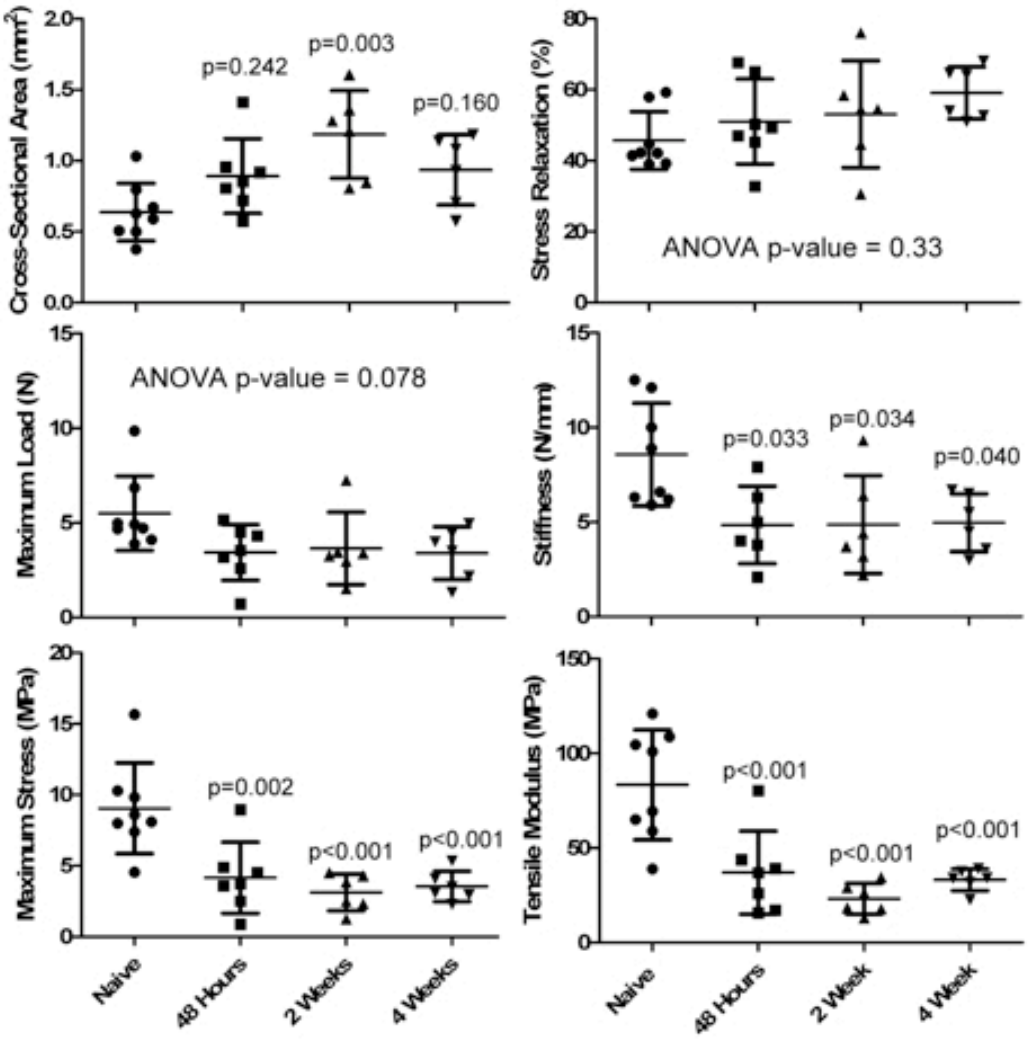

Figure 5.4- Mechanical properties with the mean and standard deviation lines.

There were significant differences of the naïve mice between the wild type and *ts5*$^{-/-}$ mice in the gene expression. Aggrecan and MMP3 were more highly expressed in the *ts5*$^{-/-}$ mice compared to the wild type. Type 1 and 3 collagens were less expressed in the *ts5*$^{-/-}$ mice. At 48 hours post injection, there were no significant differences in the gene expression of aggrecan and type 1 collagen in the wild type mice and the *ts5*$^{-/-}$ mice at the same time point. Type 3 collagen, fibronectin, and MMP3 were slightly more

expressed in the *ts5*$^{-/-}$ mice. At the 2 weeks time point, the *ts5*$^{-/-}$ mice and wild type mice with mechanical stimulation had similar gene expression in the type 1 collagen and fibronectin, however there was decreased in expression for the *ts5*$^{-/-}$ mice in aggrecan and type 3 collagen. At 4 weeks, aggrecan was more than four fold higher expressed in the *ts5*$^{-/-}$ mice compared to the treated wild type mice, where as the both type 1 and 3 collagens were highly down regulated. There were no significant differences in fibronectin and MMP3 at 4 weeks.

Table 5.2- *ts5*$^{-/-}$ Naïve and 48 Hours Post injection compared to Wild Type Naïve and 48 Hours Post Injection. ΔCT is the change from GAPDH and standard deviations in parentheses. Fold values are from Cage Wild Type at the same time point. Student's T-test was run comparing the same time point of cage activity to treadmill treated. Significance values: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, NS= no significance.

	Wild Type Naïve	***ts5*$^{-/-}$ Naive**		**Wild Type 48 Hour**	***ts5*$^{-/-}$ 48 Hour**	
Gene	**ΔCT**	**ΔCT**	**Fold**	**ΔCT**	**ΔCT**	**Fold**
Col 1	-0.14	1.86**	0.60	-1.61	-1.93	NS
	(0.16)	(0.60)		(0.38)	(0.06)	
Col 2	16.65	10.93***	36.72	16.95	15.69	NS
	(0.39)	(0.75)		(0.64)	(0.23)	
Col 3	4.04	8.27***	0.43	2.08	1.28 *	1.74
	(0.07)	(0.32)		(0.19)	(0.21)	
Col 5	7.11	9.94***	0.48	12.19	11.63	NS
	(0.13)	(0.19)		(0.58)	(0.25)	
Fibronectin	1.38	1.13	NS	0.35	-0.23 **	1.50
	(0.03)	(0.57)		(0.11)	(0.15)	
Aggrecan	15.91	13.79**	2.80	10.56	7.70	NS
	(0.61)	(0.49)		(0.08)	(0.46)	
MMP3	11.35	9.64**	2.40	6.58	5.57 *	2.04
	(0.20)	(0.37)		(0.14)	(0.26)	
MMP13	16.76	16.80	NS	ND	ND	ND
	(0.77)	(n=1)				

Table 5.3- *ts5*$^{-/-}$ 2 weeks and 4 weeks post injection compared to Wild Type 2 weeks and 4 weeks post injection. ΔCT is the change from GAPDH and standard deviations in parentheses. Fold values are from Cage Wild Type at the same time point. Student's T-test was run comparing the same time point of cage activity to treadmill treated. Significance values: * p<0.05, **p<0.01, ***p<0.001, NS= no significance.

	Wild Type 2 Weeks Treadmill	*ts5*$^{-/-}$ 2 Weeks Treadmill		Wild Type 4 Weeks Treadmill	*ts5*$^{-/-}$ 4 Weeks Treadmill	
Gene	**ΔCT**	**ΔCT**	**Fold**	**ΔCT**	**ΔCT**	**Fold**
Col 1	-4.47 (0.10)	-4.32 (0.32)	NS	0.11 (0.15)	-0.44* (0.19)	0.08
Col 2	9.96 (0.02)	12.72 (0.36)	0.16	15.66 (0.18)	ND	ND
Col 3	0.94 (0.08)	6.00* (0.13)	0.35	8.96 (0.04)	16.08*** (0.73)	0.03
Col 5	6.12 (0.57)	14.33*** (0.85)	0.003	12.71 (0.18)	ND	ND
Fibronectin	-3.19 (0.23)	-3.67 (0.33)	NS	-1.04 (0.47)	1.56 (0.15)	NS
Aggrecan	7.33 (0.29)	8.76** (0.36)	0.38	13.44 (0.42)	11.44** (0.66)	4.29
MMP3	4.97 (0.02)	4.60** (0.05)	1.29	8.85 (0.14)	8.55 (0.20)	NS
MMP13	1.63 (0.20)	2.47 (0.46)	NS	10.36 (0.12)	9.58 (0.80)	NS

