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A Better Treatment for Tendinopathy: Molecular Insights from Tendon Development, Injury, and Exercise Studies

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A Better Treatment for Tendinopathy: Molecular Insights from Tendon Development, Injury, and Exercise Studies

By

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DISSERTATION

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Committee in Charge

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Declaration

I declare that this dissertation is my own unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of California, Davis.

It has not been submitted for any degree or examination at any other University.

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This dissertation is dedicated to those working to improve quality of life for everyone.

Abstract

The ability to move without pain is essential to many occupations and sports. When chronic tendon pain becomes limiting, it is diagnosed as tendinopathy. At a minimum, this tendon pain limits quality of life. For athletes engaged in professional sport, tendinopathy can be devastating and potentially career ending. The exact etiology of tendinopathy is unknown. However, it is especially common in activities that involve repetitive movements and high jerk (typing, jumping, etc.). Since this describes many sports and professions, it should not be surprising that tendon sprains, strains, and pulls are the leading cause of time away from work. The prevalence of tendon injury is highest in professional basketball with ~75% of players having patellar tendon lesions (Benítez-Martínez *et al.*, 2019). Despite the prevalence and high personal and economic costs, there has been no progress in treatments for chronic tendon injuries over the last 25 years. This is in part due to a lack of understanding about basic tendon biology and adaptations that occur with growth, injury, and repetitive loading. This dissertation investigated post-natal development of the Achilles and patellar tendons, developed a rat model of patellar tendinopathy, determined the molecular response of different types (isometric and dynamic) of loads on a tendinopathic tendon, and characterized spatial gene expression in a healthy adult rat patellar tendon.

The molecular and mechanical changes that occur during rat post-natal Achilles and patellar tendon development were characterized at P7 (before walking), P14 (shortly after the onset of walking), and P28 (motor maturity). The results are presented in Chapter 2. From P7 to P28, The Achilles increased 3-fold in length, whereas the patellar tendon increased largely in cross-sectional area. Despite the different modes of growth there was a ~10-fold increase in ultimate tensile strength of both the Achilles and patellar tendons. The increase in Achilles length resulted in a greater increase in modulus, whereas the greater cross-sectional area of the

patellar meant a larger increase in maximal tensile load. The tendons shared transcriptional similarity at P7 and P14 but diverged at P28. However, there were still many shared processes indicative of tendon post-natal development. These changes included increased extracellular matrix (ECM) gene expression and decreased cell cycle and mitochondrial gene expression. Ribosomal gene expression also significantly decreased in the Achilles tendon and tended to decrease in the patellar tendon. From the genes that were highly expressed at P28 in the patellar tendon, STAT signaling was identified as a downregulated pathway. We hypothesized that these genes contributed to tendon radial growth. STAT inhibition in engineered human ligaments caused an increase collagen content and maximal tensile load (MTL). These results suggest that our developmental transcriptomic data may provide insight into targets to improve tendon function both after injury and during development/training.

There are many models of rodent tendon injury; however, no molecular comparison had been made to the human condition. We modeled tendinopathy in the rat using a biopsy punch to remove the central third of the patellar tendon, followed by two weeks of cage activity to allow scar formation. The scar tissue was sequenced with 3' Tag RNA-seq. Differential gene expression analysis of the healthy versus scar tissues identified an increase in ECM gene expression, decrease in mitochondrial gene expression, and no change in inflammatory pathways. The increase in ECM and decrease in mitochondrial genes meant that the scar was not recapitulating the developmental pattern of gene expression observed in Chapter 2. There was also a visible increase in vascularity and cellularity in the scar tissue. These changes within the tendon are nearly identical to human tendinopathy (Jelinsky *et al.*, 2011). Next, we studied the effect of two distinct types of mechanical load (dynamic and isometric) on gene expression in tendinopathy. The isometric load (4 x 30 s) caused an increased in scleraxis and collagen Ia1 expression relative to the contralateral control leg. In contrast, the time under tension-matched dynamic load (360 x 0.33 s) caused in increase in type II collagen expression relative to the

isometric loaded group. These data suggest that isometric loads may be used to treat a tendon injury and that the type of load, rather than the time under tension, is an important consideration when exercising a tendinopathic tendon.

The last chapter of the dissertation presents the first complete report of spatial gene expression in tendon. I used the 10X Visium Platform to profile cell type and gene expression location in two adult rat patellar tendon samples. The cell type classification was performed with an anchoring analysis to a single cell RNA sequencing data set from mouse Achilles tendon (de Micheli *et al.*, 2020). There were four cell populations identified: tendon fibroblasts (two main subpopulations), red blood cells, immune cells, and pericytes. The tendon midsubstance contained one tendon fibroblast subpopulation, while the other tendon cell subpopulation was located to the periphery of the main population and within the strip of connective tissue to the side of the tendon. This loose connective tissue also contained red blood cells, immune cells, and pericytes. The top expressed spatially variable genes in both samples were genes with known function in tendon (*Col1a1*, *Dcn*, *Fmod*, *Sparc*, and *Comp*). Interestingly, the two most common collagens 1a1 and 3a1 were expressed in separate populations of fibroblasts. I also identified one gene (*AABR07000398.1*) without a known function that was highly expressed in a pattern opposite to *Col1a1*. These data are important because they describe the spatial separation of two distinct fibroblast populations and provide the basis for better understanding spatial gene expression following injury.

This dissertation provides novel insight into tendon post-natal development and spatial gene expression. It also validates a rodent model of tendinopathy and identifies molecular changes that occur after two different types of exercise on a tendinopathic tendon. Together, these data support a model where force is passed through the center of a healthy tendon and this force is transmitted through a matrix that is maintained by a population of tendon fibroblasts. Following injury, the scarred portion of the tendon is shielded from stress/strain and this may allow the

infiltration of the tendon by a second population of fibroblasts. In order to return normal function of the tendon after injury, long holds are necessary to overcome stress shielding. Further, my data suggest that JAK/STAT inhibitors may improve tendon regeneration after injury. These data fill important knowledge gaps in the field of tendon biology, provide evidence for the first drug to treat tendinopathy, and establish the basis for future studies to reverse tendinopathy.

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Chapter 1. Loading in Tendon Development, Adult Tendon, and Tendinopathy

Introduction

Tendons connect muscles to bones and function to transfer force within the musculoskeletal system. However, tendons are subjected to acute and chronic injury that, if not properly repaired, could impair the ability to transfer force. Tendinopathy is considered an imbalance between these small injuries and the cell's ability to repair the matrix. Tendinopathy is one of the most common injuries in the general population accounting for around 20% of all musculoskeletal complaints (Forde *et al.*, 2005). In the United States, musculoskeletal strains, sprains, and tears have accounted for at least a third of all missed workdays for over 50 years (Robinson, 1988; U.S. Department of Labor, 2015). These injuries not only affect productivity, but also have a considerable effect on quality of life. This chapter will focus on the influence of load on the biology and mechanics of healthy and tendinopathic tendons and will use this information to develop a hypothesis as to the optimal strategy to treat tendinopathy.

Tendon

Tendon Composition, Structure, and Cellularity

A tendon is composed of longitudinal bundles of collagen fibers that connect muscle to bone (Figure 1). Type I collagen forms the tendon backbone and is responsible for force transmission. Other components of the tendon extracellular matrix include other collagen isoforms and ground substance: glycoproteins, proteoglycans, and water. The total collagen content of a tendon accounts for approximately 70-80% of adult tendon dry weight (Rumian *et al.*, 2007; Zhang *et al.*, 2020) and type I collagen accounts for 65-80% of the total collagen content (Riley *et al.*, 1994; Little *et al.*, 2014). Water accounts for 55-70% of the wet mass of a tendon (Elliott, 1965; Ristaniemi *et al.*, 2020).

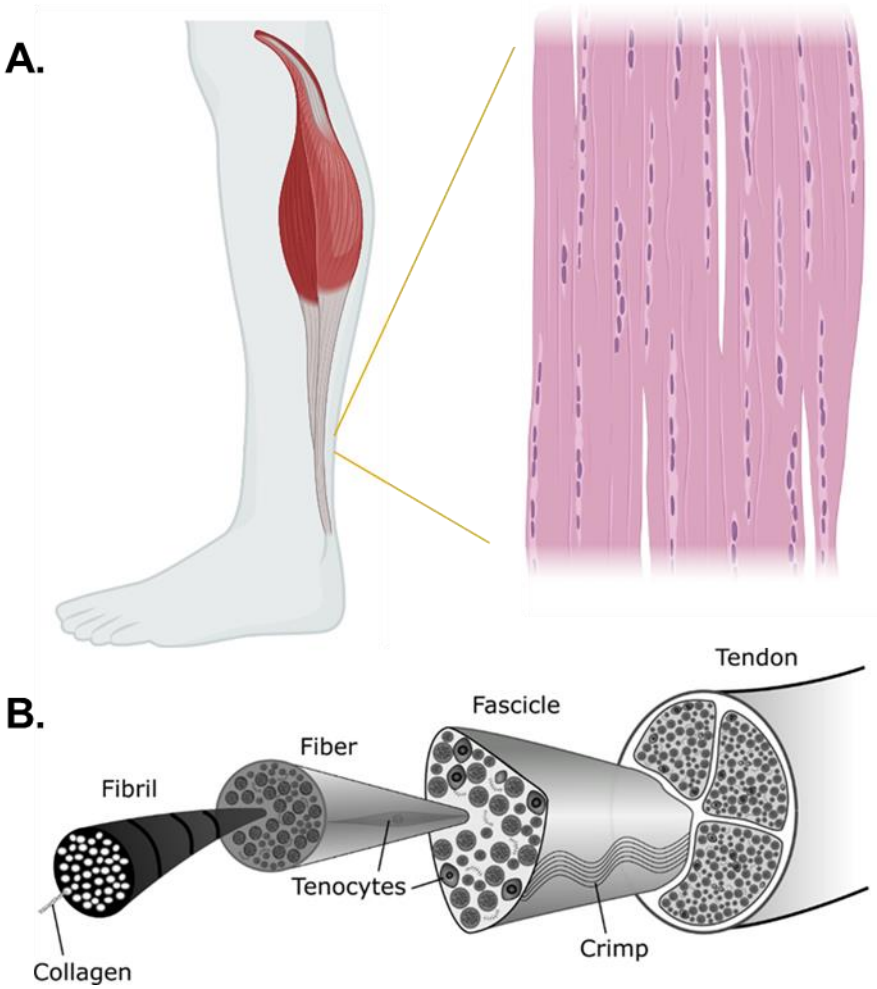

Figure 1. Tendon Structure and Collagen Organization. Tendon connects muscle to bone and is composed collagen fibrils/ fibers/ fascicles. The primary cell population are collagen-synthesizing tenocytes that are located in between collagen fibers and fascicles.

Force transmission within a tendon relies on the organization of long rope-like collagen I proteins that are packed into hierarchical bundles along the long axis of the tendon (Handsfield *et al.*, 2016; Lee & Elliott, 2019) (Figure 1B). Collagen I is the most common type of collagen and is formed as a heterotrimeric molecule of two alpha 1 and one alpha 2 chains (DiChiara *et al.*, 2018). The triple helix region of the collagen chain contains the characteristic amino acid motif (Gly-X-Y). Glycine occupies every third residue to fit into the center of the triple helix while the X & Y amino acids are most frequently identified as proline and hydroxyproline (Ramshaw *et al.*,

1998). Other functionally relevant second and third residues include lysine, hydroxylysine, and arginine (Ramshaw *et al.*, 1998; Gaar *et al.*, 2020). Each chain is synthesized in the endoplasmic reticulum as a pre-pro-protein and undergoes multiple post-translational modifications before forming a mature triple helix procollagen molecule in the matrix. Important post-translational modifications include hydroxylation of proline residuals for helix stability (Berg & Prockop, 1973; Rosenbloom *et al.*, 1973) and intermolecular crosslinking of adjacent lysine residues (Siegel, 1976) (Figure 2). The three procollagen chains assemble into a right-handed triple helix to form procollagen I, and this procollagen molecule is deposited into the extracellular matrix along the line of force (Kapacee *et al.*, 2008). The procollagen I protofilament is processed into a mature collagen molecule after the cleavage of the N and C terminal propeptides (Ricard-Blum & Ruggiero, 2005). The collagen fibrils are then free to assemble in the pericellular space where the N and C-termini telopeptides are enzymatically crosslinked by lysyl oxidase (Siegel, 1976). These collagen fibrils pack into fibers that are surrounded by an endotenon and bordered by a population of resident tendon cells to help maintain the matrix. Individual collagen fibers can slide past each other during movement, but this movement is resisted by intermolecular crosslinks. Bundles of collagen fibers can be further organized into fascicles, which are surrounded by an epitenon sheath and separated from neighboring fascicles by the interfascicular space where nerves and blood vessels pass (O'Brien, 1997; Khan *et al.*, 1999). Fascicles are not present in smaller organisms such as rodents (Lee & Elliott, 2019). In large animals, fascicles can slide independently to accommodate excursion differences in tendon regions during complex movements, such as those seen in the supraspinatus tendon during shoulder adduction and abduction (Fallon *et al.*, 2002). The fascicles within a single tendon are encased by a synovial sheath (i.e. hand and foot tendons) or a loose fibrillar tissue known as the paratenon (i.e. Achilles) (Benjamin *et al.*, 2008).

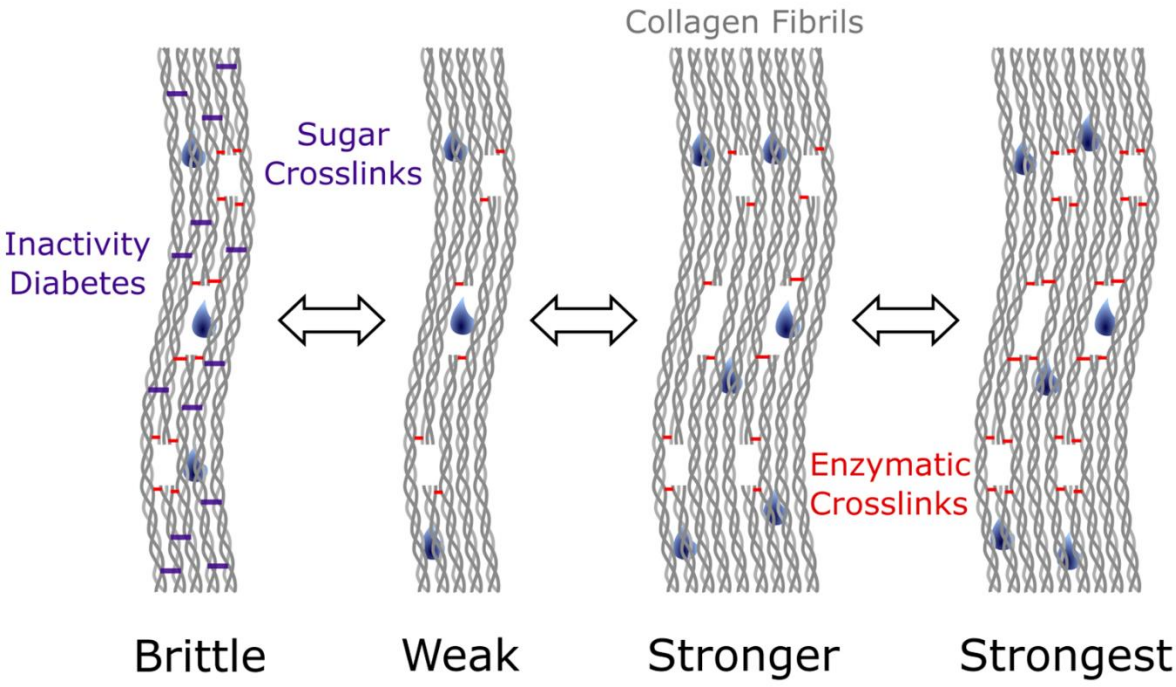

Figure 2. **Collagen Crosslinks in Tendon.** Collagen fibrils are enzymatically crosslinked together at the collagen telopeptide. AGE (sugar crosslinks) can be made anywhere along the collagen molecule. There is a positive relationship between the number of crosslinks and tendon stiffness.

Besides type I collagen, the tendon matrix contains numerous other collagen isoforms and non-collagenous proteins. It is important to note that the concentration of these proteins will vary regionally within a tendon and between tendons with distinct functions (Thorpe *et al.*, 2016). Type III collagen accounts for up to 10% of the dry mass of a normal tendon (Williams *et al.*, 1980; Little *et al.*, 2014). Little is known about the function of type III collagen in healthy tendon. *In vitro*, collagen III tends to form smaller fibrils (Wiedemann *et al.*, 1975; Lapiere *et al.*, 1977) and assists with the longitudinal growth of type I collagen fibrils by limiting lateral growth (Eryilmaz *et al.*, 2017). The patellar tendon and anterior cruciate ligament can be distinguished with the patellar tendon having a greater type I collagen and lower type III collagen concentration (Little *et al.*, 2014). Other minor collagens such as V, VI, XI, XII and XIV are suggested to have a role in collagen fibril and fiber organization (Taye *et al.*, 2020). Type II collagen is most common in cartilage but can occur as an adaptation to compression in tendon fibrocartilage such as in

wrap-around tendons, under an aponeurosis, or at the enthesis (Benjamin & Ralphs, 1998; de Palma *et al.*, 2004).

Glycoproteins (GAGs) are a large class of non-collagen proteins that are characterized by a protein core and small covalently linked carbohydrate side chains. GAGs are linear polysaccharides with an amino sugar. Tenomodulin (TNMD) and thrombospondin 4 (THBS4) are two glycoproteins that are highly expressed in tendon and ligament compared to other musculoskeletal tissues (Jelinsky *et al.*, 2010). Tenomodulin is a transmembrane glycoprotein (Shukunami *et al.*, 2001) with unknown binding partners (Taye *et al.*, 2020) that is important for proliferation of tendon stem/progenitor cells (Alberton *et al.*, 2015). *Tnmd* KO mice have reduced cell proliferation in development and abnormal collagen fibril diameter (Docheva *et al.*, 2005). Thrombospondin 4 is an adhesive glycoprotein that forms complexes with cartilage oligomeric matrix protein (COMP) (Södersten *et al.*, 2006). COMP belongs to the thrombospondin protein family (Adams, 2001). Within weight bearing tendons, COMP protein is more common in regions that lack tension (Smith *et al.*, 1997) and may play a role in collagen fibrillogenesis (Sodersten *et al.*, 2005; Halász *et al.*, 2007). Tenascin C is another glycoprotein that is associated with areas of compression in tendon (de Palma *et al.*, 2004) and its levels regulated by mechanical load (Järvinen *et al.*, 2003).

Proteoglycans make up a subgroup of glycoproteins and are defined by the covalent attachment of long carbohydrate glycosaminoglycan side chains (GAGs) to a core protein. Proteoglycans (PGs) are embedded within the collagen matrix and help regulate collagen organization and tissue hydration. The abundance of specific PGs is also dependent on the biomechanical environment. Canonical small leucine rich proteoglycans (SLRPs) are a class of PGs with collagen binding domains that are characterized by a leucine-rich repeat region (McEwan *et al.*, 2006). Decorin and biglycan are modified with one or two, respectively, dermatan sulfate GAG side chains (Vogel, 2004). Immediately after birth, biglycan content is nearly twice that of

decorin. As animals begin to walk (Williams & Scott, 1954) gene expression and protein levels of decorin increase while those of biglycan decrease (Birk *et al.*, 1995; Zhang *et al.*, 2006). However, the amount of decorin associated with collagen fibrils decreases during the period of rapid fibril growth (increased fibril length and diameter) (Birk *et al.*, 1995). Decorin binds procollagen to assist with collagen fibril assembly (Keene *et al.*, 2000), and it is suggested that it may prevent the association of collagen fibrils with each other. Decorin is moderately expressed into adulthood, while biglycan expression is minimal in mature tendons (Birk *et al.*, 1995; Zhang *et al.*, 2006). In adult tendons, decorin and biglycan are required to maintain collagen fibril diameter and mechanical properties (Robinson *et al.*, 2017). Fibromodulin and lumican are two other SLRPs common in tendon that contain two keratan sulfate GAG chains (Vogel, 2004). Lumican and fibromodulin prohibit collagen fibril assembly early in post-natal tendon development, then transition to assist with fibril growth with fibromodulin continuing to promote fibril growth throughout adolescence (Ezura *et al.*, 2000). SLRPs are expressed to a greater degree in cells under tension, whereas tendon cells loaded in compression synthesize more large PGs (Ehlers & Vogel, 1998). Large PGs like aggrecan hold substantially more water, helping to resist matrix compression (Kiani *et al.*, 2002). For this reason, aggrecan is more prevalent in compressed tendon regions and cartilage (Evanko & Vogel, 1990; Vogel *et al.*, 1994; Perez-Castro & Vogel, 1999; Kiani *et al.*, 2002). Versican, another large proteoglycan, is present in tendon but is higher in concentration in ligaments (Little *et al.*, 2014; Kharaz *et al.*, 2018).

Tendons have relatively few cells compared to other tissues, yet there are multiple cell types present in a tendon each with their own distinct function. Over 70% of cells in a tendon are classified as tenocytes which synthesize high levels of collagen and function to maintain the matrix. Endothelial cells are the next most abundant cell population with the remaining cell fractions comprising of pericytes, nerve cells, red blood cells, and immune cells (De Micheli *et al.*, 2020; Kendal *et al.*, 2020). Tenocytes are elongated spindle-shaped fibroblast tendon cells

with extended cell processes that are located between collagen fibers in the endotenon and exterior to collagen fascicles in the epitenon (O'Brien, 1997). In larger organisms with fascicles, the bulk of cells are located in the interfascicular matrix (Banes *et al.*, 1988; Thorpe & Screen, 2016). Lastly, tendons are surrounded by a paratenon which contains a thin layer of cells that do not express scleraxis (Dyment *et al.*, 2013). The cell population for the tendon proper cells and peritenon cells have different transcriptional characteristics and important expression differences, such as increased expression of *scleraxis* (*Scx*) and *mohawk* (*Mkx*) in the tendon proper progenitor cells (Mienaltowski *et al.*, 2019).

Multiple studies have demonstrated the existence of stem/progenitor cells within a tendon. However, the exact location and markers vary from study to study. In 2007, Marian Young's group was first to show that cells isolated from human (8-12 years old, hamstring) and mouse (6-8 week old, patellar tendon (without sheath) met the stem cell classification criteria: capacity for self-renewal, clonogenicity, and multipotency (Bi *et al.*, 2007). Shortly after, a population of tendon stem cells from the middle region of the rabbit patellar and Achilles tendon was identified (Zhang & Wang, 2010). Mienaltowski and colleagues later showed that the (P30 mouse) stem/progenitor cells within the tendon proper and peritenon are regionally distinct (Mienaltowski *et al.*, 2013, 2019). Tendon stem cells were identified in the patellar tendon sheath of adult rats (3 month) as a population of *Tppp3*$^{+}$*Pgdfra*$^{+}$ cells (Harvey *et al.*, 2019).

Tendon cells, like many other cells, are linked to each other and the extracellular matrix through cell junctions. The main classes of cell junctions are occluding junctions (e.g. tight junctions), anchoring junctions (e.g. cadherins and integrins), and communicating junctions (e.g. gap junctions) (Alberts B *et al.*, 2002). These cell junctions in developing and adult tendons help facilitate communication between tenocytes. In embryonic and early-post natal development, there is a denser cell network in the tendon due to the relatively higher cellularity and lower

matrix composition (Evanko & Vogel, 1990; Curwin *et al.*, 1994). However, this cell-cell communication network still persists in adult tendon (McNeilly *et al.*, 1996).

Gap junctions in vertebrates are composed of proteins from the connexin protein family. They allow for cell-cell communication via the diffusion of ions and small molecules between cells. Connexin-32 and -43 are present at high levels in embryonic tendon and decline in the post-natal period (Stanley *et al.*, 2007). In the adult tendon, the localization of the junctions is specialized. Connexin 43 is found at meeting points between cell processes and cell bodies, whereas connexin 32 is only found between cell bodies (McNeilly *et al.*, 1996).

Cadherins are a type of cell-cell anchoring junction. Cadherin-11 and N-cadherin are the two most commonly studied cadherins and more is known about their role in tendon development than the adult tendon. Embryonic tendons require cadherin-11 and N-cadherin to form properly, yet the N-cadherin is localized to the tendon periphery and cadherin-11 is restricted to the midsubstance (Luo *et al.*, 2001; Richardson *et al.*, 2007). In adult tendon, N-cadherin is found at cell-cell junctions. *In vitro* radial stretch of adult tendon cells has been shown to increase the amount of N-cadherin (Ralphs *et al.*, 2002).

Integrins have an essential role in the transmission of mechanical information as they function as a physical link between the extracellular matrix and the intracellular actin cytoskeleton. There are many types of integrin subunits: α1 and α2 are the most widely expressed (Docheva *et al.*, 2014). Knockout of integrin α1 did not affect tendon collagen content but did reduce *Col1a1* expression in muscle (Bayer *et al.*, 2020). However, only integrin α11 is specifically localized around dense type I collagen and binds type I collagen at a high affinity (Tiger *et al.*, 2001; Docheva *et al.*, 2014). It was also observed that the integrin α11 promoter contains multiple consensus sequences for binding of a tendon/ ligament specific transcription factor, scleraxis, suggesting that this integrin may be more relevant to mechanotransduction in tendon (Tiger *et*

al., 2001). Mechanical stretch of tendon stem/ progenitor cells increased the expression of multiple integrin subtypes (including α1/2/11) and also the phosphorylation of ERK and p38 which are kinases downstream of integrin signaling (Popov *et al.*, 2015). The notion that integrins have a role in tendon mechanotransduction is supported by another study that demonstrated that fibroblasts with ligand- bound integrins were able to stiffen their cytoskeleton in response to a restraining force (Choquet *et al.*, 1997). Overall, the data suggest that tendon cells can form a chemically connected syncytium, modulate the production of specific ECM components in response to load, which in turn affects tendon mechanics.

Tendon Mechanics (viscoelasticity, stiffness/ crosslinks, biomechanical types)

Tendons are commonly appreciated for their mechanical function: they anchor or transfer force from muscle to bone. The composition of a tendon underlies its mechanical and material properties. Tendons are a non-linear viscoelastic material meaning that they have different mechanical behavior depending on the rate of loading. The non-linearity of the tendon stress-strain curve is due to the uncrimping of collagen fibers that occurs until ~2% strain. Further strain causes linear elongation of the collagen until rupture (Figure 3A). When a tendon is loaded slowly, it is more compliant and able to elongate more before failure. During fast movements, tendons are stiffer and more effective at transferring force (Clemmer *et al.*, 2010). In addition to the speed of the movement altering the mechanical response of a tendon, the “smoothness” or jerk of the movement is also important to consider. Jerk is the rate of movement acceleration and high jerk movements have been hypothesized alter the microstructure of tendons (Lapinski *et al.*, 2019).

Tendon viscoelasticity results from the combined effects of the mesh-like matrix, the water trapped within the matrix, and the isovolumetric nature of the tissue. The faster the movement, the less water can escape the matrix, resulting in greater resistance to radial compression that occurs when an isovolumetric substance is stretched. Individual collagen fibrils (Svensson *et al.*,

2010; Baldwin *et al.*, 2014) and fascicles (Clemmer *et al.*, 2010) are also viscoelastic indicating that this is a basic mechanical property of the interaction between collagen protein and the ground substance.

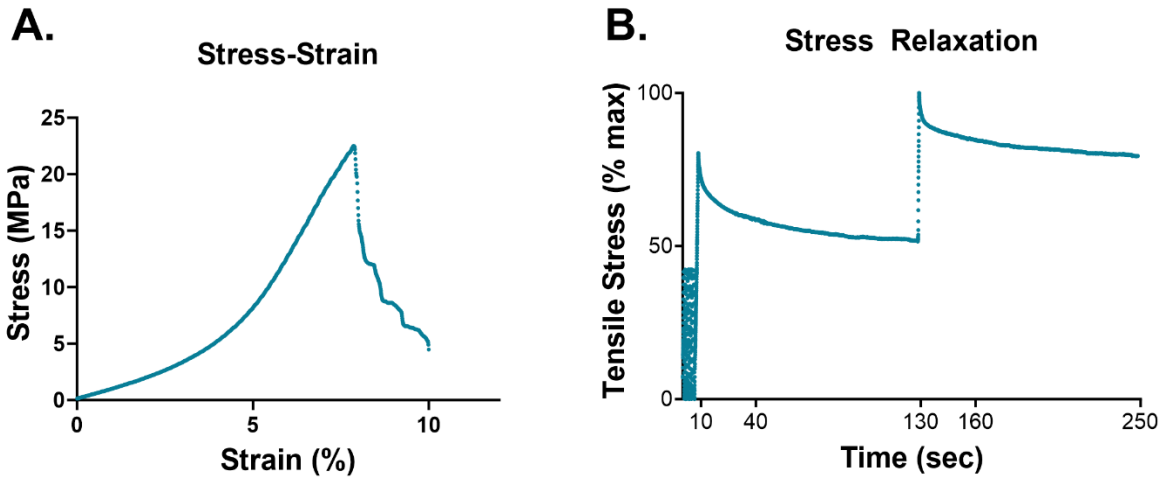

Figure 3. Tendon Stress-Strain & Stress Relaxation Graphs. Tendon is a non-linear viscoelastic material. A) When a tendon is elongated, collagen fibers uncrimp (toe region), extend (linear region), then rupture (yield point). B) Holding a tendon at a set length causes a decrease in tissue stress. The majority of stress relaxation occurs within the first 30 seconds.

Given that tendons are a viscoelastic material, they exhibit two important non-linear behaviors under load: stress relaxation and creep. Stress relaxation occurs when a tendon is strained and held at a set length and the stress within the tissues decreases (Figure 3B). Creep is the opposite phenomena, where a tendon must be increasingly strained to maintain the same amount of stress. Both behaviors depend on the amount of stress or strain applied to the tissue, the loading history, and numerous biological parameters, which makes these properties hard to compare across studies unless they are conducted under the exact same experimental conditions.

In the human patellar tendon, over half of total stress relaxation occurs within the first 30 seconds, as demonstrated by stress relaxation tests conducted at 2% strain. The stress relaxation response is more pronounced in larger (>4 mm^2 CSA) than smaller (~1 mm^2 CSA)

specimens (Atkinson *et al.*, 1999). The amount of stress relaxation can also vary with tendon type: energy storing tendons exhibit less stress relaxation than positional tendons (Shepherd *et al.*, 2014). One possible explanation for these differences is the GAG content of the tendon, since GAGs help resist stress relaxation (Legerlotz *et al.*, 2013). Interestingly, there is a positive correlation between tendon water content and stress relaxation (Atkinson *et al.*, 1999). If the GAGs in the tendon were helping increase tissue hydration, tendons with more water and GAGs should exhibit more stress relaxation. Therefore, these data suggest other parameters, possibly crosslinking can contribute to resistance to stress relaxation.

The amount of tendon creep depends on the number of loading cycles and the behavior of the contracting muscle. Creep increases with force and is reduced with a faster contraction at a given force (Pearson *et al.*, 2007). In the human Achilles tendon, the amount of creep reaches a plateau after 5 min of activity at approximately 3.5% in adults during cyclic loading to 25-35% of MVC (Hawkins *et al.*, 2009).

Tendon stiffness is dictated by the content, orientation, and crosslinking of collagen and modulated by the GAG and water content of the tissue (Figure 2). Collagen crosslinking occurs both enzymatically and non-enzymatically. Enzymatic crosslinking occurs between mature collagen molecules at the collagen telopeptide while non-enzymatic crosslinks occur at any place along the collagen molecule at lysine and arginine residues (Figure 2). Collagen fibrils form in the ECM and are stabilized lysyl oxidase (LOX) mediated crosslinks at lysine and hydroxylysine residues at the telopeptide (Gaar *et al.*, 2020). Enzymes belonging to the LOX family oxidize lysine or hydroxylysine residues into aldehydes like allysine, the lysine derived aldehyde, or hydroxyallysine, the hydroxylysine derived aldehyde (Siegel, 1976; Eyre, 1984). The allysine reaction dominates in the relatively unloaded connective tissues of skin and rat tail tendon (Bailey & Peach, 1968; Davis & Bailey, 1971). Interestingly, in tissues with a high mechanical load such as bone, cartilage, and the Achilles tendon the crosslinks follow the hydroxyallysine

route (Mechanic & Tanzer, 1970; Mechanic *et al.*, 1971; Davis & Bailey, 1971). End products of the hydroxyallysine route are trifunctional 3-hydroxypyridinium crosslinks that connect three collagen molecules together. Hydroxylysl pyridinoline (HP) is the major hydroxyallysine crosslink and contains three hydroxylysines. Lysyl pyridinoline (LP) is less abundant and contains two hydroxylysines and one lysine (Eyre, 1984). In contrast to the enzymatic crosslinks that link the telopeptide of three collagen molecules together, advanced glycation end products (AGEs) are non-enzymatic crosslinks that can link any region between two adjacent collagen molecules. The AGE reaction begins with addition of glucose to the amino group of typically lysine or arginine, leading to the formation of AGEs such as glucosepane, pentosidine, N(epsilon)-(carboxymethyl)lysine (CML), and N(epsilon)-(carboxyethyl)lysine (CEL). Functionally, AGEs limit fibril and fiber sliding (Hammes *et al.*, 1991; Corman *et al.*, 1998; Li *et al.*, 2013; Gautieri *et al.*, 2014) and results in collagen that turns over slower (Verzijl *et al.*, 2000).

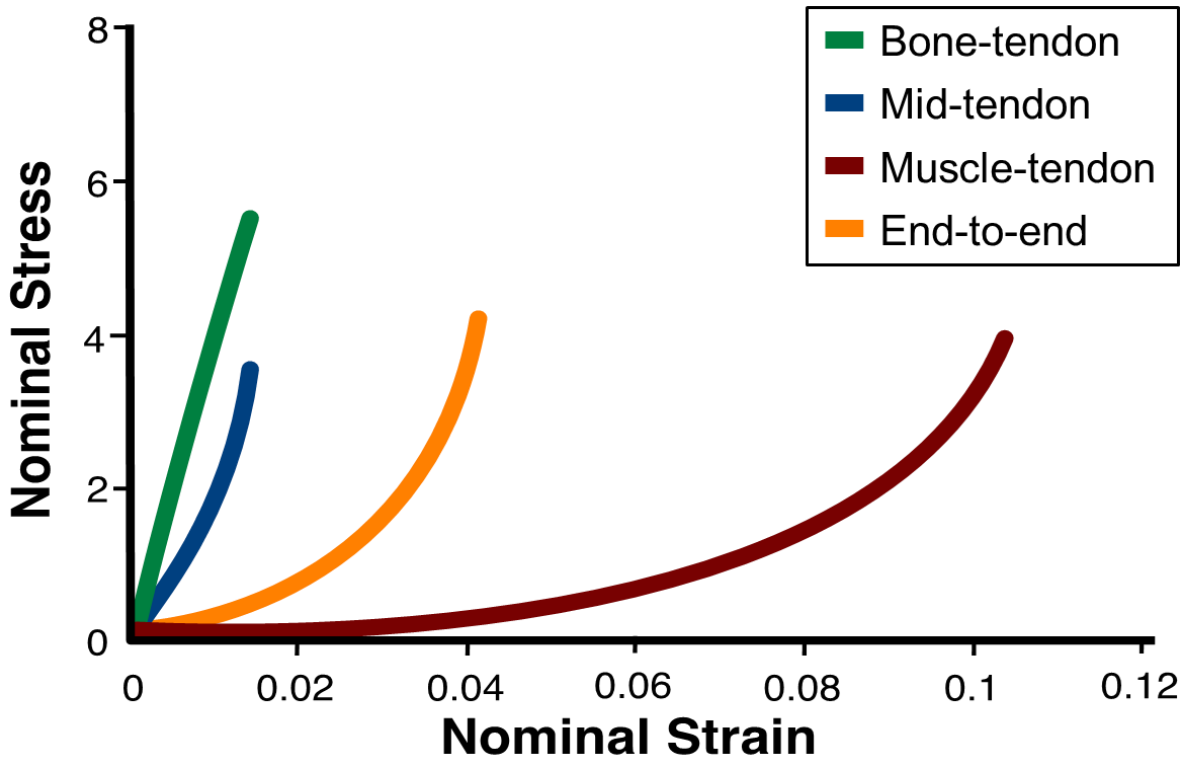

Figure 4. Tendon Regional Stiffness. The muscle-end of the tendon is more compliant than the bone-end of the tendon.