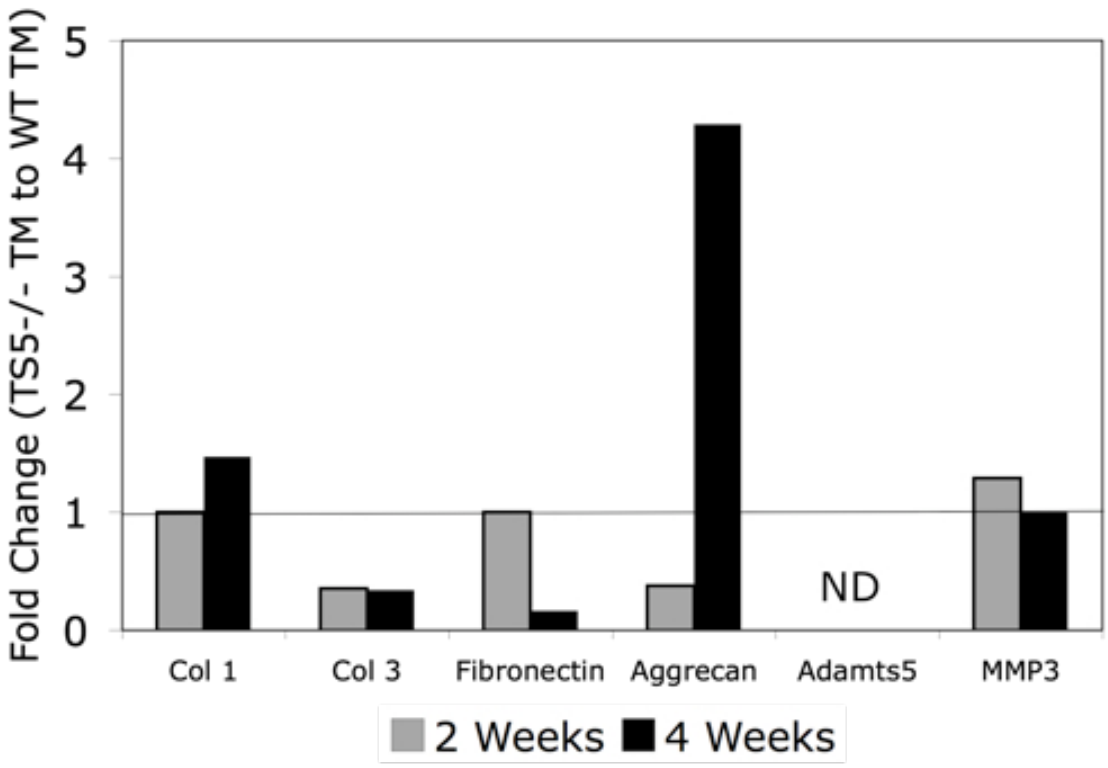

Figure 5.5- Fold changes compared to the wild type with treadmill.

The naïve mice had a significant lower expression compared to the wild type naïve for all integrins but β5. At 48 hours, there was higher expression in the *ts5*$^{-/-}$ mice compared to the wild type mice at the same time for all of the integrins except for β3 and α5. For 2 and 4 weeks, the *ts5*$^{-/-}$ mice had a much higher expression than wild types at the corresponding time of all the integrins; especially β5 who had 27 fold increase at 2 weeks and 10 fold increase at 4 weeks.

Table 5.4- *ts5*$^{-/-}$ Naïve and 48 Hours Post injection compared to Wild Type Naïve and 48 Hours Post Injection. ΔCT is the change from GAPDH and standard deviations in parentheses. Fold values are from Cage Wild Type at the same time point. Student's T-test was run comparing the same time point of cage activity to treadmill treated. Significance values: * p<0.05, **p<0.01, ***p<0.001, NS= no significance.

Integrin		Wild Type Naïve ΔCT	*ts5*$^{-/-}$ Naive ΔCT	Fold	Wild Type Acute ΔCT	*ts5*$^{-/-}$ Acute ΔCT	Fold
Central	**β1**	1.69 (0.15)	5.30*** (0.06)	0.45	3.25 (0.04)	2.20* (0.30)	2.09
RGD	**β3**	11.17 (0.21)	14.02*** (0.23)	0.48	10.90 (0.57)	9.17 (0.21)	NS
	β5	11.47 (0.13)	11.13 (0.18)	NS	12.73 (0.67)	9.37* (0.30)	10.36
	α5	7.44 (0.14)	11.16 *** (0.33)	0.45	6.19 (0.06)	4.29 (0.43)	NS
	αV	5.28 (0.15)	10.36 *** (0.20)	0.42	6.62 (0.19)	5.40** (0.15)	2.33
Collagen	**α1**	8.42 (0.15)	11.02*** (0.13)	0.50	8.75 (0.12)	7.86** (0.05)	1.85
	α2	9.48 (0.08)	13.31** (0.45)	0.44	12.33 (0.26)	10.81* (0.42)	2.93

Table 5.5- *ts5*$^{-/-}$ 2 weeks and 4 weeks post injection compared to Wild Type 2 weeks and 4 weeks post injection. ΔCT is the change from GAPDH and standard deviations in parentheses. Fold values are from Cage Wild Type at the same time point. Student's T-test was run comparing the same time point of cage activity to treadmill treated. Significance values: * p<0.05, **p<0.01, ***p<0.001, NS= no significance.

Integrin		Wild Type 2 Weeks Treadmill ΔCT	*ts5*$^{-/-}$ 2 Weeks Treadmill ΔCT	Fold	Wild Type 4 Weeks Treadmill ΔCT	*ts5*$^{-/-}$ 4 Weeks Treadmill ΔCT	Fold
Central	**β1**	1.19 (0.28)	-0.36** (0.27)	2.96	2.58 (0.08)	-0.25*** (0.04)	7.12
RGD	**β3**	8.90 (0.17)	7.18** (0.29)	3.34	12.06 (0.17)	7.90*** (0.05)	17.94
	β5	10.40 (0.56)	5.66*** (0.50)	27.67	9.52 (0.41)	6.17* (0.17)	10.18
	α5	6.50 (0.13)	3.43** (0.37)	8.57	8.76 (0.06)	5.85*** (0.09)	7.57
	αV	5.27 (0.26)	3.34** (0.02)	3.80	6.34 (0.69)	4.29* (0.39)	4.25
Collagen	**α1**	8.75 (0.23)	5.61*** (0.17)	8.87	8.87 (0.12)	6.41** (0.20)	5.57
	α2	9.68 (0.27)	7.61*** (0.29)	4.27	10.43 (0.24)	8.82** (0.13)	3.05

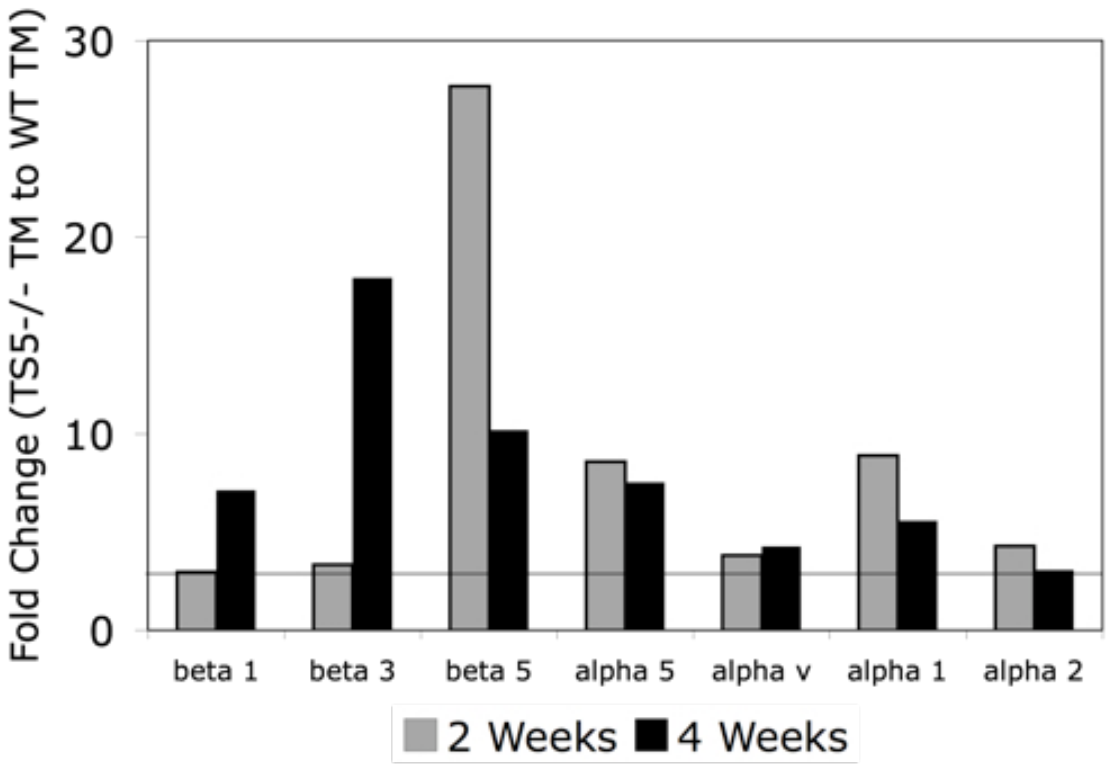

Figure 5.6- Integrin expression compared to wild type treadmill.

Discussion

When the ADAMTS5 was knocked out of the mice, the tendinopathy could no longer be successfully treated by mechanical stimulation alone. Also it appeared the running could have possibly worsened the problem since at 3 weeks the mice struggled to run and 22% mice were not able to complete the 4 week protocol. This was seen to be a similar to the horses in the Plaas et al.[47] study where the show horses develop DSLD once they begin to exercise and the ADAMTS5 enzyme in these horses are bounded by hyaluronic acid and not able to cleave aggrecan.

Corps et al.[78] looked at different stages of tendinopathy in the posterior tibialis tendon. In the stage 2 level of the disease they saw fiber disorganization, increased

cellularity, cell rounding and increased GAG content, which was similar to what we see in our mice tendons. When they looked at the mRNA expression of the stage 2 posterior tibialis tendon, they saw significant decrease of the ADAMTS5 again proving the need of ADAMTS5 enzyme for healthy tendons.