Tendons exhibit a regional variation in stiffness across their length (Arruda *et al.*, 2006; Wood *et al.*, 2011) to improve phase-matching between a compliant muscle and stiff bone (Paxton & Baar, 2007) (Figure 4). The increase in stiffness as the tendon moves from the muscle to the bone is mediated by an increase in the orientation and the crosslinking of collagen. For example, Curwin and colleagues have shown that mature hydroxypyridinium crosslinks increase ~70% from the proximal to the distal region of the tendon (Curwin *et al.*, 1994). Lastly, the regional variation in stiffness from the muscle to the bone-end of the tendon is dependent on muscle contraction. We have hypothesized that muscle contraction breaks crosslinks at the muscle end of the tendon, resulting in the gradient observed by Curwin. Consistent with this hypothesis, inactivation of the muscle results in stiffening of the muscle-end of the tendon, likely due to the inability to break crosslinks near the muscle end of the tissue (Arruda *et al.*, 2006).

Molecular Regulation of Tendon Cell Specification: Markers of Tendon Cell Identity

Tendons begin to form in embryonic development and in altricial animals continue to mature throughout the post-natal period (Figure 4). There are three major groups of tendons: trunk (axial), limb, and head. In all groups, tendon cells originate from embryonic mesenchymal tissues that are shared with cartilage progenitors but distinct from muscle. Mesenchymal stem cells are induced into tendon progenitor cells (TPC) early in embryonic development. TPCs express scleraxis, whereas MSCs do not. It is important to note that the TPC induction signal is distinct for limb tendons, which require a signal from the overlaying ectoderm, whereas tendon progenitor induction in axial and cranial tendons depends on muscle (Schweitzer *et al.*, 2010; Gaut & Duprez, 2016). Throughout the later embryonic and post-natal period, tendon progenitor cells differentiate into tendon cells (tenocytes) that express higher levels of tendon associated genes (Huang *et al.*, 2015*a*). Tendon growth/maturation occurs throughout the post-natal period and a tendon is not considered mature until it contains a heterogenous population of small and large collagen fibrils (Huang *et al.*, 2015*a*) (Figure 5). Given that there is no single molecule that

is unique to tendon, nor can completely drive tendon formation, the markers of tendon cell identity related to the induction and differentiation of tendon cells will be discussed below (Schweitzer *et al.*, 2010; Huang *et al.*, 2015*a*; Gaut & Duprez, 2016).

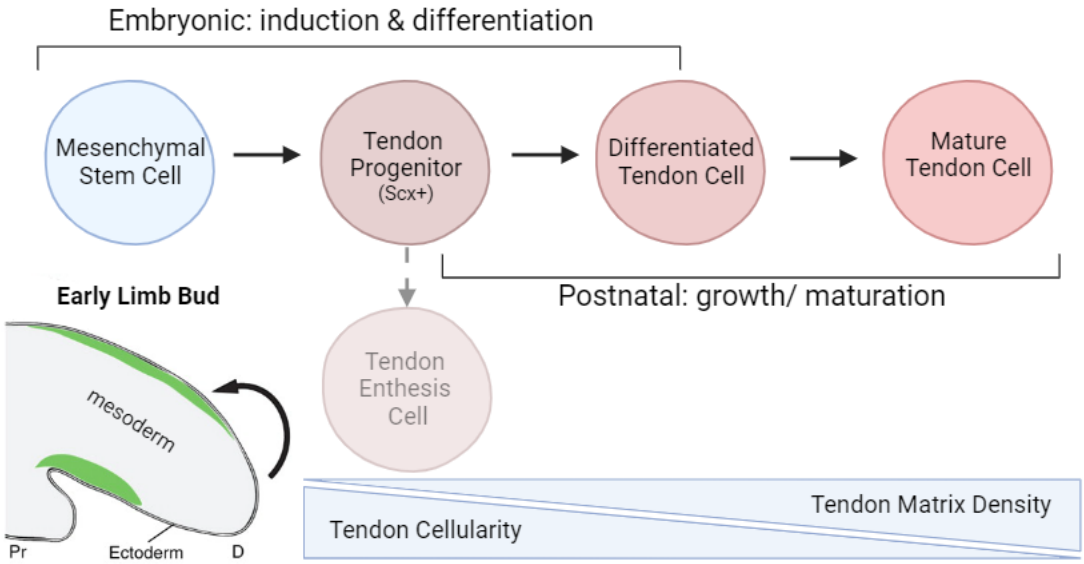

Figure 5. Tendon Induction, Differentiation, and Maintenance (adapted from Huang et al 2015a).

Scleraxis (SCX) is a basic helix loop helix (bHLH) transcription factor that was the first tendon/ligament specific marker discovered to have a major role in tendon formation (Schweitzer *et al.*, 2001). *Scx* is the earliest expressed marker in tendon development (Schweitzer *et al.*, 2001; Huang *et al.*, 2015*a*). Two years after the identification of scleraxis, the somitic compartment that gives rise to axial tendons (in chick) was identified. This compartment was termed “syndetome” and it contains *Scx* positive cells and is functionally distinct from other somitic compartments that contain similar looking mesenchymal cells (Brent *et al.*, 2003). Limb tendons arise from the limb lateral plate from a population of *Scx* positive cells (Schweitzer *et al.*, 2001; Gaut & Duprez, 2016).

The role of scleraxis in tendon growth has been shown to not depend on the embryonic origin of a tendon, but possibly the biomechanical function of the tendon. Ronen Schweitzer’s group in

2007 were first to draw attention to the considerable diversity in the amount of load tendons receive, and the distance that load must be transmitted. His group discovered that when they knocked out a key tendon transcription factor, scleraxis, there were severe defects in the development of long force transmitting tendons (e.g., flexor digitorium profundus, tail tendons; the Achilles tendon was not reported). There were deficits in tendon morphology at E13.5, which is the same time that tendon progenitors condense and align between muscle and cartilage. These mutant tendons had fewer cells and less matrix yet were able to maintain attachments to muscle and bone. In the long forearm tendons of E16.5 of *Scx* -/- mice, *Col1 (*no chain specified) expression was reduced, and no *Col4α1* or *Tnmd* expression was detected using *in situ* hybridization (Murchison *et al.*, 2007).

The reduction in expression of these tendon-associated genes in the *Scx* -/- mice is unsurprising since scleraxis is a transcription factor for *Col1α1/2* (Léjard *et al.*, 2007; Bagchi & Czubryt, 2012; Paterson *et al.*, 2020) and *Tnmd* (Shukunami *et al.*, 2018; Paterson *et al.*, 2020). SCX along with all bHLH proteins, bind DNA as heterodimers but occasionally do form homodimers to regulate gene expression (Murre *et al.*, 1989). SCX has been shown to heterodimerize with E12 (Cserjesi *et al.*, 1995; Shukunami *et al.*, 2018) or E47 (Léjard *et al.*, 2007; Shukunami *et al.*, 2018) to enhance expression of *Col1α1* (Léjard *et al.*, 2007) and *Tnmd* (Shukunami *et al.*, 2018). The *Col1a1* promoter contains two tendon specific elements (TSE1/2) that preferentially bind scleraxis and NFATc proteins, respectively (Léjard *et al.*, 2007).

In tendons that undergo substantial lengthening concomitant with bone growth, scleraxis is required in embryonic development to recruit MSCs into the elongating tendon (Huang *et al.*, 2019). It is important to consider that the same tendons in different animals have different lengthening requirements that are related to organism size. Thus, the tendons which displayed growth defects in $Scx^{-/-}$ mice such as the patellar tendon, may undergo substantial lengthening in a larger animal and have a similar molecular mechanism of growth as a longer, force transmitting

tendon (Murchison *et al.*, 2007). For tendon growth in adults, scleraxis is required as conditional knockout of scleraxis followed by Achilles tenectomy in adult mice prevents overload induced plantaris tendon growth (Gumucio *et al.*, 2020). These data suggest that scleraxis is required to recruit cells into the tendon. The recruited cells may contribute to longitudinal growth when there is a tensile load signal, such as in development. Without a clear tensile load signal like in the plantaris overload, the extra cells recruited may accumulate and contribute to the production of scar tissue.

Mohawk is a transcription factor that belongs to the homeobox (HOX) gene family and regulates specification of muscle and tendon progenitor cells. It was first identified as a regulator of musculoskeletal development because its expression was identified as early at E9 in the precursor tissue to skeletal muscle, cartilage, and tendon (Anderson *et al.*, 2006). The HOX amino acid sequence within *Mkx* is perfectly conserved among humans, mice, rats, chimpanzees, zebrafish, pufferfish, and western clawed frogs suggesting an essential structure and function (Anderson *et al.*, 2006). In fact, the homeodomain contains a DNA recognition helix that binds to the *MyoD* promoter (Chuang *et al.*, 2014) and can inhibit MyoD-induced transdifferentiation of 10T1/2 fibroblasts into muscle cells. The carboxy-terminal region of Mkx forms a complex with the SinH3A/HDAC co-repressor complex and regulates the epigenetic state of the associated promoter (Anderson *et al.*, 2009). *Mkx*$^{-/-}$ mice have a severe tendon phenotype with small collagen fibrils, lower collagen content, reduced thickness, and impaired mechanical strength (Ito *et al.*, 2010). The expression of *Colα1/2, Tnmd, Dcn, Fmod* are reduced in *Mkx*$^{-/-}$ mice at P0 compared to wild-type littermates. Collagen fibrils develop a bimodal distribution at P21 in wild type mice, yet *Mkx*$^{-/-}$ mice only have a population of smaller collagen fibrils in the tail and FDP tendon, indicating impaired maturation (Liu *et al.*, 2010).

Early growth response proteins 1 and 2 (EGR1/2) belong to a family of zinc finger transcription factors that regulate matrix production in connective tissues (Havis & Duprez, 2020). *Egr1* is

expressed after *Scx* suggesting that it is not necessary for tendon cell induction. However, it has a role in tendon cell differentiation as there is a 15-fold increase in Egr1 and 10-fold increase in Egr2 from tendon progenitor (E11.5) to differentiated tendon cell state (E14.5). *Egr1*$^{-/-}$ and *Egr2*$^{-/-}$ mice have deficits in *Col1a1* expression, collagen fibril density and organization, suggesting an essential role for *Egr* activity in tendon structure and function (Lejard *et al.*, 2011). *Egr1/2* can have different localization in differentiating tendon cells, yet either *Egr1* or *Egr2* is sufficient for expression of *Col3a1, Col5a1, Col12a1, and Col14a1.* Interestingly, only *Egr1*$^{-/-}$ mice have reduced *Scx, Mkx, and Tnmd* expression (Lejard *et al.*, 2011). *Egr1* overexpression in C3H10T1/2 mesenchymal stem cells increased expression of tendon markers and the size of engineered tendon constructs (Guerquin *et al.*, 2013). *Egr1* expression is also responsive to mechanical load as *in vivo* muscle immobilization or *in vitro* MSC construct tension release will reduce *Egr1* expression (Gaut *et al.*, 2016; Herchenhan *et al.*, 2020).

In summary, tendons are formed through a process that begins with induction of mesenchymal tissue into scleraxis-expressing tendon progenitor cells. These TPCs align and differentiate between muscle and cartilage, and tendons that continue to lengthen into force transmitting tendons require scleraxis(Murchison *et al.*, 2007; Huang *et al.*, 2019). Mohawk is another tendon lineage transcription factor that has expression which occurs alongside tendon cell specification. *Mkx* may contribute to tenogenesis by repressing the myogenic fate(Anderson *et al.*, 2006, 2009). Egr1/2 are not necessary for tendon induction but are required for collagen synthesis and tendon differentiation(Lejard *et al.*, 2011).

Signaling of Tendon Induction, Differentiation, and Maintenance

The signaling pathways that guide limb and axial tendon formation are better defined than those that underlie cranial tendon formation. In both axial and limb tendons, FGF and TGFβ signaling have a major role in controlling limb tendon development. Early work on the molecular regulation of tendon induction suggested that fibroblast growth factor (FGF) signaling was necessary for

tendon induction (Edom-Vovard *et al.*, 2002; Brent *et al.*, 2003), yet this was only shown to be true for chick embryo (Havis *et al.*, 2014). In mammals, the transforming growth factor-β (TGFβ) superfamily includes the major activators (TGFβ) and repressors (BMP signaling) of tendon development. In mouse, robust expression of scleraxis/induction of tendon progenitors occurs at E10.5 (Brent *et al.*, 2003; Perez *et al.*, 2003), with recognizable tendon organization at E12.5 (Huang *et al.*, 2015*b*), functional tendons appear at E13.5 (Huang *et al.*, 2015*b*), and differentiated tendon cells at E14.5 (Havis *et al.*, 2014). Below, the role of FGF and TGFβ in tendon development will be outlined.

In chick limb, *Scx* expression is dependent on FGF signaling from the adjacent developing muscle (myotome). *Scx* is only expressed in the anterior and posterior regions of the sclerotome. Overexpression of Fgf8 in muscle induced *Scx* expression in the sclerotome regions adjacent to the myotome, but expression of the FGF receptor *Frek* was restricted to the myotome. This suggested that FGF signaling from the myotome interacts with the sclerotome to induce tendon progenitors in the syndetome (Brent *et al.*, 2003). Similarly, FGF4 treatment on E3 chick limb explants increased *Scx* expression. However, administration of the same FGF4 ligand to E9 mouse limb explants decreased *Scx* and *Col1α2* expression (Havis *et al.*, 2016) suggesting an opposite relationship between FGF signaling in mouse and chick.

FGF ligands can activate ERK/MAPK signaling. In mouse limb development, MAPK signaling was downregulated during the organization of the tendon primordia and muscle-dependent phase of limb tendon development (E11.5 to E14.5) (Havis *et al.*, 2014). During the process of tendon induction (mouse E9/9.5) where MSCs will commit to a tendon lineage and begin to express *Scx*, inhibition of ERK phosphorylation (via treatment with MEK inhibitor PD18) on mouse limb explants and mesenchymal stem cells *in vitro* increased *Scx* and *Col1α1* expression (Havis *et al.*, 2014, 2016). This suggests that in mammalian limbs, unlike their avian relatives,

inhibition of ERK/MAPK drives tendon progenitor cell induction. However, why such a strong difference in tendon development exists between chick and mouse is unclear.

Members of the TGFβ subfamily signal canonically through SMAD2/3 to elicit cellular responses including transcription of collagen genes. Tendon progenitor cell induction is not affected by TGFβ, but the ability of TPCs to differentiate and maintain the differentiated state is impacted. Loss of TGFβ signaling, via knockout of the TGFβ receptors (*Tgfb2*$^{-/-}$; *Tgfb3*$^{-/-}$), completely disrupts limb tendon formation. Prior to TPC differentiation at E11.5, *Scx* expression in *Tgfb2*$^{-/-}$; *Tgfb3*$^{-/-}$ mice is normal. However, at E12.5 when TPCs begin to differentiate and organize between the differentiating muscle and cartilage condensations, *Scx* expression was reduced in *Tgfb2*$^{-/-}$ embryos and lost in *Tgfb2*$^{-/-}$; *Tgfb3*$^{-/-}$ mice (Pryce *et al.*, 2009). TGFβ signaling was also identified in a microarray screen as once of the most altered pathways from E11.5 (tendon progenitors) to E14.5 (tendon differentiated). *In vitro* activation or inhibition of TGFβ-SMAD2/3 signaling increased or decreased, respectively, *Scx* and *Col1a1/2* expression, again suggesting the dependency of tendon formation on TGFβ-SMAD2/3 signaling (Havis *et al.*, 2014).

To determine the dependence on maintenance of the differentiated tendon cell fate on TGFβ signaling, a mouse model with the *Tgfbr2* gene floxed and targeted by *Scx*Cre was established. This model would allow TGFβ signaling to be unaffected until the stage of tendon cell induction where Scx expression would induce the conditional knockdown of the TGFβ type II receptor in tendon cells. It was noticed that *Scx* expression was gradually lost from P0 to P7 . Movement limitations were observed at P3 and tendon degeneration began at P7 in the conditional knockouts. Transfection of *Tgfbr2* into *Tgfbr2*$^{f/-}$*;ScxCre* mice at E16.5 rescued expression of *Scx* and *Tnmd* at P6 which suggests that TGFβ is necessary to maintain the differentiated tendon cell state (Tan *et al.*, 2020).

Bone Morphogenic Protein (BMP) signaling can negatively regulate tendon formation and a potently induce cartilage and bone formation. The earliest *in vivo* evidence was described

alongside the discovery of *SCX* as a tendon specific marker and identified that BMP4 overexpression restricted *Scx* expression in chick embryo. Expression of the BMP antagonist, noggin, rescued *Scx* expression and resulted in broader induction of tendon progenitors. However, this does not lead to progenitor differentiation or additional tendon formation suggesting that additional inducing signals such as TGFβ signaling, is necessary for tendon formation (Schweitzer *et al.*, 2001).

There are a few members of the BMP subfamily that positively regulate tendon development. *Bmp14* $^{-/-}$ (GDF 5) mice have irregular collagen fibril distribution in both the tail (Clark *et al.*, 2001) and Achilles tendons (Mikic *et al.*, 2001). Hydroxyproline/DNA content was reduced in the Achilles (Mikic *et al.*, 2001) but not tail tendon (Clark *et al.*, 2001) in *Bmp14* $^{-/-}$ mice as compared to *Bmp14*$^{+/-}$ mice. *Bmp12* $^{-/-}$ (GDF 7) mice do not have reduced expression of fibrillar collagens or proteoglycans in the Achilles (Mikic *et al.*, 2006) or tail tendon (Mikic *et al.*, 2008). Yet, the Achilles tendon of *Bmp12* $^{-/-}$ mice have a greater proportion of small collagen fibrils (Mikic *et al.*, 2006).

There is debate about crosstalk between signaling pathways necessary for tendon formation. BMP and TGF-β signaling both use SMAD proteins as downstream targets. As stated above, TGFβ activates Smad 2/3, whereas BMPs activate Smad1/5/8. Since Smad 2/3 compete with Smads 1/5/8 for the co-Smad4, interplay between these pathways would be expected. Further, it is possible that other pathways increase Smad7, an inhibitory Smad that prevents all Smad signaling. Blocking ERK phosphorylation in the mouse limb explants did not change expression of the TGFβ target, *Smad7* (Havis *et al.*, 2014). However, whether other signaling pathways can modify Smad signaling remains undetermined. Interestingly, administration of a TGFβ1 receptor inhibitor to rat Achilles tendon decreased pSmad2 and pSmad3, and increased pERK1/2 levels and *Col1a1* expression, suggesting crosstalk between Smad and ERK signaling (Potter *et al.*,

2017). Further, whether non-canonical TGF-β signaling and/or MAPK/ERK signaling can drive tendon formation has yet to be determined.

Adaptions to Load: Development

Tendons have a remarkable ability to adapt to load, with the best described adaptations occurring in response to tensional and compressive loads. There are three types of loads within the musculoskeletal system: tension (the tissue is pulled in one direction), compression (the tissue is pushed in one or more directions), and shear (the tissue is compressed during sliding) (Figure 6). These adaptations begin in utero/ ovo. Tendons that develop under a tensile load exhibit a dense, aligned, predominantly type I collagen matrix (Figure 6A), whereas under compressed tendons show a fibrocartilage phenotype with sparsely connected, unaligned and smaller type I collagen fibers and larger proteoglycans (Figure 6B). Tendons under shear a partially aligned matrix and surface lubricating proteins (lubricin/ proteoglycan 4 and hyaluronic acid) are produced at high levels (Evanko & Vogel, 1990; Berenson *et al.*, 1996; Nugent *et al.*, 2006; Hayashi *et al.*, 2013; Zamboulis *et al.*, 2020) (Figure 6C).

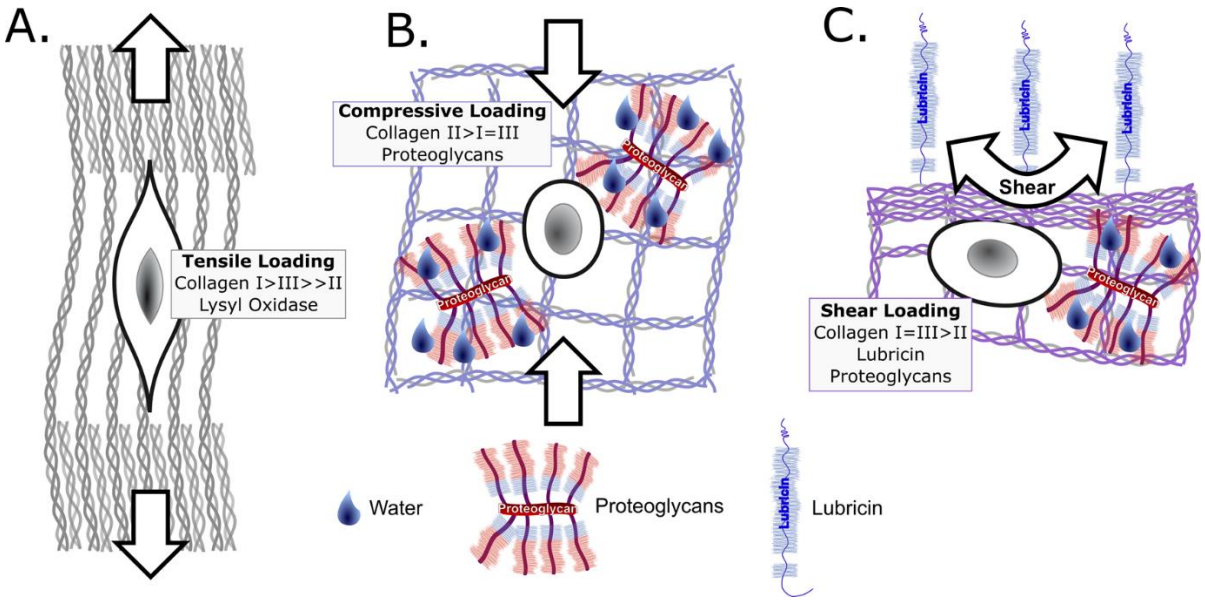

Figure 6. Three Types of Loads that Influence Tendon Composition: A) Tension, B) Compression, and C) Shear.

During embryonic and post-natal development, most tendons exhibit an increase in collagen content and a decrease in cellularity (Evanko & Vogel, 1990; Curwin *et al.*, 1994; Marturano *et al.*, 2013). These changes in tissue composition during the fetal period are associated with an increase in lysyl oxidase mediated crosslinks, resulting in greater modulus (Marturano *et al.*, 2013). Despite the fact that striking differences in collagen organization between the tensional and compressive tendon regions become apparent late in fetal development, the appearance of large proteoglycans in the compressed region does not take place until the post-natal period (Evanko & Vogel, 1990). Even before the phenotype is apparent at the tissue level, there are different cellular responses to mechanical load between cells isolated from the tensional and compressed region. In fact, cells isolated from within compressive regions synthesize more large and small proteoglycans when subjected to *in vitro* cyclic compression than those from tensional regions (Evanko & Vogel, 1993). Specifically, aggrecan expression increased 5-fold in response to cyclic compression in cells isolated from the pre-fibrocartilaginous region. In neither region did decorin protein increase; however, a larger form of decorin is made in response to cyclic compression (Evanko & Vogel, 1993). Interestingly, TGF-β treatment or cyclic compressive loading increased large PG and biglycan levels, but not decorin levels (Robbins *et al.*, 1997), suggesting that TGF- β signaling has a role in regulation tendon matrix composition in response to load.

Adaptation to Load: Adult

Once tendons have reached their adult length, changes in loading can result in circumferential growth that results in increased mechanical properties. In humans, two to three months of training is sufficient to have a moderate to large effect on tendon stiffness and cross-sectional area. High intensity training, where jerk is higher, has a bigger effect than lower intensity exercise, while type of muscle contraction has no effect on tendon stiffness (Bohm *et al.*, 2015). Intercollegiate male and female cross-country runners increased Achilles tendon CSA during the

first three and six weeks of cross-country season (Sponbeck *et al.*, 2017), and lifelong distance runners have larger Achilles tendon CSA than non- runners (Rosager *et al.*, 2002). Similarly, intercollegiate female soccer players increased their ACL volume throughout the competitive season (Myrick *et al.*, 2019) (Figure 7). In animal models, running mice for six weeks increased Achilles tendon mass, fibroblast density, and CSA (Mendias *et al.*, 2012). Swimming exercise performed one hour a day for five weeks, for seven weeks is also sufficient to increase total protein in the rat Achilles tendon suggesting that jerk is not required to drive tendon adaptations (Carvalho *et al.*, 2020). These tendon adaptations are the result of the load applied directly to the tendon, as opposed to a systemic exercise effect, as in humans the only the loaded patellar tendon increases in CSA and stiffness in response to twelve weeks of unilateral resistance training (Kongsgaard *et al.*, 2007). Further, years of training one limb more than the other results in a significantly larger and stiffer tendon in the dominant leg (Couppé *et al.*, 2008). In contrast, two weeks of immobilization in elderly men caused an 80% reduction in tendon collagen synthesis (Dideriksen *et al.*, 2017). These studies suggest that connective tissues are highly dynamic and acutely respond to load.

Figure 7. ACL Thickness Increases Throughout a Competitive Season. (Figure from Myrick et al 2019)

The collagen content in a tendon/ligament is a balance between collagen synthesis and incorporation versus degradation. Degradation can take two forms in tendon, the breakdown of an existing dense collagen matrix or the degradation of newly synthesized collagen prior to incorporation. In one of the first studies to examine collagen metabolism in response to load, Laurent and colleagues utilized a hypertrophy model where a weight was hung on a chicken wing for 45-50 hours before measuring changes in muscle collagen synthesis and degradation. Interestingly, they found that half of newly synthesized collagen is normally degraded within two hours of synthesis. With overload, collagen synthesis increased fivefold and degradation before incorporation decreased two and a half fold. This highlights the fact that, in muscle, collagen mass in response to load is due to both increased synthesis and increased incorporation of the newly synthesized collagen into the matrix (Laurent *et al.*, 1985).

The extent of collagen turnover in adult tendons is debated. Early studies using the ^{14}C bomb pulse method suggested that the central core of adult tendon does not turnover after the age of 17 (Heinemeier *et al.*, 2013, 2018). From 1955-1963 as nuclear bombs were being tested, there was a sharp rise in the ^{14}C content in the atmosphere. The levels of radioactive fallout began to decrease exponentially after the Test Ban Treaty was signed in 1963. The authors found that the ^{14}C levels in the core of the Achilles tendon correlated with the average level of atmospheric ^{14}C on the subject's seventeenth birthday, but many tendons retained higher ^{14}C at the time of sampling than expected suggesting that the tendon core only turns over during skeletal growth or regeneration (Heinemeier *et al.*, 2013). Using this method, no regional differences in collagen turnover in human PT measured were observed (Zhang *et al.*, 2020). This work is supported by data showing that collagen proteins in horses have a 100-fold longer half-life than the non-collagen proteins in the high strain superficial digital flexor tendon (SDFT), and a 10-fold longer half-life in the low strain common digital extensor tendon (CDET) as measured by the rate of aspartic acid racemization (Thorpe *et al.*, 2010).

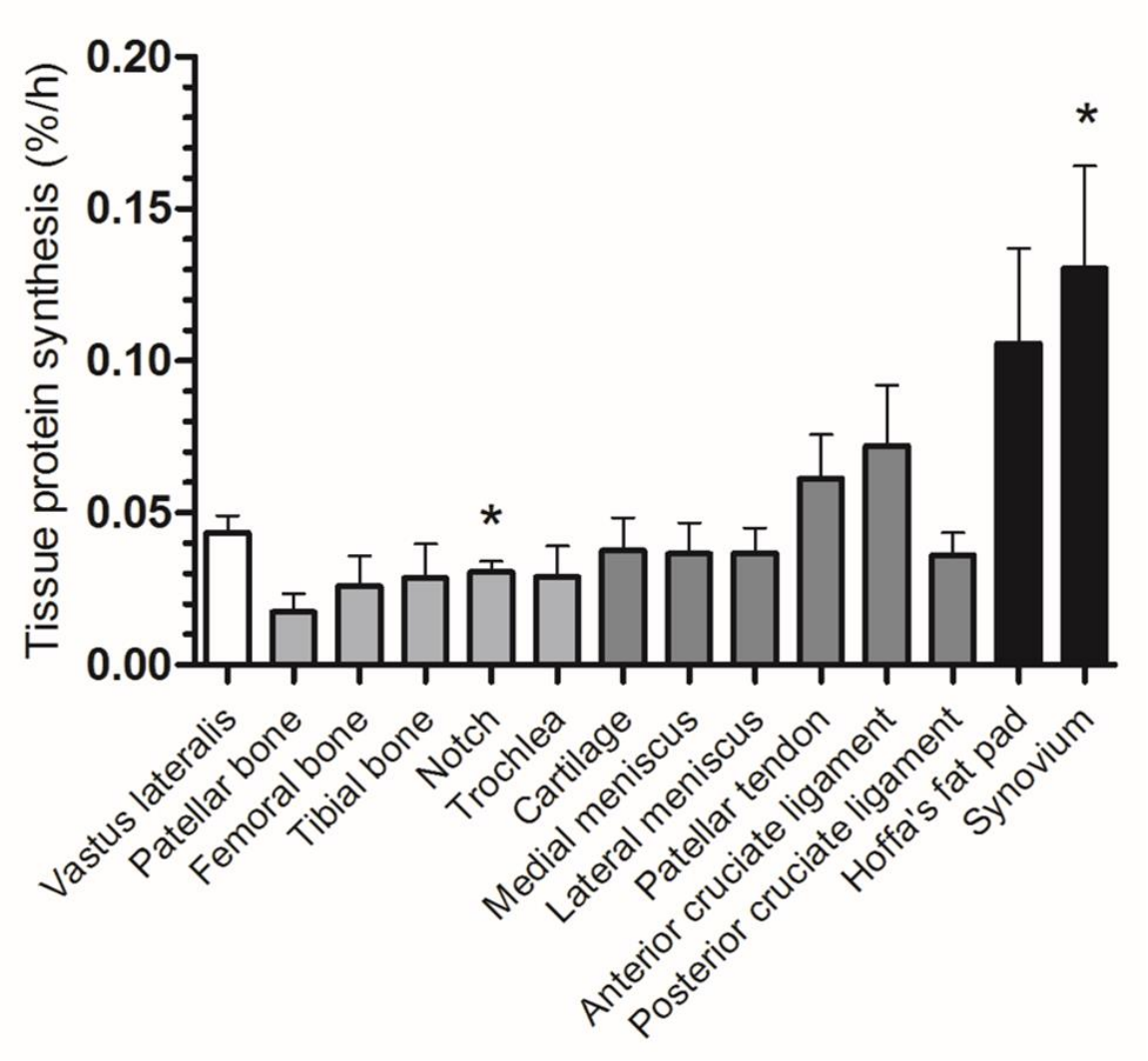

Figure 8. Protein Turnover in Tendon Compared to Other Musculoskeletal Tissues (adapted from Smeets et al 2019). Tendon has elevated protein synthesis as compared to bone and rates similar to muscle.

In contrast to this work using ^{14}C, using stable isotopes suggest that whole tendon and ligament protein synthesis rates are similar to muscle (Smeets *et al.*, 2019) (Figure 8). A potential weakness of the recent work from Smeets is that they used phenylalanine as the stabile isotope label and this amino acid may be present at a different concentration in collagen vs non-collagen proteins. Arguing against this critique, the authors measured the amino acid content of the newly synthesized proteins and found that fully a third of all the amino acids were glycine. Since collagen is the only protein with a G-X-P* repeating tri-peptide sequence, this means that the

newly synthesized proteins were most likely collagen (Smeets *et al.*, 2019). It is important to remember that stable isotope measurements only measure synthesis and do not account for protein breakdown, but these data suggest that collagen turnover within a tendon or ligament is much higher than previously thought (Smeets *et al.*, 2019). Further, if the core of the tissue doesn't turnover and the whole tissue turnover rate is 0.06%/hr, this suggests that the outer part of the tendon turns over even faster than the whole tissue rate.

The molecular and cellular mechanisms that mediate increases in collagen content with load are poorly understood. In response to tendon loading, progenitor cells are thought to migrate towards the core. Following six weeks of treadmill running in young mice, *Scx* expression localized to the epitenon and between collagen fibers suggesting that tendon cells emerge from the outer layer of the tendon and migrate towards the core (Mendias *et al.*, 2012).

Insulin-like growth factor 1 (IGF-1) was the first load-responsive molecule suggested to have a role in tendon hypertrophy. Loading or unloading the rat Achilles tendon increased or decreased, respectively, the concentration of IGF-1 within the tendon cell cytoplasm (Hansson *et al.*, 1988). Further, *Igf-1* expression in the rat Achilles tendon was increased 24 hours after concluding four days of training regardless of the type of muscle contraction (concentric, eccentric, isometric), suggesting that tendons respond similarly regardless of whether the muscle shortens, lengthens, or remains the same length (Heinemeier *et al.*, 2007). In an overload model of plantaris tendon hypertrophy, mice with *Igf1r* knockout in scleraxis positive cells experienced half the total tendon hypertrophy after fourteen days of loading. *In vitro* application of IGF1 on tenocytes activates both P13K/Akt and ERK signaling to promote tenocyte protein synthesis and cell proliferation (Disser *et al.*, 2019). Further, application of IGF-1 to engineered ligaments results in a dose-dependent increase in collagen and mechanics (West *et al.*, 2015).

ERK can also by activated directly by load in tendon/ligament cells (Paxton *et al.*, 2012). As little as 10 minutes of loading (regardless of the strain and frequency of load) is sufficient to activate

ERK, arguing against the need for IGF1 in the acute response to loading. Further, a five-day intermittent loading protocol optimized for ERK1/2 phosphorylation increased collagen content in engineered ligaments more than a continuous loading protocol (Paxton *et al.*, 2012). ERK may signal to increase collagen content through EGR1, since ERK1/2 are transcriptional activators of the EGR1 promoter (Gregg & Fraizer, 2011). Since EGR1 is a transcription factor for collagen Iα1 (Guerquin *et al.*, 2013), load-induced activation of ERK1/2 could increase EGR1 expression and subsequent production of COLIa1(Gregg & Fraizer, 2011).

Both male and female tendons hypertrophy with load yet the mechanism of increasing collagen content within the tissue may be mediated in a sex-dependent fashion due to the role of estrogen. For a detailed review on the role of estrogen in musculoskeletal tissue composition and performance, please see (Chidi-Ogbolu & Baar, 2019). In men, Miller and colleagues used stable isotopes and patellar tendon biopsies to show that a leg-kicking protocol increased patellar tendon collagen fractional synthesis rates (FSR), peaking 24 hours post exercise, and remaining elevated 72 hours later (Miller *et al.*, 2005). In women in their follicular phase, the same kicking exercise did not increase patellar tendon collagen FSR 24 hours post-exercise (Hansen *et al.*, 2009*b*). However, in another study using the same model that did not control for menstrual cycle, two out of three female participants in the exercised group increased patellar tendon collagen FSR (Dideriksen *et al.*, 2013). Interestingly, post-menopausal women — who naturally have lower estrogen – have lower tendon collagen FSR (incorporation) but higher PINP (synthesis) 24 hours following resistance exercise than post-menopausal women who use estrogen replacement therapy (ERT). At rest, the ERT users had more medium-sized and fewer larger collagen fibrils than those who were not taking ERT (Hansen *et al.*, 2009*a*). Estrogen's beneficial effects on collagen incorporation into the tissue is supported by a proteomics study that reported a greater type I collagen content of both the ACL and PT in pre-menopausal females than males (Little *et al.*, 2014). However, in a study on monozygotic twins where only one took ERT, the twin

taking estrogen had a smaller Achilles tendon CSA compared with the twin who did not take estrogen (Finni *et al.*, 2009) and a similar smaller Achilles CSA was found in a separate study in a large group of active women taking ERT (Cook *et al.*, 2007). Together, these data indicate that estrogen can modulate collagen synthesis and incorporation into tendon in response to load. Further the response changes as women transition through menopause.