Integrin expression was significantly increased in the *ts5*$^{-/-}$ mice compared to the wild type mice at all time points. TGFβ increases integrin expression and integrin expression increases TGFβ[23]. By the injection of TGFβ and the lack of ts5 enzyme the tendon gets into a pSMAD 2/3 pathway and stay. This in turns creates more integrin expression which leads to more TGFβ expression and the cycle continues.

When the *ts5*$^{-/-}$ mice are used, the tendinopathy model could represent the part of the population that does not recover from tendinopathy by the conservative treatment of eccentric exercise.

Chapter 6

Looking at the cage active mice's FDL and Achilles tendons in thorough way with mechanical, histological, and biochemical assays allowed us to analyze the effects of *Adamts5*$^{-/-}$ mice and how tendon specific our results were. Mechanical testing of the FDL had no significant differences in the structural properties but since the *ts5*$^{-/-}$ FDL was 33% larger, the material properties of maximum stress and optical modulus were significantly lower. However these differences were not seen in the Achilles tendons but there was a change of failure location. The *ts5*$^{-/-}$ failed 60% of time at the insertion site, where the wild type tendons failed 100% in the tendon. TEM data of the *ts5*$^{-/-}$ FDL tendon had changes of the packing density of the fibrils, which were different than the wild type tendon, possibly explaining the changes of the cross-sectional area. The Achilles tendons had differences in the histology and the immunohistochemistry at the insertion site with the *ts5*$^{-/-}$ mice having an increase amount of aggrecan compared to the wild type. This could explain the failure locations. Once the phenotype of the *adamts5*$^{-/-}$ and wild type mice was established, the next step was to create a tendinopathy model.

We have created a new tendinopathy murine model by injecting TGFβ directly into the tendon. This model had an increase amount of aggrecan and deposits in the intrafibrillar region similar to the human tissues from epicondylitis biopsies. The model also fit the criteria suggested by Lui et. al [25] for a successful tendinopathy animal model of having proteoglycan accumulation, changes in gene expression, defective long-term healing, increased cross-sectional area and decreased mechanical properties. By adding mechanical stimulation to the tendinopathy model, the mice were able to recover from the initial injury of the injection. When the *Adamts5*$^{-/-}$ mice were used, the recovery was not

able to happen even with the mechanical loading proving the need for both mechanical stimulation and aggrecanase-2.

We hypothesized that using TGFβ we would be able to create a chondrogenic or pSmad 1/5/8 response in the tendon, which would need both signaling from mechanical stimulation and ADAMTS5 in order to change pathways into a fibrogenic or pSmad 2/3 response. The initial response of a tendon wound is assumed to be like any wound repair setting. It will go through the initial inflammatory phase, a longer-term proliferative and matrix synthesis phase to an ongoing homeostatic control phase. TGFβ is most active in the inflammatory phase and helps regulates cell migration, proteinase expression, fibronectin binding interactions, termination of cell proliferation and stimulation of collagen production [10].

Mechanical stimulation was used to treat the tendinopathy and at two weeks the histology already showed improvement with less chondrocyte-like cells. At 4 weeks, the tendon appeared to be back to having naïve characteristics. This was consistent with the mechanical properties. At 2 weeks, the tendons were damaged similar to the model tendons but by 4 weeks the tendons were able to recover back to their naïve properties. Other studies have shown similar beneficial results in humans with mechanical treatment[68,69]. However, those studies have shown that not everyone benefits from mechanical stimulation.

When *Adamts5*$^{-/-}$ mice had the same treatment as the wild type mice, the mice had histological changes still at 4 weeks. The mechanical properties had maximum stress decreased at all time points compared to the naïve. Gene expression was reduced at 4 weeks to even lower levels of expression than the non-injured tendons. We proposed that

the *Adamts5*$^{-/-}$ mice had a failed healing response and the gene expression data showed a lack of expression at 4 weeks. The integrin expression was increased at 4 weeks and that was due to the response of TGFβ and the integrin response to the initial response which caused a never-ending cycle. Aggrecanase was needed to progress to the next step of the healing and remodeling process.

The naïve and 48 hours post-injection mice had higher standard deviations due to the variability in the mice as well the range of initial inflammatory response seen at 48 hours. At two weeks, the variations decreased due to the uniformity of the injury to the tendons. With the tendinopathy model and the *ts5*$^{-/-}$ mice, the standard deviations increased at 4 weeks with the variations of the success of the healing.

Aggrecan and type 3 collagen were the two genes that varied the most between the tendons that were able to heal and the tendons that were not able to heal. In both the tendinopathy model and the *Adamts5*$^{-/-}$ mice, there was an extremely high expression of aggrecan at 4 weeks where the healed tissue had return closer to naïve levels.

Our other studies[47,21] have produced similar results. The ADAMTS5 enzyme was needed in the dermal wound healing in order for the fibrogenic tissue to be healed properly. The diseased equine ligaments had an increased amount of aggrecan similar to this present study. When the ligaments of the equine were looked for the cleaved portions of the aggrecan they showed the diseased tendons had less aggrecanase cleaved aggrecan.

Our new model of tendinopathy presents an improvement on previously described chemical models in that it uses a single dosage injection of TGFβ1, which has been demonstrated to be the critical biological factor translating mechanical overuse injury of tendon cells into a biological response[66]. In this study we have showed the importance of

mechanical stimulation and the role of ADAMTS5 in order to prevent and heal tendinopathy.

Limitations

As in all animal studies, there is a limitation on the conclusions one can make. Even though the model provides strong evidence of the human tendinopathy it has to be confirmed using human tendinopathic tissues. Our lab is in the process of collecting tissue in order to compare this study's outcomes to human tissue outcome using the same assays and methods.

Knockout mice are a valuable tool to study the effects of the particular gene that was removed, however one has to be cautious with the results. Since the gene was knocked out before development, the mouse has adapted to the missing gene and may have compensated for it in an unpredictable way. Future studies can examine conditional gene knockout to eliminated the development variable. By using many assays and two different tendons we were able to become very knowledgeable in the phenotype of this mouse and our confident in the results.

Future Work

The *Adamts5*$^{-/-}$ mice have the enzyme of ADAMTS5 fully knocked out of them so future work would be to look how much of the enzyme is really needed. To look into this case, we bred wild type mice with the *Adamts5*$^{-/-}$ mice to create heterozygous mice (*Adamts5*$^{+/-}$). These mice went through the same process as Chapter 4 and 5 with injection of TGFβ and 2 or 4 weeks of treadmill running. Histological and biomechanical

analyses were the only two assays that were completed. In both assays the heterozygous mice acted similar to the wild type mice. As seen in Figure 6.1, the maximum stress had similar pattern with the reduction of stress at 2 weeks but the recovery of the properties at 4 weeks.

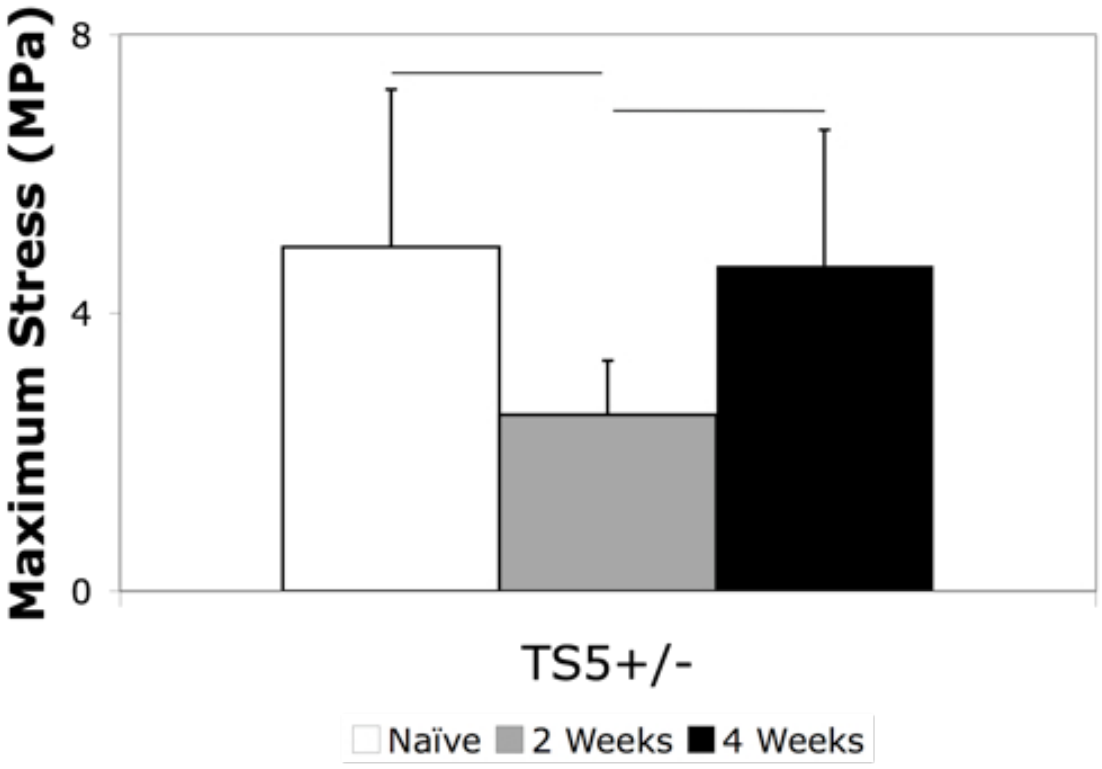

Figure 6.1- Maximum stress of the heterozygous mice. Bars indicate $p<0.05$. n=6 per group.