Adult tendons with distinct mechanical microenvironments (tension vs compression) exhibit regional variation in gene expression. Energy storing tendons such as the rat Achilles and patellar tendon have more similar transcriptomes than the supraspinatus tendon that is subjected to a compressive load as it passes underneath the acromial arch (Disser *et al.*, 2020). In the adult bovine flexor tendon, the tensile region had no signal (northern blot, less sensitive than PCR for detecting expression) for collagen I, II, decorin, and biglycan. Expression of these genes in the compressed region depended on the distance from the tendon surface. Decorin and biglycan were expressed near the surface, whereas collagen I and aggrecan were expressed in the core, and collagen II had peak expression deep within the compressed region (Perez-Castro & Vogel, 1999). With time, the difference in gene expression resulting from the loading history of the cell is epigenetically encoded. For example, tendon cells cultured from the tensional region of a tendon synthesize proportionally more small proteoglycans and fewer large proteoglycans than cells from the compressed region or differentiated chondrocytes (Ehlers & Vogel, 1998). However, acute changes in load (from tension to compression) results in dramatic changes in gene expression. *Ex vivo* compressive loading of tendons for 72 hours is enough to increase the large proteoglycan aggrecan mRNA 4.5-fold and protein 2-fold (Robbins *et al.*, 1997).

These studies highlight the role of tensional loads promoting a tendon cell phenotype and compressive loads promoting fibrocartilage gene expression. Tendon cells in a normally structured healthy tendon experience more tensile loads. Tendon cells near the enthesis or where tendons wrap around bones experience compressive loads and take on a more

fibrocartilage-like phenotype. Over time, the cells become epigenetically tuned to their loading environment; however, acutely switching from tension to compression (or *vice versa*) can lead to a phenotype shift.

Adaptation to Unloading: Adult

The fact that extended immobilization or disuse reduced mechanical strength of a tendon or ligament was first recognized in 1977. In an ethically questionable study, primates were subjected to eight weeks of total body plaster immobilization (no weight bearing) or single lower limb immobilization. With eight weeks of total body immobilization, ACL maximal load decreased 40% from 78 kg to 46.79 kg and the linear stiffness decreased ~30%. With five months of reloading, MTL recovered to 79% of baseline. 12 months of reloading was sufficient to return MTL to 91% of baseline which was not statistically different from the control group. In contrast, linear stiffness had recovered to 90% of baseline with 5 months of reloading and was fully recovered by twelve months. This suggests that complete lack of weight bearing for eight weeks causes a disproportional loss in tissue material that contribute to maximal load (i.e., collagen content) that cannot completely be recovered within a year, while tissue structures that contribute to stiffness (i.e., collagen crosslinks) are lost less quickly and can be recovered more rapidly during the reloading period.

In the 8-week single leg immobilization component of the study, ACL maximal load and stiffness decreased by 22%. The single leg immobilization was a distinct stressor from the full body immobilization because in this situation the animal's overall activity was maintained and the non-immobilized leg was loaded normally. This suggests that there is a systemic signal from loading that helps alleviate the reduction in tissue mechanics. It is possible that tissue stiffness is less sensitive than MTL to a direct mechanical load signal on the tissue of interest because it is similarly reduced under full body (30% reduction) and single leg (22% decrease) immobilization. These data suggest that a systemic signal from loading may better mitigate the reduction in MTL

than stiffness that occurs with immobilization, because MTL decreases by half the amount in the single leg (22% decrease) versus the full body condition (40% decrease) (Noyes, 1977).

Tendinopathy

Definition and History

Tendinopathy is common musculoskeletal condition characterized by persistent tendon pain and loss of function related to mechanical loading. It is debated whether diagnostic imaging of a tendon is necessary because an experienced clinician can diagnose tendinopathy in the in the absence of imaging (Scott *et al.*, 2020). However, ultrasound is a relatively inexpensive imaging tool that can be used to identify at-risk individuals for developing tendinopathy because structural abnormalities increase the risk of developing symptoms (McAuliffe *et al.*, 2016). Magnetic resonance imaging (MRI) is more costly and invasive but can be helpful in diagnosing and monitoring repair. Importantly, not all tendon lesions seen on imaging are symptomatic or impair function. However, individuals with asymptomatic tendon pathology have a higher pain pressure threshold, suggesting that they may be ignoring the pain associated with a long-term issue (Rio *et al.*, 2018). There are a number of chemicals and drugs (aromatase inhibitors, fluoroquinolone antibiotics, glucocorticoids, ACE inhibitors, and statins) (Bolon, 2017; Nyyssönen *et al.*, 2018) that may cause tendon rupture or exacerbate tendinopathy, but this review will focus on tendinopathy that is primarily related overuse.

Treatments for tendinopathy aim at pain reduction and have been hindered by an inadequate understanding about the underlying pathology. The poor understanding of the pathology is reflected in the history of the terminology used to describe the disease. It was initially assumed that all tendon injuries were the result of excessive inflammation. Thus, tendinitis was first used to describe the condition. Non-steroidal anti-inflammatory drugs were therefore used to treat tendinitis without success (Khan *et al.*, 2002). In 1976, a much more limited role of inflammation in chronic tendon pathology was suggested since the tendon core is relatively avascular and it is difficult for inflammatory cells to enter the tendon. Peritendinitis was then used to describe inflammation occurring within the peritendon (i.e. Achilles tendon) and distinct from tenosynovitis

to describe inflammation occurring in tendons with synovial sheaths. Importantly, degenerative alterations to the tendon matrix (formerly described as tendinosis) in the absence of active inflammation can produce symptoms and often precede tendon rupture (Puddu *et al.*, 1976). The fact that early after an acute tendon injury inflammation may contribute to pain, but that in many individuals with chronic tendinopathy there are no signs of active inflammation led to a re-evaluation of the condition. In 2019, an international consensus paper proposed that the term tendinopathy should be used in place of tendinitis or tendinosis as a way to describe to persistent tendon pain related to mechanical loading regardless of inflammation or structural damage (Scott *et al.*, 2020).

Epidemiology

Tendinopathy is a frequent musculoskeletal complaint that affects a wide range of individuals. The exact incidence and prevalence depend on the tendon. However, one of the largest (>10,000 participants) recent cross-sectional studies on a general population reported an incidence of 1.05 % and prevalence of 1.18 % for lower extremity tendinopathy. There was no difference between men and women in that population; however, it was more prevalent among older (>45 years) individuals (Albers *et al.*, 2016). Estrogen may have a protective effect against certain tendon pathologies, and this is best demonstrated by the peak incidence of tendon rupture in women occurring after menopause (Maffulli *et al.*, 1999).

In sport, tendon rupture and tendinopathy are more common in men. In a cohort of players belonging to the Dutch Volleyball Association, men had twice the odds of developing patellar tendinopathy compared to women (van der Worp *et al.*, 2012). Even among high school athletes, boys have at least a three times greater risk of developing tendinopathy than girls (Visnes & Bahr, 2013). Elite male Norwegian soccer and handball players reported a 13.5% prevalence of tendinopathy compared to 5.6% prevalence in sport-matched women (Lian *et al.*, 2005). Given that structural degradation precedes rupture (Kannus & Jozsa, 1991), it is unsurprising that two

studies in Denmark covering a thirty-year period reported a three to one male/female Achilles tendon rupture ratio (Houshian *et al.*, 1998; Ganestam *et al.*, 2016). Over the first thirteen-year period, 75% of these ruptures were related to sport with almost half of the ruptures occurring while playing badminton (Houshian *et al.*, 1998).

Types of Tendinopathy

Although tendinopathy occurs in both athletes and non-athletes, this portion of the review will focus on the different types of tendinopathy and their prevalence in sport. The prevalence of tendinopathy in all sports can range from 20-50% (Florit *et al.*, 2019; Goes *et al.*, 2020) and the exact percentage depends on the sport, level, and age of the athlete. Most tendinopathies do not usually result in time lost from sport (Florit *et al.*, 2019), Thus, epidemiology studies which only use time lost from sport to track injuries are likely underreporting the incidence of tendinopathy. If time is lost because of tendinopathy, the average is seven days. Patellar and Achilles tendinopathy have the most variable duration of return to play (Florit *et al.*, 2019). While tendinopathies are not catastrophic injuries, they can severely reduce quality of life with over 90% of athletes reporting sleep disturbances, >20% anxiety and depression (Wong *et al.*, 2020).

The Achilles tendon is loaded with forces greater than seven times body weight while running (Giddings *et al.*, 2000) and is the highest stressed tendon in the body with an estimated maximum stress near 70 MPa (KER *et al.*, 1988). Unsurprisingly, Achilles tendinopathy is most common in runners (Kujala *et al.*, 2005). Achilles tendinopathy can occur at the mid substance or insertion. Insertional tendinopathy can be further categorized into a pre-insertional component, two centimeters above the calcaneus, or right at the calcaneal insertion (Del Buono *et al.*, 2013). In elite male Finnish athletes, the lifetime incidence of Achilles tendinopathy is over 50% in middle-long distance runners. Interestingly, rupture is more common in sprinters and decathletes (17% incidence each sport) given the more explosive nature of the sport and the resulting higher jerk of training and competition. The lifetime incidence of tendinopathy among elite athletes is

lowest in cross-country skiers at 4%, with no ruptures reported, likely due to the lack of high impact and high jerk movements (Kujala *et al.*, 2005). Risk factors for developing Achilles tendinopathy include increased leg stiffness (Lorimer & Hume, 2016) and training volume (Lagas *et al.*, 2020).

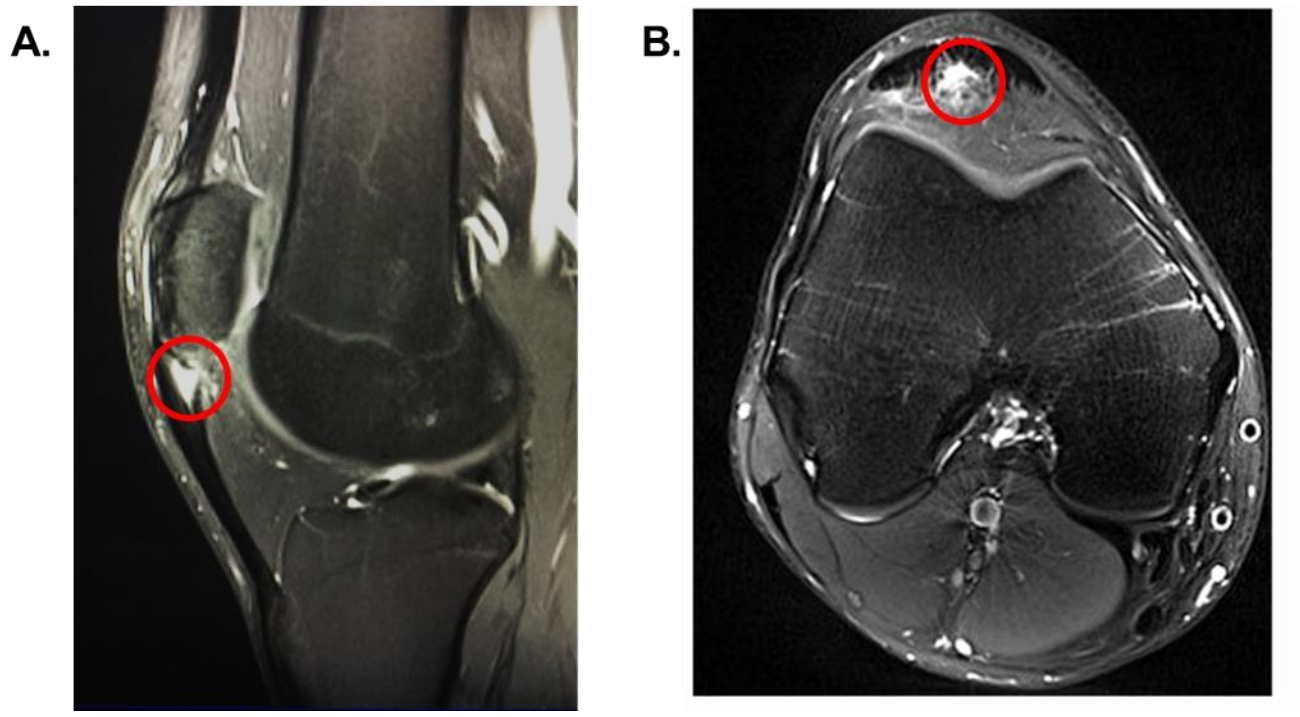

Figure 9. MRI of Patellar Tendinopathy in Saggital (A) and Transverse (B) planes (Baar 2019). Notice the MRI reactivity (outlined in red) just distal to the patella indicating disrupted collagen structure.

Patellar tendinopathy or “jumpers knee” is most common in jumping sports, such as basketball and volleyball (Lian *et al.*, 2005; Florit *et al.*, 2019). The patellar tendon connects the patella to the tibia and is technically a ligament because it runs bone-to-bone, yet because its matrix composition and collagen fibril diameter is more similar to tendons it is referred to as a tendon (Rumian *et al.*, 2007). The most common location of patellar tendinopathy is the central core proximal to the enthesis (Figure 9) (Johnson *et al.*, 1996; Baar, 2019). In Dutch male elite athletes, the prevalence of patellar tendinopathy was at 45% in volleyball players and 32% in basketball players (Lian *et al.*, 2005). Non-elite Dutch volleyball players report a much lower

prevalence of 15% (Zwerver *et al.*, 2011), but this population included both male and female players who were about four years younger than the elite players. Among volleyball players, outside hitters and middle blockers are at greater risk than setters suggesting again that the volume and intensity of jumping contributes to the development of patellar tendinopathy (van der Worp *et al.*, 2012). In the same study, no risk factors were reported for elite basketball players (van der Worp *et al.*, 2012). Even though the incidence of reported pain was less than 50%, in professional Spanish basketball players approximately 77% had structural abnormalities (91/146 patellar tendons) and almost all these abnormalities included sonographic detection of a focal area of hypoechogenicity suggesting the presence of a tear (Benítez-Martínez *et al.*, 2019). Because of the difficulty in regenerating tendon, it is unsurprising that the recurrence rate of patellar tendinopathy is reported at 12-27% in professional soccer players (Hägglund *et al.*, 2011).

Lateral epicondylitis, also known as tennis elbow, is pain at and around the lateral aspect of the elbow. Despite the name, tennis players make up the minority of the individuals with lateral epicondylitis (De Smedt *et al.*, 2007). Tennis elbow is the second most common injury in tennis players with 22% over players affected (Minghelli & Cadete, 2020). There is a 35-50% career incidence of lateral epicondylitis in Finnish players of all levels (Abrams *et al.*, 2012). Overuse is considered a contributing factor because the incidence and recurrence rate increase with age, and increased playing time increases the relative risk. Interestingly, changing stroke technique and type of racquet were successful in reducing recurrence suggesting that the amount jerk on the elbow contributes the of development of lateral epicondylitis (Gruchow & Pelletier, 1979). In the general population, repetitive movements of the hands or wrist ≥2 hours/day increases the odds of developing epicondylitis which suggests that overuse is the underlying cause of the development of this type of tendinopathy (Shiri *et al.*, 2006).

Unlike tennis players where backhand shots cause lateral epicondylitis, golfers and throwing athletes develop medial epicondylitis, known as golfer's elbow. The only real difference is which tendons experience the jerk of the movement. In a golf swing, the inside of the trailing arm is the site of maximal jerk when the accelerating club contacts the ball or the turf, whereas in throwers the snap of the throwing arm during the cocking phase puts maximal jerk on the flexor carpi radialis and pronator teres tendons and ulnar collateral ligament. The result is that medial epicondylitis occurs in the non-dominant arm in golfers and the dominant arm in throwers.

Shoulder pain is one of the most common musculoskeletal complaints in the general population (Bergman *et al.*, 2001; Nakamura *et al.*, 2011). Rotator cuff tendinopathy involves at least one of the four tendons from the major rotator cuff muscles: supraspinatus, infraspinatus, teres minor, and subscapularis. The prevalence of rotator cuff tendinopathy in the general population is 18% and tends to be higher in females (Holdaway *et al.*, 2018). Risk factors that increase the risk of developing rotator cuff tendinopathy include age over 50, diabetes, and overhead activities (Leong *et al.*, 2019). Despite high complaints of shoulder pain, there is still a disconnect between pain and pathology. Asymptomatic elite male and female volleyball players all have abnormal shoulder pathology on MRI despite the absence of symptoms. Ninety percent of players that were asymptomatic at the time of the study and had not done rehab within the prior year had tendon degeneration and 65% had partial rotator cuff tears (Lee *et al.*, 2020). Given that tendon degeneration precedes symptom development, "prehab" exercise should be a core component of training with a specific focus on the tendons that receive the greatest volume of high jerk loads in training.

Structural, Biological, and Mechanical Changes in Tendinopathy

Repetitive tendon overload with a failed repair process can lead to a weaker, painful tendinopathic tendon (Xu & Murrell, 2008). Pain is a defining feature of tendinopathy but is not

always present in tendons with structural damage (Lee *et al.*, 2020). This section will focus on the histopathological, cellular, molecular, and mechanical changes that occur in tendinopathy.

Major structural changes in tendinopathy include neovascularization (Alfredson *et al.*, 2003), collagen disorganization, decreased collagen fibril size, and increased total cellularity with cell rounding (Soslowsky *et al.*, 2000; Pingel *et al.*, 2012, 2014). In horses, the total collagen content in a tendon scar has been reported to be 37% of that of a healthy tendon (Williams *et al.*, 1980). The population of collagen fibrils within the scar is heavily biased towards smaller fibrils (<70 nm) and notably missing fibrils of a larger (>130 nm) diameter (Pingel *et al.*, 2012, 2014). In contrast to the near parallel arrangement of collagen fibrils in a healthy tendon, the tendinopathic collagen matrix is disorganized (Pingel *et al.*, 2014). There is more total and type III collagen in the matrix (Pingel *et al.*, 2014; Heinemeier *et al.*, 2018). Type III collagen accounts for up to 10% of a normal tendon matrix, but this concentration is increased in scar tissue (Williams *et al.*, 1980; Little *et al.*, 2014). Tendon cells are typically elongated fibroblasts, but cell rounding and loss of cellular extensions can occur in tendinopathy (Pingel *et al.*, 2014). Increased tendon thickness is a structural change that has been implicated in Achilles tendinopathy (Docking *et al.*, 2015) likely in an attempt to maintain the mechanical properties of a tissue with lower material properties.