With this data, it would be interesting to further analyze it with gene expression, however since the mechanical and the histological data mimics the wild type mice, it could be reason the expression the data to be similar to the wild type data as well.

Since an animal model was established for tendinopathy, the next step in this work would be to develop treatment plans that would be able to speed up the healing in

the wild type mice and to heal the *Adamts5*$^{-/-}$ mice. Also as mentioned above the key to establishing this model is to be able to relate it to the human condition of tendinopathy.

Appendix
Protocols

FDL Mechanical Testing
-Thaw out the mouse
-Dissect out both of the FDL's
-Refreeze the mouse
-Scrape off muscle from the superior side of the tendon
-Measure sample's length with calipers
-Measure sample's width at 5 locations along the length with calipers
-Measure sample's thickness at ~same locations with laser displacement
-Set gauge length to 8 mm
-Keep sample moist during set-up
-Lay tendon out on sandpaper grips, try to align tendon best as possible (can adjust side to side, but make sure it isn't twisted)
-Glue is not needed, but can be used
-After tightening the grips, let tendon dry for ~1 min
-Mark the tendon in 3 locations with a sharpie maker
-Let the marker dry for ~2 minutes
-Transfer grips to MTS, carefully put in the machine, easier to do when tendon is very lax
-Soak in 37^{o}C saline bath for 15 minutes prior to testing
-Zero out load cell
-Slowly increase elongation
-When the load hits 0.01N, zero out the elongation
-Continue to add elongation until load is 0.05
-Preload- Keep the load ~0.05N for 2 minutes
-At the end of 2 minutes, write down the elongation, that value plus 8mm is the initial length
-Zero out the elongation (Not the load)
-Preconditioning- 0.05 N to 0.15 N for 20 cycles at 0.10 N/sec
-Rest for minimum 5 minutes
-Stress-Relaxation- Strain 5% at 1mm/sec, hold for 600 secs.
-Record peak load and final load
-Failure Test- Rate of 0.05 mm/sec (3 mm/min)
-Record peak load, elongation at peak load, stiffness, work, and failure location

Laser Displacement

-Connect the power pack to the display, using the wires and tiny screwdriver
-Connect the laser to the display
-Attach the laser to the plastic holder
-Level the laser using a balance
-Zero the laser
-Once level, use the calibration plates (1mm or 2mm) to check accuracy
-Lay the tendon same way the width was measured
    -Make sure the tendon does not have excess saline on it
-Have the laser measure in the center of the tendon
-Measure the thickness at approximately the same location along the length as the width measurements

Achilles Mechanical Testing

-Thaw out the mouse
-Dissect out both of the Achilles
-Refreeze the mouse
-Scrape off muscle from the superior side of the tendon
-Measure sample's length and width with calipers
-Measure sample's thickness with laser displacement
-Pot the calcaneus bone
-Set gauge length to 6 mm
-Wite-out the top of the tube and the cement, avoid getting any on the tendon
-Let wite-out and tendon dry
-Using a black Sharpie marker, mark the tube, the cement, the exposed bone/tendon, the midportion of the tendon, and the tendon just below the grip (still be able to see a little tendon before the grip starts)
-After the marker dries, soak sample with saline
-Soak in 37^{o}C saline bath for 15 minutes prior to testing
-Zero out load cell
-Slowly increase elongation
-When the load hits 0.01N, zero out the elongation
-Continue to add elongation until load is 0.05
-Preload- Keep the load ~0.05N for 2 minutes
-At the end of 2 minutes, write down the elongation, that value plus 6mm is the initial length
-Zero out the elongation (Not the load)
-Preconditioning- 0.05 N to 0.55 N for 20 cycles at 0.10 N/sec
-Rest for minimum 5 minutes
-Stress-Relaxation- Strain 5% at 1mm/sec, hold for 600 secs.
-Record peak load and final load
-Failure Test- Rate of 0.05 mm/sec (3 mm/min)
-Record peak load, elongation at peak load, stiffness, work, and failure location

Histology

All tissue processing protocols used freshly harvested, normal flexor digitorum longus (FDL) tendons from 12 week-old C57BL-6 mice, and freshly harvested patellar tendons from 12 week-old C57BL-6 mice. Tendons were harvested immediately following animal euthanasia under approval of our institution's Animal Care and Use Committee. During dissection and preparation for histologic processing, specimens were kept moist with isotonic saline. Following tissue harvesting, samples were laid flat and affixed to acrylic plates to maintain tendon tension during fixation. Tendons were fixed with 10% neutral buffered formalin or 4% paraformaldehyde. Fixation times were dependent on the tissue volume (range: 24 h for FDL tendons to 96 h for patellar tendons with entire tibiae intact). Samples were rinsed well with deionized water after primary fixation. Cite Laudier et al.?

Procedure	Reagent/Media	Processing Time (FDL)	Processing Time (PT)
Fixation	Formalin	>24 h	>72 h
Decalcification	Formic Acid or EDTA	N/A	~2 weeks
Dehydration I	EGME	>24 h (1 change)	>24 h (2 changes)
Dehydration II	Acetonitrile	>24 h	>24 h (1 change)
Clearing	Methyl salicylate	Overnight	>24 h
Embedding	MMA	6 days	7 days

Immunohistochemistry

DAB/Methyl Green Immunohistochemistry Staining with Vecastain Elite ABC Kit

Reagents

Normal Blocking Serum

Add 3 drops (150 ul) of stock normal goat serum (yellow label) to 10 ml PBS buffer in mixing bottle (yellow label)

1% BSA in PBS buffer

Add 0.01 g BSA in 10 ml PBS buffer

Biotinylated Secondary Antibody

Add 3 drops (150 ul) of stock normal goat serum (yellow label) to 10 ml buffer (PBS or 1% BSA in PBS) and 1 drop (50 uL) of biotinylated antibody stock (blue label) to mixing bottle (blue label).

3% H_2O_2 in Tap Water

1 ml of ~30% H_2O_2 to 9 ml tap water

ABC Reagent

Add 2 drops of Reagent A and 2 drops of Reagent B to 5 ml buffer (PBS or 1% BSA in PBS) in mixing bottle (gray label). Mix immediately. Allow to stand for 30 minutes.

DAB Substrate (Vectastain Kit)

Add 2 drops of buffer stock solution to 5 ml DI water and mix well. Add 4 drops of DAB stock solution and mix well. Add 2 drops of hydrogen peroxide and mix well. If gray-black stain is desired, add 2 drops of nickel solution and mix well.

Procedure

1. Deparaffinize and hydrate tissue sections through xylenes (2 times for 3 minutes each) and graded alcohols (100% EtOH for 1 minute and 95% EtOH for 1 min) and rinse in DI water. (This is done in the hood with the lined up containers of each group)
2. If working with bone or cartilage, incubate in 10% formalin for 30 minutes at room temperature.
3. Wash in DI water for 5 minutes.
4. Wash in buffer for 5 minutes.
5. Proteinase K Treatment (optional)
    a. Stock is at 10 mg/ml (check that). Dilute to 20 ug/ml.
    b. Incubate on sections for 20 minutes at 37^{o}C
    c. Rinse with PBS buffer
    d. Incubate sections with 0.1mM AEBSF (protease inhibitor) for 5 minutes at room temperature
    e. Wash sections for 5 minutes with PBS buffer
6. Incubate sections for 20 minutes with diluted normal blocking serum (normal goat serum diluted in PBS buffer OR 1% BSA in PBS buffer)
7. Tip excess serum from the sections

8. Incubate sections overnight in 4^{o}C with primary antiserum diluted in blocking serum (Be sure to keep slides moist, can place in a sealed tuberware container with wet PBS gauze on the bottom)
9. Wash slides for 5 minutes in buffer
10. Incubate sections for 30 minutes with diluted biotinylated secondary antibody serum
11. Wash slides for 5 minutes in buffer
12. Quench endogenous peroxidase by treating sections with 3% H_2O_2 in tap water for 10 minutes
13. Wash slides for 5 minutes in buffer
14. Incubate sections for 30 minutes with ABC re
15. Wash slides for 5 minutes in buffer
16. Incubate sections in DAB until desired stain intensity develops. (5-10 minutes)
17. Rinse sections in tap water for 5 minutes.
18. Counterstain in methyl green (be sure it is fresh) at 60^{o}C for 10 minutes.
19. Wash in DI water until clears
20. Dehydrate in graded alcohols (2x in 95% EtOH for 1 min each and 2x in 100% EtOH for 1 minute each) and xylenes (2-3x for 3 minutes each).
21. Mount and coverslip.