While the amount of collagen turnover within a healthy tendon is debated, there is consensus that tendinopathic tissue has increased turnover that is in part due to increased proteoglycan turnover. Individuals with midportion Achilles tendinopathy have an increased GAG concentration and collagen concentration in the injured region (Heinemeier *et al.*, 2018). The large PGs aggrecan and versican increase within the pathologic tissue (Samiric *et al.*, 2009; Parkinson *et al.*, 2010) and turnover significantly faster (Parkinson *et al.*, 2010). Versican increases in fibrocartilage zones, hypervascular, and hypercellular regions in human patellar tendinopathy (Scott *et al.*, 2007). Certain small PGs are also increased such as fibromodulin (Samiric *et al.*, 2009; Parkinson *et al.*, 2010) and biglycan (Samiric *et al.*, 2009). The increased PG content in

tendinopathy may be responsible for the associated tendon pain. PGs can have sulfated (heparan sulfate, chondroitin sulfate, keratin sulfate) or non-sulfated (hyaluronic acid) GAG side chains. Greater sulfated GAG content is associated with a worse Victorian Institute of Sports Assessment (VISA) score in human patellar tendinopathy (Attia *et al.*, 2014).

There are genetic associations with tendinopathy, but the research in this field is limited and the cause-and-effect relationships are poorly understood. Mutations in collagen 5α1, tenascin C, matrix metalloproteinase-3, and estrogen-related receptor beta have the strongest evidence for an association with tendinopathy (Vaughn *et al.*, 2017).

The first polymorphism associated with tendinopathy was identified as tenascin-C. It was observed that individuals with alleles containing either 12 and 14 guanine-thymine dinucleotide repeats within tenascin-C had an increased risk of Achilles tendon injury, while individuals with 13 or 17 repeats had fewer Achilles tendon injuries (Mokone *et al.*, 2005). Before the identification of tenascin-C as a genetic link to tendon injury, individuals with O blood type were identified as having an increased risk of tendon injury (Jozsa *et al.*, 1989*a*, 1989*b*; Kujala *et al.*, 1992). Interestingly, the tenascin-c and *ABO* genes are located near each other (genetically linked) on the long arm of chromosome 9 (Rocchi *et al.*, 1991; Bennett *et al.*, 1995), suggesting that tenascin-C may be one of the genes which explains the relationship between ABO blood type and tendon injury.

The most comprehensively studied genetic factors associated with tendinopathy are collagen 5A1 polymorphisms. Mokone and colleagues were first to demonstrate that *COL5A1* polymorphisms were associated the Achilles tendon pathology (Mokone *et al.*, 2006). *COL5A1* also maps near the same location as the *ABO* gene on chromosome 9q34 (Caridi *et al.*, 1992; Bennett *et al.*, 1995). Recently, a meta-analysis of *COL5A1* polymorphisms suggested that the rs12722, rs71746744, and rs3196378 polymorphisms are correlated with an increased risk of musculoskeletal soft tissue injury (Guo *et al.*, 2022). Since collagen V is mutated in classical

Ehlers-Danlos syndrome, resulting in skin hyperelasticity and joint hypermobility (Michalickova *et al.*, 1998; Wenstrup *et al.*, 2000), it is not surprising that *COL5A1* is related with tendon injury. *COL5A1* associates with collagen I and assists in fibrillogenesis (Kadler *et al.*, 2008) and could therefore directly affect tendon stiffness.

There are a few, but not many, examples of epigenetic alterations associated with tendinopathy. DNA methylation is an epigenetic alteration associated with downregulated gene expression because promoter methylation can interfere with the ability of transcription factors to access DNA. Both MMP11 and ADAMTS4 have CpG regions within the promoter that are more methylated in patellar tendinopathy than healthy controls (El Khoury *et al.*, 2018; Rickaby *et al.*, 2019).

Impaired tendon mechanics are an important functional consequence of tendinopathy because of the impaired force transfer between the muscle and bone. Because the material properties of a tendon (e.g. modulus, ultimate tensile strength) are relative to the direction, amount, and crosslinking of collagen it is not surprising that tendinopathy alters material properties. Tendinopathic tendons, with small fibrils and lower orientation, show lower material properties than healthy tendons (Maeda *et al.*, 2009; Pingel *et al.*, 2014). One way the body compensates for the lower material properties in a tendinopathic tissue is to increase the size of the tissue. The bigger tissue would be able to meet the absolute demand (i.e. 7-times body weight) on the tissue even though the material is weaker relative to its size. One meta-analysis showed reduced mechanical and material properties in Achilles tendinopathy, but the difference was less clear for patellar tendinopathy (Obst *et al.*, 2018). Only one patellar tendinopathy study within the meta-analysis compared the mechanics of the symptomatic limb to the asymptomatic contralateral limb. This study used shear wave elastography which estimates tissue modulus based on the propagation velocity of the shear wave that is generated perpendicular to the ultrasound pulse. This technique was more accurate than ultrasound at identifying tendinopathy and found a

reduced modulus in symptomatic patellar tendons (Dirrichs *et al.*, 2016). Another recent study that measured tendon biomechanics during isometric contractions found a lower patellar tendon stress, stiffness, and modulus in tendinopathic tendons. Further, the decline in modulus correlated with a worse VISA-P score (Wiesinger *et al.*, 2020).

Role of Stress Shielding in the Development of Tendinopathy

The etiology of tendinopathy is usually attributed to repetitive mechanical load on a tendon with a failed remodeling response (Cook *et al.*, 2016). However, this model fails to appreciate how/why the initial injury fails to remodel. It is our hypothesis that stress shielding contributes to the development of tendinopathy (Figure 10). Stress shielding is when a tissue (or a region of a tissue) with lower material properties is shielded from mechanical stress by stronger surrounding tissue.

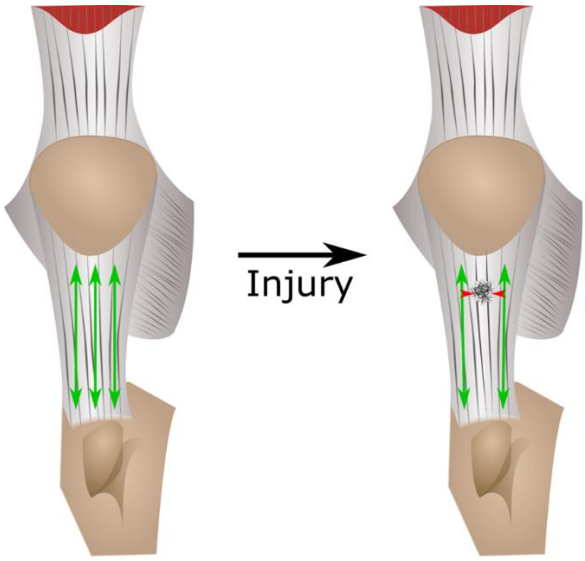

Figure 10. Force Transfer in Tendon and Tendinopathy. In a healthy tendon, force is transferred through healthy, strong collagen. In tendinopathy, we hypothesize that the weaker scar tissue is shielded from tensile load.

The best evidence for the role of stress shielding in the development of tendinopathy was performed in a series of studies by a group led by Prof Hayashi from Hokkaido and Osaka University. They experimentally stress shielded a tendon by removing tension from the rabbit

patellar tendon and studying the resulting structure and function of the patellar tendon. In their model, the researchers placed a stainless-steel wire around a pin inserted into the patella and a screw in the tibial tubercle. The researcher then shortened the distance between the patella and tibia by 6.2 mm (~22%). The result was that the load of the quadriceps muscles passed through the stainless-steel wire and not the biological tendon. Functionally, researchers had stress shielded the patellar tendon, shielded it from load, without injuring the tissue directly. The rabbits were then allowed to resume normal cage activities until sacrifice at 1, 2, 3, or 6 weeks later. Within a week of unloading, the ultimate tensile strength of the PT decreased nearly 50% and UTS continued to decrease out to three weeks (Yamamoto *et al.*, 1993). The decrease in material properties can be partially attributed to the decrease in collagen fibril density and size (Tsuchida *et al.*, 1997).

Histologically, the number of fibroblasts in the mid-substance of the tendon increased four-fold over the control/sham within two weeks of stress-shielding. The cells in the mid-substance also changed morphology from spindle to round, and the collagen fibers became smaller and less aligned (Yamamoto *et al.*, 1993). When the cells in the patellar tendon were destroyed by freezing prior to stress shielding, it was noted that proliferative round and ovoid cells appeared three weeks later, suggesting that the increase in cellularity arises from outside the tendon (Ohno *et al.*, 1993). Freezing the patellar tendon also reduced tendon tensile strength and there was a further reduction in tensile strength with six weeks of stress shielding (Yamamoto *et al.*, 2000). These data suggest that tenocytes contribute to maintaining the mechanical integrity of the matrix, and this necessity persists under stress shielded conditions.

The tensile strength of collagen fascicles decreased more than the bulk tendon under stress shielded conditions. Stress shielding the entire patellar tendon decreased tensile strength of the collagen fascicles by 74% (one week), 44% (two weeks), and 19% (three weeks) which is more than that observed for the bulk tendon which decreased by 50%, 13% and 9% during the same

period (Yamamoto *et al.*, 1993, 1999). Together, these data suggest that interactions between ground substance and collagen fascicles contribute to the mechanical integrity of the tendon and that tendon cells negatively impact tendon strength under stress shielded conditions.

Restressing the patellar tendon for six weeks following a one-week stress shielding period, or for twelve weeks after a two-week stress shielding period was not able to rescue ultimate tensile strength. This suggests that long term changes to the tendon substance occur because of stress shielding (Yamamoto *et al.*, 1996). Interestingly, the optimal load (cyclic stress of 4 Hz – 1 hr/day, 2 MPa) needed to maintain tendon mechanics in culture mimics the *in vivo* stress and frequency experienced by the rabbit patellar tendon while running (Yamamoto *et al.*, 1992, 2005).

Other evidence that stress shielding contributes to the development of tendinopathy is supported by studies that demonstrate that tendons with weaker material properties can heal better following injury. Having a weaker material with similar stiffness throughout would allow load to be distributed throughout the tissue and prevent stress shielding of weaker regions. Fetal tendons injured with a partial transection completely heal within seven days with no noticeable scar tissue (Beredjiklian *et al.*, 2003). This is true even when the fetal tendon is transplanted into an adult animal, suggesting that there is not a circulating substance that prevents tendon regeneration (Favata *et al.*, 2006). It is either the weak material of the fetal tendon or the epigenetic status of the fetal tendon cells that is permissive for regeneration.

Further support that material properties of the tendon dictate regeneration are corroborated by studies that used that beta-aminopropionitrile (BAPN) during early tendon healing. BAPN inhibits collagen crosslinking, which decreases tendon stiffness, and BAPN treatment after a tendon injury resulted in better collagen fiber alignment in repaired tissue (Alves *et al.*, 2001). The problem with reducing the tendon stiffness for a brief period of time with BAPN is that the tendon

does not regain mechanical strength (Craver *et al.*, 1968) and there is no difference in subsequent injury risk (Dyson, 2004).

The first suggestion that stress shielding contributed to the etiology of tendinopathy was made by Almekinders and colleagues in 2002. They found that in the human patellar tendon, strain increased at all regions in the patellar tendon throughout the knee flexion cycle except for the central proximal posterior region (Almekinders *et al.*, 2002). The central proximal region is also the most common location of patellar tendinopathy (Johnson 1196, Shalaby 1999). However, the idea that tendinopathy occurs in regions where load is minimal is contrary to the notion that tendinopathy results from tensile overload.

In a native tendon, it is possible that a small amount of damage results in a matrix that is weaker than surrounding regions and this results in stress shielding by the healthier part of the tendon. This situation occurs in tendinopathy. After an acute injury to a tendon, the regenerated scar tissue is weaker than the surrounding tendon (Maeda *et al.*, 2009). This imbalance between the material properties of the scar tissue and the healthy tendon could result in stress shielding of the scar tissue because load will preferentially pass through stiffer regions of tendon, in much the same way that the stainless-steel wire shielded the patellar tendon in the Hayashi work. Considering that the hallmarks of tendinopathy (collagen disorganization, increased cellularity, and decreased mechanics) (Pingel *et al.*, 2012, 2014) appear with experimental de-tensioning of the rabbit patellar tendon (Ohno *et al.*, 1993; Yamamoto *et al.*, 1993), there is a clear argument for the role of stress shielding in the development of tendinopathy.

Tendinopathy: Translational Models & Perspective

Translational models of tendinopathy aim to understand the basic science of tendon degeneration and regeneration and relate these changes to the human condition. For humans, improved pain and function are the goal of any treatment, yet they serve as biased endpoints because pain assessments do not provide an accurate assessment of tissue structure/function.

There are few translational models that help delineate the role of load as an etiological factor and treatment for tendinopathy. However, understanding the basic mechanisms behind tendon degeneration and regeneration is essential to advance treatments for tendinopathy. There are many ways to injure a tendon, in attempt to create tendinopathy in a model organism. The most common methods involve a physical injury (transection, laceration, biopsy punch), overuse (i.e., treadmill running), or chemical intervention (collagenase, prostaglandins, cytokines, and substance P) (Warden, 2007). This section is not meant to be a complete review of each model, rather an introduction to the models and discussion of their translatability to human tendinopathy.

The most extreme model used to study tendon healing are tendon transections. In tendon transection models, the tendon is cut in the transverse plane and two stumps of the tendon on either end are left unrepaired. This model therefore represents a tendon rupture rather than tendinopathy. In a complete tendon transection, the newly formed scar tissue is characterized by exclusively small collagen fibrils (Frank *et al.*, 1992, 1997). Regardless of the clinical applicability, following Achilles tendon transection, daily short loading bouts are sufficient to increase the tensile strength of the healing tendon. There was no difference in mechanics between rats that were injured then loaded for 15 min compared to 60 minutes a day for 12 days, suggesting that only a short loading bout is necessary to increase tendon strength (Andersson *et al.*, 2009). Full time cage activity had a bigger effect on tendon strength than the 60 min bout. However, with full time cage activity there was a 4-fold increase in mechanical properties (MTL) and only a 1.5-fold increase in material properties (UTS) compared to the injured and unloaded control suggesting that the newly formed tendon scar tissue was poor quality (Andersson *et al.*, 2009).

Tendon injury healing is also commonly modeled using full thickness, partial width defects. These injures can be induced using a blade to cut an incision in the tendon, or a using a biopsy punch to remove part of a tendon. These models produce a significant reduction in tendon

mechanics, with the least variation observed using a biopsy punch compared to an incisional injury (Beason *et al.*, 2012). Beason suggested that the patellar tendon was the most consistently reproducible injury model, when compared with the FDL and Achilles tendons (Beason *et al.*, 2012). However, reproducibility of the patellar tendon biopsy punch model could result from the familiarity of the lab with this particular injury model (Lin *et al.*, 2006). In the biopsy punch model, mechanical properties (maximum stress & modulus) of the healing PT at 3 weeks post injury are decreased by roughly 50% in the in $IL6^{-/-}$ mice (compared to the injured wild type mice) suggesting the necessity of an inflammatory response in tendon repair (Lin *et al.*, 2006).

Overuse models such as treadmill running have been studied as an attempt to physiologically induce tendinopathy. In the supraspinatus tendon, downhill running for an hour a day, five days a week increased tendon CSA yet decrease tendon material properties (stress and modulus) relative to control. Changes in CSA were apparent after 4 weeks of running and continued to increase over 16 weeks, whereas changes in material properties plateaued after 4 weeks (Soslowsky *et al.*, 2000). Similar changes in tendon morphology were noted in the Achilles tendon following a 12-week uphill running protocol. Interestingly, the increase in cellularity was attributed to endothelial cells and fibroblasts because immunohistochemistry showed no signal for CD45 (labels immune cells- leukocytes) but a robust signal for vimentin (labels endothelial cells and fibroblasts) (Glazebrook *et al.*, 2008). However, when this uphill running model was repeated, the changes with running noted in the Achilles tendon were largely positive. No region of the Achilles tendon displayed pathologic histology or metabolism. The runners had increased Achilles tendon failure stress relative to body weight, which was not exclusively driven by a significantly lower body weight in the runners but also a trend for higher absolute failure stress. Gene expression changes were not characteristic of tendinopathy except for increased *Col3a1* and decreased *Timp3* and *Adamts-5* (Heinemeier *et al.*, 2012). Overall, these data suggest that

overuse models have the potential to cause tendinopathy, but do not always result in a robust, repeatable changes in tendon pathology because of the potential for a beneficial tendon adaptation from loading.

Collagenase injections are the most commonly used chemical to induce tendinopathy. There is no standard dose or duration of collagenase-induced tendinopathy, and the resulting changes to the matrix depend on the collagenase dose and time after injection (Orfei *et al.*, 2016). The typical sequence of events following a collagenase injection include a robust immune cell infiltration immediately after injection (Marsolais *et al.*, 2001; Orfei *et al.*, 2016). Within a day after treatment with 30 uL of collagenase (10 mg/ mL) in the Achilles tendon, there was a 46-fold increase in the number of neutrophils and an 18-fold increase in a macrophage sub-population. Neutrophils begin to subside within a week following injury. By two weeks, the macrophages present in the tendon have shifted towards a different sub-population (Marsolais *et al.*, 2001). The collagen fiber alignment is disrupted within a day of injection (Marsolais *et al.*, 2001). This occurs alongside a decrease in type I collagen content and increase in collagen I and III gene expression (Dahlgren *et al.*, 2005). Considering that collagenase injections have a large immune response and there is considerable degradation of the quality of the tendon matrix, the changes induced may be too aggressive to be useful as a model of human tendinopathy.