FACE Protocol with Proteniase K

Day 1

- Take tendons out of mice and put into tubes. Can combine more than one tendon into tube (ideally 4-8 tendons per tube)
- Add 250ul (or enough to cover tendons) of 0.1M Ammonium Acetate (AA) ph 7.4
    -0.1 M Ammonium Acetate= 1ml of 1 M AA + 9ml of water
- Add 10 ul of Protease K (10mg/ml) per 2 tendons
- Incubate overnight at 55^{o}C

Day 2

- Take tubes out of incubator
- Punch holes into the lids (with needle)
- Heat at 100^{o}C for 15 minutes
- Remove from heat and let cool
- Centrifuge for 25 minutes at 15,000 RPMs
- While centrifuging, make solutions
    - 1 ml of AEBSF (1mM)=> 10ul of 0.1 M of AEBSF + 990 ul of 0.1 M of AA
    - 10 ml of 0.1M of AA ph 7.4- 1 ml of 1M of AA + 9 ml of water
- After running centrifuge, transfer liquid (leave pellet) to the filtering tube
- Centrifuge for 25 minutes at 15,000 RPMs
- (1) Rinse with 500 ul of 0.1 M AA ph 7.4
    - Centrifuge for 25 minutes at 15,000 RPMs
    - Discard liquid (or keep for DNA assay)
- (2) Rinse with 250 ul of 1mM of AEBSF solution
    - Let sit for 15 minutes
    - Centrifuge for 25 minutes at 15,000 RPMs
    - Discard the liquid
- (3) Rinse with 500 ul of 0.1 M AA ph 7.4
    - Centrifuge for 25 minutes at 15,000 RPMs
    - Discard the liquid
- Take 2 vials of 10 ug CS standard & 2 vials of 5ug HA standard out of freezer
- Add 200 ul of 0.1 M of AA ph 7.4 to the standards and all of the sample vials
- Add 7.5 ul of enzyme Chondroitinase ABC (regular) to all standards and sample vials
- Incubate at 37^{o}C overnight

Day 3

- Take tubes out of incubator

SAMPLES ONLY

- Spin down in centrifuge at 15,000 RPMs for 25 minutes
- Take the liquid out of filter tubes and save in new tubes
- Add 250 ul of water to the filter tubes
- Spin down in centrifuge at 15,000 RPMs for 25 minutes
- Take the liquid out of filter tubes and save in the same tubes

BOTH SAMPLES AND STANDARDS

- Put all tubes into the speed vac to dry overnight (about 4 hours, can be done in same day)

Day 4
- Take tubes (standards & samples) out of speed vac
- Add 5ul of AMAC (dark brown stuff) solution
- Vortex it, centrifuge briefly
- Leave at room temperature for 15-30 minutes
- Add 5ul of sodium cyanoborohydride solution
    - 10 mg of cyanoborohydride + 160 ul of DI water
- Vortex it, centrifuge briefly
- Incubate at 37^{o}C overnight

Day 5
- Take tubes out of incubator
- Add 30 ul of 25% glycerol to all tubes and HA standards
- Add 90 ul of 25% glycerol to CS standards
- Can now either store at -20^{o}C or electrophorese

FACE Protocol with Sodium Hydroxide

Day 1

- Pulverize samples in liquid nitrogen (if needed) and place samples into a tube
- Add 400 ul of 0.1 M ammonimum acetate (AA) to the tube
    - 0.1M AA = 1 ml of 1M of AA + 9 ml of water
- Add 3.33 ul of 12 M NaOH for a final concentration of 0.1M NaOH to degrade sample
- Incubate overnight at room temperature

Day 2

- Next morning, spin samples down for 15 minutes at 15,000 RPMs
- Remove supernatant to a new tube and discard pellet
- Add 200 ul of water
- Centrifuge for 15 minutes at 15,000 RPMs
- Remove supernatant to same tube and discard pellet
- To the supernatant, add 5 ul of 0.1% of phenol red to each sample
- Adjust ph using HCl to neutral- Phenol red is yellow when ph is low and red when high, Neutral is orange
- Once all samples are neutral, load on to filter tubes
- Centrifuge at 15,000 RPMs for 15 minutes or until liquid has passed through
- Discard flowthrough, wash 3-4 times with 500 ul of AA, discarding flow through
- Add 200 ul of AA to filter tube
- Add 7.5 ul of chondroitnase ABC to each sample
- Set-up the standards
    - 10 ug of chondroitinase sulfate
    - 200 ul of 0.1 M AA
    - 7.5ul of chondroitnase ABC

  and
    - 5 ug of hyaluronan
    - 200 ul of 0.1 M AA
    - 7.5 ul of chondroitnase ABC
- Incubate both your samples and standards overnight at $37^{o}C$

Day 3

- Next morning, spin down samples for 20 minutes at 15,000 RPM
- Keep flow through in new labeled tube
- Wash membrane once with ~200 ul of deionized water,
- Spin down water through membrane for ~20 minutes at 15,000 RPM
- Combine flow with original flow through
- Speed vac the samples and standards until dry
- Resuspend each sample & standard in 5ul of AMAC solution and incubate at room temperature for 20 minutes
- During this time, make cyanoborohydride solution
    - 10 mg cyanoborohydride in 160 ul of ultra pure water
- After 20 minutes, add 5 ul of cyanoborhydride solution to each sample and standard
- Incubate at $37^{o}C$ overnight

Day 4
- Next morning, add 30 ul of 25% glycerol solution to each sample and the hyaluronan standard
- Add 90 ul of 25% glycerol to the chondroitin sulfate standard
- Can store at -20^{o}C or electrophorese

FACE Protocol- Gel Casting

- Clean Plates and assemble with spacers, seal sides with electrical tape and insert in clean non-leaking gel pouches
    -Make sure plates and spacers are aligned at bottom
- Place 2 per gel cast (back to back)
- Prepare separating gel solution at room temperature
    - 2 20% Gels (Keratan Sulfate & Monosaccharides)
        -11 ml of Acrylamide/Bis
        -2.25 ml Tris Acetate buffer
        -2.25 ml 25% glycerol
        -6.25 ml water
        -*120 ul Ammonium persulfate
            (100 mg of ammonium persulfate + 500 ul of water)
        -*15 ul TEMED
    * once added these 2 ingredients, must move quickly

-Fill plates with solution to about ¾" from top of notched plate, using 10 ml pipette
-Tap on benchtop several times to expel air bubbles
-Overlay top of gel with water (using a plastic Pasteur pipette)
-DO QUICKLY

-Let gels set at room temperature, check completion by watching polymerization of extra solution in beaker
-Discard water layer from top and use filter paper to get remaining water
-Prepare stacking gel solution at room temp
    -1.5 ml of acrylamide/Bis
    -1.5 ml Tris Acetate buffer
    -1.5 ml 25% glycerol
    -3.0 ml water
    -*50 ul Ammonium persulfate
    -*5 ul TEMED
-Overlay separating gel with stacking gel solution, fill to overflow
-Insert 8-well combs (avoid trapping air bubbles)
-Let set at room temperature
-Place gels in refrigerator for cooling to $4^{o}C$
-Can be made 24 hours in advance

FACE Gel Electrophoresis

-Fill tub with ice around the electrophoresis tank
-Fill tank with buffer & activate stir bar
-Remove plates from casting frame & from plastic bag (use running water)
-Clean excess gel from plates using spatula (Dispose in garbage)
-Carefully remove combs. Wash wells out
-Place gels into electrophoresis frame (face to face)
-Secure with white holders
-Fill top of frame with buffer and place into tank
-Load sample (6-8ul) per well
-Attach electrodes, switch on power pack, adjust on manual voltage to 400~500 V (try to get current to be close to but not over 80 mAmps
-Electrophorese for 15-20 minutes
-Stop power and check gels under UV light, the samples should be through stacking gel
-Place gel back in the box and continue to run for 15-20 minutes

FACE Imaging

-Remove plates from electrophoresis tank/frame
-**WASH** excess AMAC from wells under running water
-Remove tape & spacers and separate the plates with the spatula
-Cut off stacking gel, rinse separating gel with water bottle & rinse UV box
-Take the separating gel from plate and place in UV box
-Turn UV light on
-Open Kodak Imaging Program
-Select camera button in the left hand corner
-Preview gel at 1 sec. then image at 1/100, 1/20, 1/10, ¼. ½, and 1 second exposures
-Turn off UV light
-Save each image after inverting, straightening, and cropping image
-When done, remove gel from UV box & wipe down box
-Discard gel in trash

Western Blotting- Ligament Pulverization, Digestion & Processing Protocol

-Incubate ligament samples in PBS+ Proteinase Inhibitors for 30 minutes
-Remove from liquid, weigh specimens, and freeze at -20^{o}C
-Ready the freezing mill by placing in liquid nitrogen until boiling slows
-Pulverize samples and place back in labeled tubes
-Add 500 ul of extraction buffer to each sample & place at 4^{o}C on a rocker to incubate overnight

DAY 2
-Next day, spin samples down, 10 min @ 15,000 RPMs
-Take the supernatant & adjust its column up to 2 ml with water
-Place samples in dialysis bags and dialyze against running water for 5 hours
-After 5 hours put the samples into clean 15ml tubes
-Adjust sample to 7M urea and 50mM tris acetate using solid urea and liquid tris
    - Done by adding 1/20th sample volume of 1 M Tris acetate to the sample itself (adjust pH to 8.0 before adding, using glacial acetic acid).
    - FOR 2 mL sample, add 0.84 g urea and 60~ 70ul of 1M tris base
-Pre-wet column tubes
-Prepare DE52 Cellulose columns by adding 1-2mL of the 50% slurry to the column tubes. This amount should correspond to 0.7mL packed column in the tubes.
-Equilibrate columns by running at least 2 mL of 7M urea 50 mM Tris acetate to them. Be sure to check saturation by dropping outflow into water
-Load the adjusted samples & be sure to collect the flow through. This volume should be ~2 ml
-Run through 2 more ml of 7M urea 50mM Tris acetate and pool with the first flow through samples
-Store these flow through samples at -20^{o}
-Elute the proteoglycan fraction by running 2 ml of 1.5 M NaCl, 7 M urea and 50 mM Tris acetate through column
    -Solution can be made by adding 1.74 NaCl to each 20 ml portion of 7 M urea 50uM Tris acetate
-Dialyze for 5 hours against running distilled water
-Divide samples into four 500ul portion and dry in speed vac overnight
-Next day, dissolve the samples in 25 ul of chase buffer + 3 ul of ABCPF and incubate at 37^{o}C for one hour ---ONLY do this step on samples you are doing westerns on, can store the remaining 3 portions in -20^{o}C
-Dry samples in speed vac for ~30 minutes or until dry
-Ready to proceed to do western protocol

Western Blot Protocol

-Make sample buffer, antibody (blocking) solution (40ml), running buffer, and transfer buffer

-Add 20 ul of sample buffer to each sample and boil for 10 minutes

-Set-up gel (4-12% gel tris-glycine) loading side facing each other (TAKE OFF TAPE)

-Load 20 ul of molecular weight standard, samples, then sample buffer (if needed to fill out gel)

-Run gels for 40 minutes or until blue line reaches the bottom of the gel at 200 volts at $4^{o}C$

-Have 2 containers with transfer buffer in it

-Place Black clip on the bottom, soak gauze and place on black clip, then filter paper, gel, membrane, and filter paper. Roll out bubbles then place on gauze and clear clip

Clear Clip

↑

Gauze

↑

Filter Paper

↑

Membrane

↑

Gel

↑

Filter Paper

↑

Gauze

↑

Black Clip

-Put membrane unit into transfer unit with transfer buffer at 100 volts for 1 hour at $4^{o}C$

-Unload gel, check to see if transfer is complete

-Cut filter paper & membrane down to size

-Take membrane and place into container with 10 ml of blocking solution

-Shake at room temperature for 1 hour

-Incubate in 1^{o} Antibody solution at $4^{o}C$ overnight, shaking

-(10 ml => 1:500 = 20 ul Antibody => 1:200 = 5 ul- Both with 10 ul of blocking solution)

-Next day, wash in 1xTBST a total of 3 times, 15 minutes each

-Incubate in 2^{o} antibody solution at room temperature for 1 hour, shaking

-Wash in 1xTBST- 3 times, 15 minutes each

-Develop with Amersham reagents :

-Combine 2.4~3 ml of each reagent from ECL kit for each gel

-Submerge membrane in mixture for ~3 minutes (Shake)

-Remove membrane allowing excess liquid to drip off

-Place membrane in plastic sleeve and begin exposure process

-Image membrane

Genotyping

Checklist

-ethanol- to clean scissors between mice
-scissors
-tube for each tail clipping
-ear tags and tagger

-Tag mouse
-Let mouse hang into cage by tail
-Clip <5 mm of the tail
-Place tail clipping into the tube
-Add 700 ul NTES buffer and 20 ul of Proteinase K (10mg/ml)
-Heat at 55^{o}C for ~30 minutes
-Shake each tube
-Continue to heat overnight
-Next day, add 700 ul of Phenol/chloroform (stored in 4^{o} C) solution
-Make sure tip goes into lower chloroform layer
-Vortex for 15 seconds or until it turns froth white
-Centrifuge at max speed (~20,000 rpms) for 10 minutes
-Pipet 300 ul of the upper phase into new tube
-Add equal volume (300 ul) of isopropanol (on desktop)
-Mix several times by inverting
(Can store at -20^{o}C if you need to stop here)
-Centrifuge at max speed for 10-15minutes
-Discard supernatant, be careful not to disturb pellet
-Wash pellet once using 1 ml cold 70% EtOH
-Centrifuge at max speed for 5 minutes
-Discard supernatant, air dry for 30 minutes, be careful not to lose pellet
-Lay flat on paper towel, can use towel to soak up excess liquid
-Dissolve pellet in 200 ul of 1xTE Buffer (TE=tris EDTA buffer on desktop)
-can add more if there was a large tail clipping
-Store overnight in 4^{o}C
-Next day, make master mix for each primer needed

For Agg2 (ADAMTS5):

	1x	20x	35x
10x PCR buffer (Qiagen)	1.0 ul	20 ul	35 ul
dNTPs (1.25 mM)	1.6 ul	32 ul	56 ul
Agg2 Primer mix at 20uM	1.0 ul	20 ul	35 ul
Taq Poly (Qiagen 5 units/ul)	0.1 ul	2 ul	3.5 ul
$MgCl_2$ (25mM)	0.4 ul	8 ul	14 ul
Rediload 5x (diluted with glycerol)	2 ul	40 ul	70 ul
H_2O	2.9 ul	58 ul	101.5 ul
DNA	1ul	---	---

Each tube gets 9ul of master mix + 1ul of DNA for a total of 10 ul

Cycling condition for Agg2:
1 x (94°C for 2 minutes)
40 x (94°C for 30 sec, 63°C for 30 sec, 68°C for 90 sec + 5 sec auto extension)
1 x (72°C for 5 min)
4°C for infinite

For CD44:

	1x	20x	35x
10x PCR buffer (Qiagen)	1.2 ul	24 ul	42 ul
dNTPs (1.25 mM)	0.96 ul	19.2 ul	33.6 ul
CD44 Primer mix	2.4 ul	48 ul	84 ul
Taq Poly (Qiagen 5 units/ul)	0.06 ul	1.2 ul	2.1 ul
$MgCl_2$ (25mM)	0.96 ul	19.2 ul	33.6 ul
Rediload 5x (diluted with glycerol)	1.66 ul	33.2 ul	58.1 ul
H_2O	2.76 ul	55.2 ul	96.6 ul
DNA	2ul	---	---

Each tube gets 10ul of master mix + 2ul of DNA for a total of 12 ul

Cycling condition for CD44:
1 x (94°C for 2 minutes)
40 x (94°C for 30 sec, 63°C for 30 sec, 68°C for 90 sec + 5 sec auto extension)
1 x (72°C for 5 min)
4°C for infinite

-Make 2% agarose gel
    -6g agarose in 300 ml 1xTBE
    -Add stir bar, and stir for 5 minutes
    -Remove stir bar, microwave for 3-4 mins, do not let slurry boil over
    -If agarose is not completely dissolved, stir using bar until dissolved
    -Let cool for ~5minutes
    -Pour into gel cassette
-Let gel set for 30-40 minutes
-Load gel
    -8 ul ladder
    -8 ul sample
-Run gel at 150 V for 30 minutes (Black up top, Red on bottom "Run to Red")
-Image gel using UV transilluminator
-Save gel image
-Print gel image with ID # and sex on hard copy
-Fill out tail clipping ID chart with genotypes

ATD Surgery

Mice were anesthetized by intraperitoneal injection of xylazine and ketamine (50%: 50%) 0.14 ml. Surgery was performed in 12 week old male mice. A five-mm incision was made on the dorsal side of the lower leg, exposed Achilles tendon, surrounding soft tissues were separated with Achilles tendon. A small supporter was inserted underneath Achilles tendon; a punch was made in the middle of the tendon by using a sharpened 22-gauge needle. The incision was closed by using 6-0 coated vicryl suture. All procedures were under sterile conditions. Mice were housed, 5-6 per cage, change cage daily up to 1 week.

TGFβ Injections

Checklist

- -Syringes
- -Pipet and tips
- -Ketamine
- -Iodine
- -Alcohol
- -Swabs
- -Plastic weigh boat something for the TGFβ
- -Blue pad
- -TGFβ
- -Clean cages
- -Ear punch
- -Ear tags
- -Sheet for Ear Tag numbers
- -Scale

-Inject 0.12 ml of Ketamine IP
-Inject a whole cage at a time and transfer them into a clean cage
-Use pipet to get 6 ul of TGFβ
-Pipet TGFβ onto weigh boat
-Take a syringe and pump it a couples of times so it is less "sticky"
-Draw the 6ul into syringe, don't worry about air bubbles but try to minimize them
-Take a mouse out
-Hold mouse with head towards you and grip right foot
-Swab Achilles with iodine, then alcohol
-Insert syringe into midtendon going towards heel
-Inject TGFβ and hold syringe down for a second after all is in
-Ear punch the mouse and weigh mouse

Treadmill Running

-Set angle of treadmill at 3rd notch from the back to give an uphill angle of 15o
-Place each mouse into lane
-Turn on treadmill power box
-Set the speed to 32 cm/s
-Press "Run"
-Run the mice for 25 minutes
-For 5 lane treadmill
    -Keep treadmill running
    -Hold cage in front of treadmill
    -Grab mouse's tail as he runs to take him out
-For 4 lane treadmill
    -Shut off treadmill
    -Hold cage in front of treadmill
    -Use plastic lane divider to push mouse into cage
-Clean each lane
-Turn off treadmill's power box

TIPS

-4 lane treadmill is better for "bad" runners, can coax them easier
-Switching lanes occasionally helps