Loading Protocols for Tendinopathy

There are a wide range of treatments for tendinopathy including rest, ice, nonsteroidal anti-inflammatory medications (NSAID), corticosteroid injections, platelet rich plasma injections, growth factors, stem cell treatment, extracorporeal shock wave therapy, laser therapy, ultrasound, and physical therapy (Andres & Murrell, 2008). Despite all the adjuvant therapies, physical therapy has the best outcomes. Given the clinical nature of all these interventions, outcome measurements are usually based on pain reduction and functional improvements. The Victorian Institute of Sport Assessment (VSIA) questionnaire is the most common assessment of

tendon injury. The VISA questionnaire was first developed for patellar tendinopathy (VISA-P) and later adapted for other tendinopathies (e.g. VISA-A for Achilles tendinopathy) (Visentini *et al.*, 1998; Robinson *et al.*, 2001). It assesses symptoms, function, and ability to participate in sport (Visentini *et al.*, 1998). Loading protocols such as eccentrics (Alfredson), combined concentric-eccentric (Silbernagel), heavy slow resistance (Kongsgaard), and isometrics can improve VISA-A scores in as little as two weeks, yet a substantial improvement is usually reported to take twelve weeks on average (Murphy *et al.*, 2018). Whether or not decreased tendon thickness is a sign of clinical improvement is debated. Reductions in Achilles tendon thickness do not correlate with clinical outcomes, but only eccentric exercise interventions have been used for these measures. It is possible that with a different tendon (i.e patellar) or intervention (i.e. HSR exercise), there is a more substantial reduction in tendon thickness that corresponds to better outcomes (Beyer *et al.*, 2015; Färnqvist *et al.*, 2020; Sprague *et al.*, 2022).

Eccentric exercises for tendinopathy are clinically considered the most effective treatment for tendinopathy (Irby *et al.*, 2020). Eccentrics are a type of physical rehabilitation exercise where the associated muscle is performing a lengthening contraction. Eccentrics were first proposed as a treatment for tendinopathy by William Stanish and Sandra Curwin in 1984 (Curwin & Stanish, 1984). Their protocol was a comprehensive program that included stretching, eccentric drop squats, and icing of the patellar tendon. The patient progressed through the program by increasing speed of the drop squat, then increasing resistance (Stanish *et al.*, 1986). In 1998, Alfredson proposed heavy eccentric exercises performed daily in three sets of fifteen repetitions as a treatment option for Achilles tendinopathy. In contrast to the Stanish and Curwin protocol, slow movements were encouraged in the Alfredson protocol and patients progressed through the protocol by increasing weight. Interestingly, Alfredson developed the protocol in an effort to rupture his tendinopathic Achilles tendon, reasoning that since ruptured tendons often heal more completely than partial tears this would help cure his chronic pain (K Baar, personal

communication). The heavy slow eccentric loading did not rupture the tendon, but twelve weeks of the protocol decreased pain and improved the isokinetic concentric and eccentric peak torque in the injured Achilles tendon (Alfredson *et al.*, 1998). Although VISA scores improve with eccentric training, twelve weeks of eccentric training does not change collagen content or crosslinking of tendinopathic patellar tendons (Kongsgaard *et al.*, 2009).

The eccentric overload protocol was modified by Silbernagel et al in 2001 for proximal Achilles tendinopathy with an increased focus on concentric moves. Her protocol consists of three phases of a progressive toe-raise protocol. The first week/phase includes: toe extension/ flexion and plantar extension/ flexion, calf muscle stretching, walking on toes, walking on heels, and two sets of fifteen double-leg toe raises. The second phase (weeks 2-3) is similar to phase 1, except an increase to two sets of twenty toe-raises and the addition of single leg toe raises and single leg heel drops. The finals phase (week 4-12) is like phase two, yet with an increased number of repetitions of double and single leg toe-raises, single leg toe raises on a step, and the addition of quick rebounding toe-raises. The initial cohort showed reduced pain and improved performance following three months of treatment (Silbernagel *et al.*, 2001). There was no difference in improvement in VISA-A score if the participants participated in sport for the first six weeks. However, there is a significant correlation between initial VISA-A score and improvement in VISA-A as a result of the program. This suggests that it is best to start rehab before symptoms become severe (Silbernagel *et al.*, 2007). A five year follow up of these patients reported a similar VISA-A score between the exercise/rehab and rest/rehab groups confirming that initial participation in sport did not alter long-term recovery (Silbernagel *et al.*, 2011).

Heavy slow resistance (HSR) first emerged as an alternative treatment for tendinopathy in 2009 (Kongsgaard *et al.*, 2009). HSR was known to increase healthy tendon CSA and stiffness, and therefore was proposed as a treatment for tendinopathy (Kongsgaard *et al.*, 2007). The first randomized control trial on HSR compared HSR (four sets of three exercises, three times a

week), an Alfredsen eccentric protocol of decline squats (two sets of fifteen slow repetitions, twice daily), and corticosteroid injections. HSR and eccentric exercises improved pain and function after twelve weeks, and these improvements were maintained after half a year. Corticosteroid treatment had the worst long-term outcome with a similar VISA-P to baseline and significantly worse VISA-P score than HSR and eccentric exercises. PT CSA decreased with HSR and corticosteroid treatment. However, tendon collagen content increased with HSR and declined with corticosteroid treatment (Kongsgaard *et al.*, 2009). This suggests that a more aligned dense tendon matrix is produced with HSR compared with eccentrics. Another randomized controlled trial that compared HSR and eccentric exercises for Achilles tendinopathy reported improved pain and function after twelve weeks. This was accompanied by a better treatment compliance and patient treatment satisfaction with HSR at the half year follow up (Beyer *et al.*, 2015). For the exercise to be effective progressive resistance is required, as no improvements in shoulder pain or function were noted in a systemic review and meta-analysis with non-progressive or non-resisted exercise (Naunton *et al.*, 2020).

It is important to note that all successful exercise interventions have a common component: the use of a slow velocity. While it has not been directly tested, performing the Alfredsen protocol at a high velocity would likely exacerbate the tendinopathy. This suggests that there is something significant about low velocity movements for tendon health.

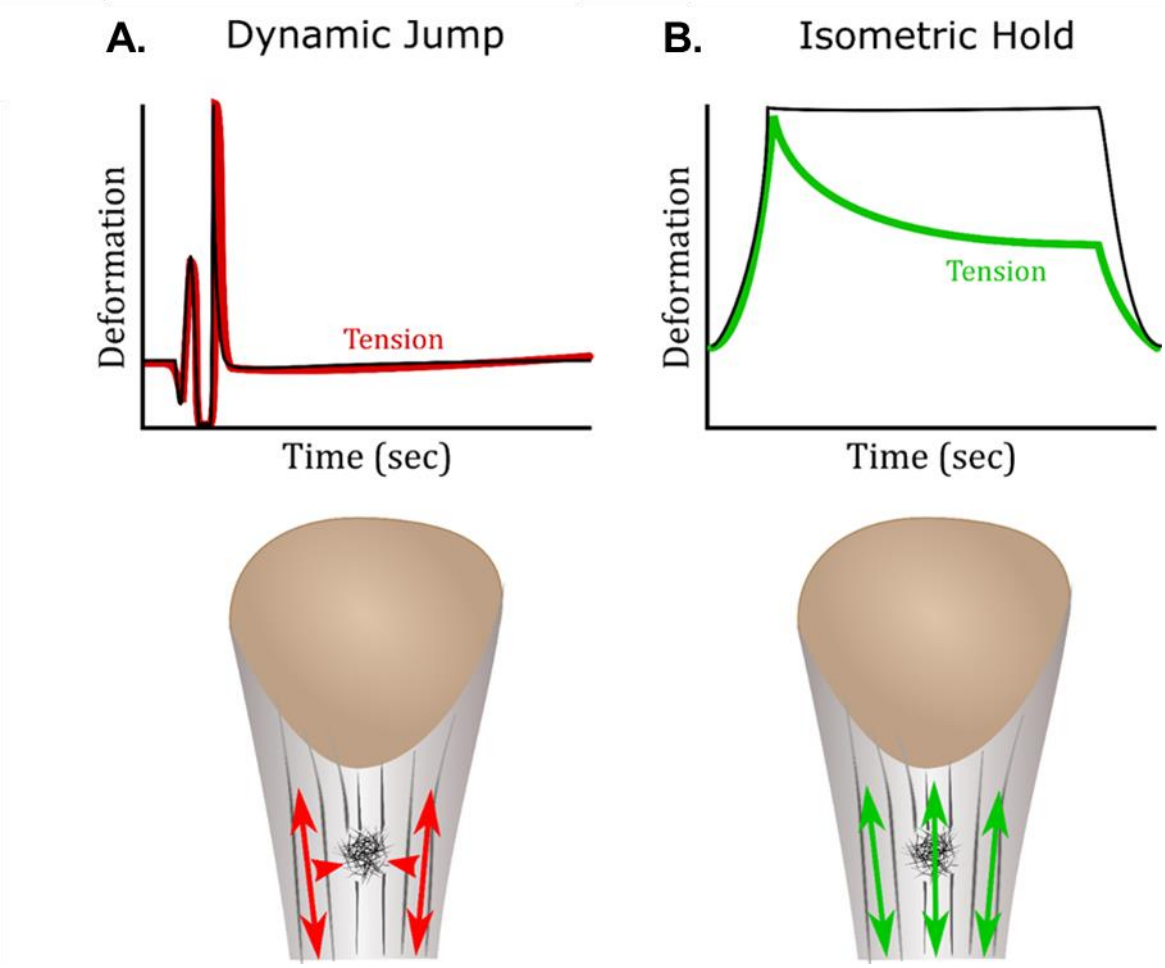

Figure 11. Isometric (Stress Relaxation) Exercises to Treat Tendinopathy. A) Short duration loads rapidly extend a tendon and compress the scar tissue. B) Isometric holds for 30 seconds cause stress relaxation within the tendon and potentially direct a tensile load signal through the scar tissue.

Extending the idea of slow movements to its maximum (i.e. decreasing the velocity of movement to zero) isometrics - muscle contractions without a change in joint angle - are the slowest form of HSR training. Isometrics provide immediate pain relief and long-term structural improvements (Rio *et al.*, 2017; Baar, 2019). Both isometric and heavy slow isotonic loading programs reduce patellar tendon pain and improve function in jumping athletes over a four-week intervention period during the competition season (van Ark *et al.*, 2016)**.** However, the subset of these athletes that had baseline and 4-week follow-up ultrasound tissue characterization (UTC) scans had no changes in tendon structure (van Ark *et al.*, 2018). Additionally, HSR and isometric

protocols provide an immediate analgesic effect following each training session, but the effect is larger with isometrics (Rio *et al.*, 2017)**.** During rehab, pain subsides much quicker than the structure of the tendon improves. In a case study with a professional basketball player that had a severe central core patellar tendinopathy, a year of isometric loading resulted in regeneration within the tendon core. During the protocol, the athlete drank 15 g of gelatin in orange juice (contains ~225 mg vitamin C, a necessary coenzyme for collagen synthesis) one hour prior to a 10 min isometric loading program. The 10-minute loading program was used because short loads are optimal to increase collagen synthesis (Paxton *et al.*, 2012; Baar, 2019). Each isometric hold was performed for ~30 seconds to allow for stress relaxation within the tendon (Atkinson *et al.*, 1999). Stress relaxation allows for the relaxation of healthy, stronger collagen within the tendon. Considering that the scar tissue is weaker than the healthy tissue (Maeda *et al.*, 2009) and tenocytes within a tendinopathic matrix are surrounded by disorganized collagen fibers (Pingel *et al.*, 2014), they are likely shielded from a tensile load signal (Figure 11A). Relaxation of the stronger collagen should allow for a tensional load to reach the cells within the damaged part of the tendon (Figure 11B). The tensile load over the scar tissue is critical for directing repair because tendon cells deposit collagen along the line of force and this directed collagen synthesis is needed to regenerate the tendinopathic matrix (Kapacee *et al.*, 2008; Baar, 2019). While encouraging, this case study is the only published demonstration of complete regeneration of a tendinopathic tissue (Baar, 2019).

From the background above, a number of important questions arise:

1. What is the molecular make up of a mature tendon?
2. What molecular changes drive the development of a mature tendon?
3. Is there a model of tendinopathy that can recapitulate the human condition?
4. How do different loads (isometric v. dynamic) alter gene expression in tendinopathic tissue?
5. Can a generalized loading prescription be developed to optimize tendon regeneration?

The dissertation that follows tested four hypotheses:

1. Different tendons (Achilles and patellar) develop through a conserved genetic program.
2. Human tendinopathy can be modeled in rats using a biopsy-based injury model.
3. Overcoming stress shielding using muscle contractions that optimize stress relaxation initiates tendon regeneration.
4. Gene expression in healthy adult tendon is regionally dependent.

Chapter 2. The Rat Achilles and Patellar Tendons Have Similar Increases in Mechanical Properties but Become Transcriptionally Divergent During Postnatal Development

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Key Points

- Little is known about mechanisms of post-natal tendon growth. We characterized morphological, mechanical, and transcriptional changes that occur before (P7), and early (P14), and late after (P28) rats begin to walk.
- From P7 to P28, the Achilles tendon increased in length, whereas the patellar tendon increased in cross sectional area. Mechanical and material properties of the Achilles and patellar tendon increased from P7 to P28.
- From P7 to P28, the Achilles and patellar tendons increased expression of ECM genes and decreased mitochondrial and cell cycle gene expression. Ribosomal gene expression also significantly decreased in the Achilles and tended to decrease in the patellar tendon.
- At P28, STAT1 signaling tended to be lower in the patellar tendon which had grown by increasing cross sectional area and inhibiting STAT activation *in vitro* improved mechanical properties in engineered human ligaments.

Abstract

The molecular events that drive post-natal tendon development are poorly characterized. In this study, we profiled morphological, mechanical, and transcriptional changes in the rat Achilles and patellar tendon before walking (P7), shortly after onset of walking (P14), and at motor maturity (P28). The Achilles and patellar tendons increased collagen content and mechanical strength similarly throughout post-natal development. However, at P28 the patellar tendon tended to display a higher maximal tensile load (MTL) ($p=0.0524$) than the Achilles tendon, but a similar ultimate tensile strength (UTS; load relative to cross-sectional area) likely due to its increased cross-sectional area during development. The tendons started transcriptionally similar, with overlapping PCA clusters at P7 and P14, before becoming transcriptionally distinct at P28. In both tendons, there was an increase in extracellular matrix (ECM) gene expression and a concomitant decrease in cell cycle and mitochondrial gene expression. The transcriptional divergence at P28 suggested that STAT signaling was lower in the patellar tendon where MTL increased the most. Treating engineered human ligaments with the STAT inhibitor itacitinib increased collagen content and MTL. Our results suggest that during post-natal development, cellular resources are initially allocated toward cell proliferation before shifting towards extracellular matrix development following the onset of mechanical load and provide potential targets for improving tendon function.

Introduction

During post-natal development, tendons mature from a loose connective tissue with high cell mass to a dense extracellular matrix (ECM) with few resident cells (Greenlee & Ross, 1967; Moore & De Beaux, 1987*a*). The force-transmitting properties of the patellar (quadriceps muscles/kneecap to tibia) and Achilles (triceps surae muscles to heel) tendons arise from the composition and organization of the ECM. Load is transmitted along the long axis of a mature tendon via densely packed type I collagen fibrils/fibers/fascicles. Mechanical loading is necessary for axial tendon maturation (Huang *et al.*, 2015*b*), during which time the collagen network develops into a bimodal population of small and large collagen fibrils (Liu *et al.*, 2010). Even though the phenotypic maturation of tendons is well understood, the molecular events that guide the development of a robust matrix are largely unknown. Further, understanding the processes that transcriptionally guide post-natal tendon development may be useful in assessing strategies that improve tendon mechanics or accelerate regeneration.

Tendons are formed prior to birth through the induction and differentiation of mesenchymal stem cells (MSC) into tendon progenitor cells (Huang *et al.*, 2015*a*). Tendons begin to form as short anchors to bone and subsequent elongation depends on recruitment of additional scleraxis positive MSCs (Huang *et al.*, 2019). At the time of birth, roughly half the tendon mass is cells, and the other half is collagen (Moore & De Beaux, 1987*a*). Over the first few post-natal weeks, the number of cells decreases, and the surviving cells elongate along the line of force into long spindle shaped cells (Chen *et al.*, 2016) within a fibrillin matrix (Ritty *et al.*, 2003)

Despite known increases in mechanical strength and changes in tissue morphology during the post-natal period, the transcriptional events that govern post-natal tendon maturation are poorly defined. The collagen fibril population is exclusively small fibrils at birth (Moore & De Beaux, 1987*a*; Ansorge *et al.*, 2011; Chen *et al.*, 2016) with round tendon cells embedded in a loose collagen matrix. Throughout the early post-natal period, collagen fibrillogenesis continues and

small collagen fibrils begin to fuse to form bigger fibrils resulting in a bimodal fibril distribution at maturity (Liu *et al.*, 2010; Chen *et al.*, 2016). Mechanical and material properties also dramatically increase during the early post-natal period (Ansorge *et al.*, 2011; Theodossiou *et al.*, 2019). The maximal tensile load (MTL) of the rat Achilles tendon has been reported to increase ~8.5-fold in the first ten days after birth (Theodossiou *et al.*, 2019), yet this increase in mechanical strength prior to walking does not depend on mechanical load signals (Theodossiou *et al.*, 2021).

The purpose of this study was to better characterize the morphological, mechanical, and transcriptional properties of the rat Achilles and patellar tendons during the early post-natal period. We selected these two tendons because they have ultrastructural, biochemical, and functional similarities in the adult. In fact, the adult patellar and Achilles have nearly identical collagen concentration, crosslink profile, and collagen fibril distribution (Amiel *et al.*, 1984; Rumian *et al.*, 2007). By selecting two different lower limb tendons, we also sought to identify processes related to the onset of mechanical load that are common to tendon development and those that are unique to the different morphological changes that occur in the Achilles and patellar tendons. The first time point selected for analysis was prior to the onset of mechanical load (walking) at postnatal day 7 (P7). The latter two time points were shortly after walking begins (P14) and at motor maturity (P28) (Shriner *et al.*, 2009; Swann & Brumley, 2019; Theodossiou *et al.*, 2019). Our hypothesis was that Achilles and patellar tendon mechanics would increase similarly throughout early post-natal development and that the transcriptional profile of the two tendons would not differ based on the different loading environments.

Materials and Methods

Ethical Approval

All experiments were approved by the University of California Davis Institutional Animal Care and Use Committee (IACUC) under Protocol 22957. The UC Davis IACUC is regulated by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), NIH Public Health Service (PHS), and US Department of Agriculture (USDA). Reporting of experimental details followed ARRIVE Guidelines (du Sert *et al.*, 2020).

Animals

All experiments were approved by the University of California Davis Institutional Animal Care and Use Committee under Protocol 22957. Sprague Dawley rats (Charles River Laboratories) were housed in 12-hour light and dark cycle in a single cage per litter with *ad libitum* access to food and water throughout the experiment.