Dissections for RNA Isolation

-Instruments
    -Large forceps to grab foot and skin
    -Smaller/finer forceps for isolating tendon
    -Scissors for cutting skin
    -Microscissors for cutting tendon out
    -Petri dish, one side to hold instruments, other side to dissect mouse in
-RNase Zap- clean instruments & Petri dish
-RNALater- for 24 tendons use 750 ul
    -Use sterile pipet and tube
-Use RNase Zap and/or alcohol on gloves & mouse
-Use scissors to cut skin at heel then cut parallel to the tendon to expose tendon
-Remove superficial tendon if able to (healing tissue is hard to distinguish)
-Cut tendon at muscle junction side first
-Cut tendon then at heel
-Place into RNAlater
-Put tube in 4^{o}C for at least 30 minutes, can leave overnight
-Transfer tube to -20^{o}C

qRT-PCR Protocol (modified from Dan Gorski's protocol)

mRNA Isolation From Tissue

1. Put mortar and pestle into liquid nitrogen until boiling stops
2. Pipet out the RNAlater out of each container, be sure to avoid the tendons
3. Put all (i.e. all for 2 week Wild type right leg) containers from one group into the liquid nitrogen
4. Dump all the frozen tendons into the chute of the mortar
5. Pulverize by using mallet ~10 times
6. Open up the chute, scrape all the tendons into the center of mortar
7. Repeat steps 5-6 a total of 5 times
8. Scrape all of the tendon pieces and place into a labeled tube with 1mL Trizol at room temperature
9. Vortex Trizol and tissue for 1 minute, vortex as much as possible
10. Let sit at room temperature for 10 minutes, continue to vortex
11. Add 200ul of chloroform, vortex 2-3 minutes
12. Centrifuge samples at 14,000g for 15 minutes at 4^{o}C (or at room temperature)
13. Samples are then separated into lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains in aqueous phase.
14. Transfer **all** of the aqueous phase to separate tube, be careful not to disturb the other layers
15. Save the other phases if DNA or protein isolation is needed
16. Precipitate RNA by adding 600 uls of isopropyl alcohol (or more, need the same amount or more than the aqueous phase that was recovered)
17. Make sure all of the tubes have equal volume. Invert each tube 2-3 times or until you don't see the "stringiness" anymore
18. Incubate 5 minutes at room temperature (or overnight at -20^{o}C)
19. Centrifuge at 14,000g for 15 minutes at 4^{o}C (or room temp), RNA pellet will be gel-like on side and bottom
20. Remove supernatant, wash pellet once with 75% ethanol at least 1 ml, Mix sample by vortexing
21. Centrifuge at 14,000g for 10 minutes at 4^{o}C
22. Pour off EtOH
23. TURN ON HEATER TO 55^{o}C
24. Dry the RNA pellet with the cap open 30-45 minutes, lay on a paper towel
25. Do not let the sample dry completely, it will decrease its solubility. Partially dissolved RNA samples have an A260/280 ration <1.6.
26. Dissolve the RNA in RNAase free (DEPC) (Located on desktop) 100ul water for 5 minutes in 55^{o}C heater
27. Fast spin down (quick, on Touch Centrifuge)
28. Store at -20^{o}C (or -80^{o}C)

***Technically this mRNA prep can be used for qRT-PCR but we like to further purify it with a spin column kit from Qiagen. Using only the RNA cleanup protocol from the RNeasy Mini Kit (below is an adapted protocol referencing reagents and solutions provided in the kit, there are certain steps and precautions omitted here that must be read.)**

mRNA cleanup using RNeasy Mini Kit by Qiagen (cat no. 74104)

1. Adjust the sample to a volume of 100 ul with RNase-free water (should already be at 100 ul). Add 350 ul Buffer RLT (in kit), and mix well.
2. Add 250 ul ethanol (96-100%, not in kit) to the diluted RNA, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 3.
3. Transfer the sample (700ul) to an RNeasy Mini spin column placed in a 2 ml collection tube (supplied in kit). Close the lid gently, and centrifuge for 15 seconds at 8,000g. Discard the flow-through. Reuse the collection tube in step 5.
4. After centrifuge, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.
5. Add 500ul Buffer RPE (in kit) to the RNeasy spin column. Close the lid gently, and centrifuge for 15 seconds (use short button on centrifuge) at 8,000g to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 6.
6. Add 500ul Buffer RPE (in kit) to the RNeasy spin column. Close the lid gently, and centrifuge for 2 minutes at 8,000g to wash the spin column membrane. Discard the flow-through. Reuse tube for step 7.
7. Centrifuge spin column at max speed for 30 seconds, to complete remove any remaining Buffer RPE on the column. Discard flow-through. At this point you may discard the collection tube as well, but have a labeled 1.5 ml collection tube waiting to place the spin column in.

   The long centrifuge dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

   Note: After centrifuge, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise carryover of ethanol will occur.

8. Place the RNeasy (in kit) spin column in a new 1.5ml collection tube (supplied). Add 30 ul RNase-free water directly to the spin column membrane, wait 10 minutes.
9. Close the lid gently, and centrifuge for 1 minute at 8,000g to elute the RNA.

mRNA quantification

1. Measure the A260/280 of the mRNA sample, looking for a ration >1.8
2. Measure the concentration of the mRNA and calculate how much volume is needed to pipette 1ug of RNA.

cDNA synthesis using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen- cat. no- 11904-018

1. Add the following components to a nuclease-free microcentrifuge tube:
   1 ul of oligo$(dT)_{20}$ (50uM);

1 ul 10 mM dNTP mix (10 uM each dATP, dGTP, dCTP, and dTTP at neutral ph)
1 ug of total RNA (calculate volume from mRNA quantification, different for each sample)
Sterile water (DEPC) to 13 ul (RNA volume + water volume=13 ul)

The first 2 can be made in a 1:1 ratio for the amount needed and pipetted into tubes prior to adding the RNA. Reduces pipetting and the need of switching tips.

2. Heat mixture to 65^{o}C for 5 minutes and incubate on ice for at least 1 minute- this is done in thermal cycler
3. Pause cycler, collect the contents of the tube by brief centrifuge and add:
   4 ul 5x First-Strand Buffer
   1 ul 0.1 M DTT
   1 ul RNaseOUT Recombinant RNase Inhibitor (Cat no. 10777-019, 30 units/ul) Note: When using less than 50 ng of starting RNA, the addition of RNasOUT is essential
   1 ul of SuperScript III RT (200 unites/ul) Note: If generating cDNA longer than 5kb at temperatures above 50^{o}C using a gene-specific primer or oligo(dT)$_{20}$, the amount of SuperScript III RT maybe raised to 400 U (2ul) to increase yield
4. Mix by pipetting gently up and down. If using random primers, incubate tube at 25^{o}C for 5 minutes.
5. Restart cycler.
6. Incubate at 50^{o}C for 30-60 minutes. Increase the reaction temperature to 55^{o}C for gene-specific primer. Reaction temperature may also be increased to 55^{o}C for difficult templates or templates with high secondary structure.
7. Inactivate the reaction by heating at 70^{o}C for 15 minutes.

The cDNA can now be used as a template for amplification in PCR. However, amplification of some PCR targets (that >1 kb) may require the removal of RNA complementary to the cDNA. To remove RNA complementary to the cDNA, add 1 ul (2units) of E. coli RNase H and incubate at 37^{o}C for 20 minutes.

<u>qRT-PCR using ABI Taqman Primers</u>

1. Make 1:10 dilution in DEPC water of cDNA preps.
2. Following the protocol provided by "ABI Taqman Gene Expression Assays" for the 20 ul/well reaction add to each well:

ABI Gene Expression Mix	10ul/rxn
ABI 20x primers	1 ul/rxn
cDNA (diluted)	9 ul/rxn

Important Note: When comparing data on two or more separate plates, you must manually set the baseline threshold to the same value after the run is complete. I have been using Dan's number- 341.57, new system sets it at 0.34157.

<u>Applied Biosystems Real Time PCR System</u>

1. Turn on Computer

2. Turn on 7300 Real Time PCR System (the machine itself)
    a. Power button is on the right, press the left side of the button
3. Put in plate- by pressing the middle button
    a. A1 is in the top left corner
4. Open 7300 System Software
    a. Create New Document
        i. **Assay:** Standard Curve (Abs. Quant)
        ii. 96 Well Clear
        iii. Blank
        iv. Standard 7300
        v. 7300 User
        vi. Comments (N/A)
        vii. **Plate Name:**
5. Click Next
    a. Move Genes for plate over
        i. Make sure the reporter is FAM
        ii. Create new if not present, again make sure it is FAM
6. Click Next
    a. Highlight wells and click gene that corresponds to the well number
7. Click Finish
8. Click Instrument Tab
    a. Change sample volume to 25 ul
    b. Run Mode: Standard 7300
    c. Data Collection: Stage 3, Step 2 (60.0 @ 1:00)
9. Click START

Once Done

1. Results
    a. Plate- Highlight all wells
    b. Amplification Plot Tab
        i. Change Threshold to **0.34157**
        ii. Manual Baseline 3 to 15
    c. Analyze (Maximize program if you can't see)
        i. Threshold line should turn green if analyzed
2. Save Plate
3. File → Export → Ct
4. Back up data
5. Remove Plate
6. Shut down machine
7. Shut down computer

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Vita

Education

University of Illinois at Chicago, Chicago, IL 2007-Present

PhD Candidate Bioengineering, expected Spring 2012

Advisor: Vincent M. Wang, Ph. D., Assistant Professor of Orthopedic Surgery and Bioengineering at Rush University Medical Center

Trinity College, Hartford, CT 2001-2005

Bachelor of Science in Engineering

Concentration: Biomedical Electrical Engineering

Relevant Experience

Graduate Research Assistantship 2007-present

- Designed biomechanical, biochemical, histological testing protocols for connective tissue.
- Structure-function analysis of transgenic mice tendons using biochemical, biomechanical, and histological assays.
- Healing study of anterior cruciate ligament repair in a rabbit model.
- Characterized the human patellar tendon with biomechanical and histological assays.
- Evaluated surgical technique and equipment in a cadaveric model.
- Performed extensive statistical analysis, including a priori sample size calculation and post-hoc power assessment.

Teaching Assistantship 2007-2008

- Helped mentor groups at University of Illinois in Chicago in Clinical Problems in Bioengineering.
- Planned/graded homework, had study sessions and taught a few classes of Introduction to Bioengineering.

Research Technologist 2005-2007

- Researched at University of Chicago's Center for EPR Imaging *in vivo* Physiology under Dr. Howard Halpern.
- Worked with EPRI and MRI while studying oxygen effect on tumor biology.
- Helped tumor set-up, image acquisition, and data analysis.

Undergraduate Research Assistantship 2004-2005

- Researched in the Trinity College Electrophysiology lab studying electrical brain waves in 12-day old rats.
- Made own electrodes, implanted the electrodes in the rat's brain and recorded the signal from the brain.

Mentor and Member of a Robot Team Spring of 2002, 2003

- Led a team of freshman students through the process of making a robot that competed in the 2003 Trinity College International Fire Fighting Contest.
- Competed in the 2002 Trinity College International Fire Fighting Robot Contest by designing, constructing and programming the robot.

Co-Leader of A TIER group Spring of 2002

- Assisted in teaching high school students in basic biomedical engineering concepts.

Full Length Publications

Yanke A, **Bell RM**, Lee A, Kang RW, Mather C, Shewman EF, Wang VM, Bach BR. Human Patellar Tendon Allograft Biomechanics: Part 1- Regional Properties. American Journal of Sports Medicine. Submitted.

Yanke A, **Bell RM**, Lee A, Kang RW, Mather C, Shewman EF, Wang VM, Bach BR. Human Patellar Tendon Allograft Biomechanics: Part 2- Effect of Low-Dose Gamma Irradiation. American Journal of Sports Medicine. Submitted.

Wang VM, **Bell RM**, Thakore R, Eyre D, Galante J, Li J, Sandy JD, Plaas A. Murine Tendon Function is Adversely Affected by Aggrecan Accumulation Due to the Knockout of ADAMTS5. Journal of Orthopedic Research. Journal of Orthopedic Research. 2012.

Bhatia S, **Bell R**, Frank R, Rodeo S, Bach BR, Cole BJ, Chubinskaya S, Wang VM, Verma NN. Bony Incorporation of Soft Tissue ACL Grafts in an Animal Model: Autograft vs Allograft with Low Dose Gamma Irradiation. American Journal of Sports Medicine. 2011.

Slabaugh MM, Frank RM, Van Thiel GS, **Bell RM,** Wang VM, Trenhaile S, Provenchar CM, Romeo AA, Verma NN. Biceps Tenodesis With Interference Screw Fixation: A Biomechanical Comparison of Screw Length and Diameter. Arthroscopy. 2010 Oct 26.

Bell RM, Wang VM. (2010) Functional Importance of the ACL: Biomechanical Considerations. In Bach BR and Provenchar MT, *ACL Surgery: How to Get it Right the First Time and What to Do if it Fails* (pg. 19-29). New Jersey, USA: SLACK Incorporated.

Haney CR, Parasca AD, Fan X, **Bell RM**, Zamora MA, Karczmar GS, Mauceri HJ, Halpern HJ, Weichselbaum RR, Pelizzari CA. Characterization of Response to Radiation

Mediated Gene Therapy by Means of Multimodality Imaging. Magnetic Resonance Medicine. 2009 Aug; 62(2):348-56.

Elas M, **Bell R,** Hleihel D, Barth ED, McFaul C, Haney CR, Bielanska J, Pustelny K, Ahn KH, Pelizzari CA, Kocherginsky M, Halpern HJ. Electron Paramagnetic Resonance Oxygen Image Hypoxic Fraction Plus Radiation Dose Strongly Correlates With Tumor Cure in FSa Firbrosarcomas. International Journal of Radiation Oncology Biology Physiology. 2008 June 1;71(2):542-9.

Conference Presentations

Bell RM, Gorski D, Bartels A, Li J, Shewman EF, Galante J, Sandy JD, Plaas AH, Wang VM. Evidence that Aggrecan-Rich Deposits Cause Tendinopathies by Blocking Fibrogenesis. International Symposium of Ligaments and Tendons, San Francisco, CA 2012. Short Talk.

Bell RM, Bartels A, Vigneswaran H, Li J, Galante J, Sandy JD, Plaas AH, Wang VM. Intratendinous TGF-b1 Injection with Treadmill Running Causes A Major Loss In Tensile Properties of the Achilles Tendon of ADAMTS5 Knockout but not Wild Type Mice. Orthopaedic Research Society, San Francisco, CA 2012. Poster 2304.

Bell RM, Yanke A, Lee A, Kang RW, Mather C, Shewman EF, Wang VM, Bach BR. Comparison of Regional Biomechanical and BMD Properties Within Human Patellar Tendon Allografts and Effect of Low-Dose Irradiation. Orthopaedic Research Society, San Francisco, CA 2012. Poster 1344.

Bell RM, Li J, Shewman EF, Bach BR, Galante J, Leurgans S, Sandy JD, Plaas AH, Wang VM. Regional Analysis of Mechanical Properties of the Human Patellar Tendon. Orthopaedic Research Society, Long Beach CA 2011. Poster 920.

Bell RM, Bhatia S, Frank RM, Rodeo S, Bach BR, Chubinskaya S, Wang VM, Verma NN. Soft Tissue Healing in an In Vivo ACL Allograft Model: Comparison to Autograft and Effects of Gamma Irradiation. Orthopaedic Research Society, Long Beach CA 2011, Poster 948.

Bell RM, Slabaugh MM, Frank RM, Van Thiel GS, Wang VM, Provenchar CM, Romeo AA, Verma NN. Biceps Tenodesis With Interference Screw Fixation: A Biomechanical Comparison of Screw Size and Technique. International Society of Arthroscopy, Knee Surgery and Orthopaedic Sports Medicine Congress, Rio De Janeiro, Brazil, 2011. E-Poster 6141.

Bell RM, Thakore R, Sandy JD, Malfait AM, Plaas AH, Wang VM. Effects of ADAMTS-5 and ADAMTS4 Knockout on Biomechanical Properties. Orthopaedic Research Society, New Orleans, LA 2010. Short Talk 276.

Bell RM, Sandy JD, Malfait AM, Plaas AH, Wang VM. ADAMTS-5 Knockout Alters Tendon Ultrastructural and Biochemical Properties. Orthopaedic Research Society, New Orleans, LA 2010. Poster 1098.

Bell RM, Slabaugh MM, Frank RM, Van Thiel GS, Provenchar CM, Romeo AA, Wang VM, Verma NN. Biceps Tenodesis With Interference Screw Fixation: A Biomechanical Comparison of Screw Size and Technique. Orthopaedic Research Society, New Orleans, LA 2010. Poster 1168.

Bell RM, Sandy JD, Plaas AH, Wang VM. ADAMTS5 Ablation Alters Tendon Biomechanical, Ultrastructural, and Biochemical Properties. Midwest Connective Tissue Workshop 2009, Poster 7.

Bell RM, Sandy JD, Plaas AH, Wang VM. ADAMTS5 Ablation Alters Tendon Biomechanical Properties. Orthopaedic Research Society, Las Vegas, NV 2009. Poster 1399.

Bell R, Elas M, Barth E, McFaul C, Haney CR, Bielanska J, Pustelny K, Ahn KH, Pelizzari CA, Halpern HJ. EPR Oxygen Imaging Used to Predict Tumor Curability. EPR 2007. 2007.

Skills

Computer

- Microsoft Office, Java, C++, Matlab, LabView, GraphPad, ImageJ, G*Power

Experimental Evaluation and Analysis

- Materials Testing Systems, Digital Motion Analysis, Western Blotting, Fluorophore-Assisted Carbohydrate Electrophoresis, Histology, RT-PCR, Genotyping

Awards

Jacquelin Perry Resident Research Award funded by Zimmer/RJOS 2012

- **"**Bony Incorporation of Soft Tissue ACL Grafts in an Animal Model: Autograft vs Allograft with Low Dose Gamma Irradiation." Bhatia S, **Bell R**, Frank R, Rodeo S, Bach BR, Cole BJ, Chubinskaya S, Wang VM, Verma NN.

Thomas P. Andriacchi Fellowship for Biomechanics Research 2010

Graduate Student Council Travel Award 2009

- "ADAMTS5 Ablation Alters Tendon Biomechanical Properties." **Bell RM,** Sandy JD, Plaas AH, Wang VM.

Society of Women Engineers Best Student Award 2003

Activities

Mentor high school students, HFS Scholars, Chicago, IL 2008-present

Varsity Basketball Team, Trinity College 2001-2005

- Captain- 2003-2004

Society of Women Engineers 2002-2005

- Treasurer – 2002-2003
- Secretary – 2004-2005