The rats compared at P7, P14, and P28 for transcriptomic analysis came from a single litter, while rats compared at those same timepoints for mechanics came from a separate litter. No *a priori* sample calculation was performed because we cannot predict the litter size. While not blinded, equal numbers of male/female rats were randomly allocated to each timepoint. The ratio of male:female rats used at each timepoint are listed in Appendix Figure 1 and Appendix Table 2. P1 was considered the first day after birth. The litter used for mechanics had nine rats and three rats were randomly allocated to each timepoint. It was decided *a priori* to perform tensile tests on both limbs and report mechanical properties from the limb with the higher MTL. This is because gripping small tendons can be challenging; and the potential for inaccurate mechanics data was high for the early timepoints. The litter used for transcriptomics had fifteen rats and five rats were assigned to each timepoint. It was decided *a priori* to extract RNA from all tendons and sequence four tendons at each timepoint to optimize the ratio of male:female rats and RNA quality score. For RNAseq, because of variability in transcript abundance, a sample size of N=4 has been observed to yield sufficient power sufficient (>0.8) to determine differential expression (Ching *et*

al., 2014; Disser *et al.*, 2020). Rat pups were euthanized by cervical dislocation and pneumothorax immediately prior to tissue collection.

Mechanics

The mechanical properties of P7 (N=3), P14 (N=3), and P28 (N=3) rat Achilles and patellar tendons were tested using a Model 68SC-1 single column tensile tester containing a 100N (rodent tendons) or 10N (engineered ligaments) load cell, BioPuls Body Temperature Bath, and BioPuls Submersible Pneumatic Side Action Grips (Instron, Norwood, MA). The left and right AT and PT from each rat were isolated, wrapped in PBS-soaked gauze, and briefly stored at room temperature before testing. Care was taken to isolate each tendon with intact muscle and bone. The patellar tendons were isolated with the quadriceps muscle, patella, and tibia. The Achilles tendon was isolated away from the plantaris tendon while keeping the gastrocnemius (GTN) and soleus muscles, calcaneal bone and foot attached. Resting length and width of each tendon was measured with digital calipers prior to testing. For the Achilles, the length was measured from the calcaneus to the beginning of the myotendinous junction on the posterior aspect of the tendon. For the patellar tendon, the length was measured from the tibia to the patella bone. For the engineered ligaments, the length of each construct was measured in millimeters using a digital caliper. The width and depth of constructs were measured using a Lumedica OQ LabScope (Lumedica Inc, Durham, NC). CSA for the Achilles and patellar tendons was calculated by measuring the width at the narrowest point and assuming the tendon has a rectangular shape with a depth of 0.50 mm.

The patellar tendon was tested by gripping the quadriceps muscle and tibia, whereas the Achilles tendon was tested by gripping the GTN and soleus and foot. All muscles were wrapped in 14 AWG (American wire gauge) wire before gripping to prevent lateral spreading within the grip. The distal end of the tissue was secured by gripping either the tibia (PT) or calcaneus (AT). Grip tension was held at 80 pounds per square inch.

Samples were immersed in 0.5x PBS within the BioPuls bath held at 37°C. All tendons were preconditioned using 10 cycles (0.10-0.25 N, 0.25 mm/s), and then elongated at 0.25 mm/s until force dropped 80%. MTL was the maximal load measured prior to failure in Newtons. Ultimate tensile strength (UTS) was calculated by normalizing the MTL by cross-sectional area (CSA) and the Young's modulus was calculated as the maximal slope of the stress-strain (displacement divided by the initial length) curve.

Collagen

Collagen content was measured using a hydroxyproline assay as described by Woessner (Woessner, 1961). Briefly, following mechanical testing, the patellar tendon was cut away from the patella and quadriceps tendon and the Achilles tendon was trimmed away from the calcaneous and gastrocnemius muscle. Tissues were dried on a glass plate for 30 minutes at 120°C and dry mass was measured immediately after cooling. For the PT at P7 and P14, the dry mass was below the detection limit of the scale (Accuris Instruments W3100A-120 with a readability of 0.1 mg) so only total collagen content is reported. Dried tissues were hydrolyzed in 200 µL of 6N HCl at 120˚C for 90 minutes (engineered ligaments) or 2 hours (rat tendon). The lids of the tubes were opened and the HCl evaporated in a lamellar flow hood. The dried pellet or a standard curve of hydroxyproline was suspended in 200µL of hydroxyproline buffer (173 mM citric acid, 140 mM acetic acid, 588 mM sodium acetate, 570 mM sodium hydroxide), 150 µL of Chloramine T solution was added to each sample, before mixing, and incubating for 20 min at room temperature. Next, 150 µL aldehyde-perchloric acid containing 60% 1-propanol, 5.8% perchloric acid, and 1M 4-(dimethylamino) benzaldehyde was added to each tube and the samples incubated at 60°C for 15 min. Samples were cooled for 5 minutes and 200µl of the solution was read at a wavelength of 550 nm on an Epoch Microplate Spectrophotometer (BioTek Instruments Limited, Winooski, VT). Hydroxyproline content was calculated using the standard curve and then converted to collagen assuming hydroxyproline contributes to 13.7% of

the dry mass of collagen. This collagen content was divided by the dry mass to determine the percent collagen within the sample where possible.

3' Tag RNA-Sequencing

The patellar (without the quadriceps tendon) and Achilles (plantaris tendon excluded) tendons were dissected from both limbs of five rats at each timepoint (Figure 1) and snap frozen in liquid nitrogen. RNA isolation was performed for all patellar tendon samples together and again with all Achilles tendons samples. In each case, the left and right tendons were pooled together for RNA isolation and isolated with a phenol free RNA isolation kit using proteinase K and DNase digestion (Norgen Animal Tissue RNA Purification). RNA sample integrity was assessed using a LabChip GX (software version 5.3.2115.0) to assign RNA quality scores by the UC Davis Genome Center.

3' Tag RNA-seq libraries were generated from the four samples with the highest RNA quality scores per timepoint by the UC Davis Genome Center from RNA (> 500 ng in 20 uL) and sequenced on an Illumina HiSeq 4000 (Illumina, San Diego, CA) generating 10M single end 90-bp reads per sample (Meyer *et al.*, 2011; Steffen *et al.*, 2022).

RNA-Sequencing Data Analysis

Data processing was performed using CLC Genomics Workbench 21 (QIAGEN) similar to traditional RNA-seq protocols. Quality control assessment was performed using QC for Sequencing Reads 0.2 based upon FastQC and then trimmed with Trim Reads 2.5 within Workbench (parameters: quality limit = 0.05; trim ambiguous nucleotides = yes; maximum number of ambiguities = 2); the first 12 bp of the non-poly A reads were trimmed to remove the random primer. PCR duplicates were removed. Single reads with a minimum of 20 bp were kept and aligned on Rattus norvegicus (Ensembl Rnor_6.0) annotated reference genome using RNA-Seq Analysis 2.3 (parameters: mismatch cost = 2; insertion cost = 3; deletion cost = 3; length fraction = 0.8; similarity fraction = 0.8; strand-specific to both strands; maximum number of hits

for a read = 10). Nearly all reads were >85 base pairs with reads less than 50 bp comprising 0.02 – 0.03% of total reads. Reads were normalized and reported as number of transcripts per million (TPM) reads. Differential gene expression was determined using Differential Expression for RNAseq 2.5 with a student's t-test statistical analysis including false discovery rate multiple test correction, considering either tendon or age. Differentially expressed genes (DEGs) were defined as having log2 (fold change) >0.6 or <-0.6 and q <0.05. DEGs were applied to bioinformatics analyses. Principal component analysis plots were generated with CLC Genomics Workbench and for publication by ClustVis (Metsalu & Vilo, 2015). DEGs from Workbench were determined and then applied to volcano plots using GraphPad Prism (Windows version 9.5.1). Volcano plots – Log_2 fold change was capped to range from -12 to 12 and FDR p values were capped at 0.000001 which resulted in a maximal log(FDR) of 6. Venn Diagrams to aid in characterization of DEGs were generated with R package eulerr (R package version 6.1.1) (Larsson & Gustafsson, 2018) and then overlap and unique groups of DEGs were also applied to bioinformatics analyses.

Bioinformatics analysis was performed with clusterProfiler and Advaita iPathwayGuide. For these analyses, we did not filter out transcripts with low counts, but excluded genes that did not have any transcripts mapped to them. Additionally, in our comparisons of X vs Y (e.g. P7 vs P28), an upregulated gene/ GO term / pathway is upregulated from timepoint X to timepoint Y. GO Terms that were shared or unique to the patellar and Achilles tendon from P7 to P28 was determined by identifying genes that were differentially expressed in both tendons, or uniquely differently expressed in the patellar or Achilles tendon. Those DEG were used for an over-representation analysis (ORA) using clusterProfiler (Yu *et al.*, 2015; Wu *et al.*, 2021). The GO terms identified in the ORA were displayed in enrichment maps. To reduce bias, we first determined what was regulated (using the enrichment maps). Since enrichment maps do not display the direction of regulation, we selected representative GO terms from each node of the enrichment map and

displayed the log2 FC and p-value of those genes in a volcano plot to show direction of change. Pathways analysis was performed with Advaita iPathwayGuide which uses a proprietary method that incorporates impact analysis via over-representation of DEGs and pathway perturbation of measured expression changes across pathway topology (Draghici *et al.*, 2007; Nguyen *et al.*, 2019). Transcription factor perturbations that best mapped to the top 200 genes (ordered by $\log_2$ fold change with at least 2/4 samples containing non-zero TPM values) upregulated in the patellar tendon versus the Achilles tendon at P28 was determined with Enrichr (Chen *et al.*, 2013; Kuleshov *et al.*, 2016; Xie *et al.*, 2021).

ACL Fibroblast Culture

Cells were isolated from remnants of a human anterior cruciate ligament (ACL) collected during reconstruction surgery in October 2022, from a 19-year-old female. The human ACL was obtained following informed consent and all procedures and experiments were approved by the University of California Davis Institutional Review Board with IRB protocol number 779755. The ACL remnants were washed 5-times in phosphate-buffered saline (PBS), then placed in a 5% antibiotic/antimycotic solution for 1 hour at 4°C. Tissues were then digested in 0.1% collagenase Type II dissolved in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 20% fetal bovine serum (FBS) (Lot no. 104P22, Cat no. S1620, Biowest USA, Inc) and 1% penicillin overnight at 37°C. The next day, fibroblasts were collected by centrifugation (2,000 x g for 5 min) and washed 3 times with growth media (DMEM supplemented with 10% FBS and 1% penicillin). Fibroblasts were cultured in 15 cm plates containing 17 ml of growth media until cells were at 70-80% confluence before passage. Engineered ligament constructs were made at passage 6.

Preparation of Engineered Ligament

Brushite anchors were prepared by mixing 3.5 M orthophosphoric acid with powdered β-tricalcium phosphate (Batch No. P325S SD, Plasma Biotal Limited, Derbyshire, UK) on ice at a 1:1 (μl:mg) ratio. This mixture was pipetted into 3D printed molds containing pins and then

centrifuged at 2250 x g at 4°C for 1 minute. The anchors were left overnight to dry at room temperature, then were removed from molds and stored at room temperature until ready for use. Human ACL constructs were formed as previously described (Paxton *et al.*, 2010). Briefly, $2.5x10^5$ fibroblasts were suspended in growth medium containing 5.8 Units of thrombin (Cat. No. T4648, Sigma-Aldrich), 20 μg aprotinin (Cat. No. 194559, MP Biomedicals, LLC), and 200 μg 6-aminohexanoic acid (Cat. No. 07260, Sigma-Aldrich). The thrombin/cell mixture (714 μl) was added to a 35 mm plate containing two brushite anchors spaced 12 mm apart. Then, 286 μl of fibrinogen (20 mg/ml) (Cat. No. F4883, Sigma-Aldrich) was added to each plate and plates were incubated at 37°C with 5% CO_2 for 15 minutes to allow fibrin-gel formation. Constructs were then fed 2 ml of feed media consisting of growth media supplemented with 200 μM ascorbic acid (Cat. No. A8960, Sigma-Aldrich), 50 μM proline (Cat. No. P5607, Sigma-Aldrich), and 5 ng/ml of transforming growth factor-β1 (Cat. No. 100-21, Peprotech, Rocky Hill, NJ). Construct feed media was replaced every other day until day 8. On day 8, groups of constructs were treated with feed media supplemented either with 0.2 μl/ml 100% ethanol (control) or 10 μM Itacitinib (Cat No. S7812, Selleck Chemicals LLC, Houston, TX) for the last 6 days of a 14-day culture period.

Statistics

All statistics except RNA-seq DEG analysis was performed using GraphPad Prism, version 9.5.1 (San Diego, CA, USA). For XY plots of morphological, mechanical, and collagen data, a simple linear regression and slope comparison was performed between age (X) and outcome, e.g. MTL (Y). The correlation coefficient (R^2) and p-value for difference between the slopes are displayed directly on the graph. Group differences were compared with an unpaired t test with Welch correction because of unequal variance between the tendons at each timepoint. The FDR was controlled using a two-stage step-up (Benjamini, Krieger, and Yekutieli). Descriptive statistics are reported with mean ± standard deviation.

Results

Experimental Design and Body Weights

Two litters of rats were used to assess morphology, mechanics, and gene expression during development. Rat patellar and Achilles tendons were collected at P7, P14, and P28 Appendix Figure 1A). Throughout this early post-natal developmental period, pups displayed a linear ~6-fold increase in body weight with an r squared >0.98. There was not a difference in body weight between sexes at any age, although the males tended to be heavier at P28 (Appendix Figure 1B,C).

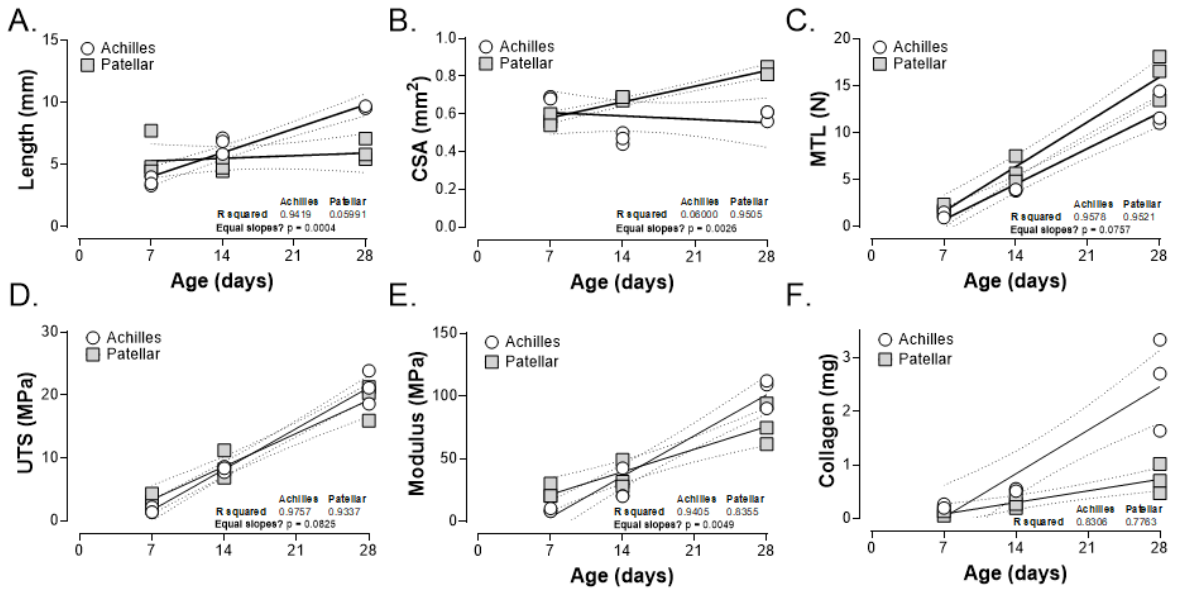

Figure 1. Achilles and patellar tendon mechanics and collagen content.

(A) The length of the Achilles tendon increased with age, while the patellar tendon remained a constant length. (B) Cross sectional area (CSA) of the patellar tendon increased with age, while Achilles CSA did not change. (C-E) There was a linear increase (R^2 > 0.8) in both the Achilles and patellar tendon mechanical and material properties with age (A-C). (F) Collagen content of the Achilles and patellar tendon increased with age. n = 3 biological replicates per timepoint for each tendon. Dotted lines correspond to 95% confidence bands.

Tendon Mechanics and Collagen Content

Morphological properties of the Achilles and patellar tendons as a function of age

Prior to tensile tests, measurements of tendon length and CSA were performed. From P7 to P28, the Achilles tendon primarily increased in length (from 3.59±0.369mm to 9.59±0.096mm; Figure 1A, Appendix Table 2). The patellar tendon, defined as the tissue attaching the patellar bone to the tibia, increased in CSA (0.57±0.03mm^2 to 0.82±0.02mm^2; Figure 1B, Appendix Table 2) without changing significantly in length.

Mechanical and material properties of the Achilles and patellar tendons increase linearly with age

Tendons were subjected to tensile tests to failure following preconditioning at a constant displacement of 0.25 mm/s at each age. There was a strong linear correlation ($R^2 > .95$) between MTL and age for both the Achilles and patellar tendons (Figure 1C). MTL of the Achilles tendon increased from 1.13 N (P7) to 12.33 N (P28). Patellar tendon MTL increased from 1.85 N (P7) to 16.04 N (P28) (Figure 1C and Appendix Table 1). The MTL of the patellar tendon tended to increase at a greater rate than the Achilles tendon (simple linear regression, tendon MTL vs age slopes; p=0.0757) (Figure 1C). The patellar tendon had a 30% greater MTL than the Achilles at P28; however, this difference did not reach statistical significance (q=0.11747) (Appendix Table 1).

The material properties (mechanical properties relative to the size of the tendon) of the Achilles and patellar tendon also increased linearly with age. UTS (MTL relative to CSA) of the Achilles and patellar tendon trended to increase at distinct rates (simple linear regression, tendon UTS vs age; slopes p= 0.0825) because the Achilles tendon began with a slightly lower UTS at P7 (AT: 1.63 MPa, PT: 3.28 MPa) but showed slightly higher UTS at P28 (AT: 21.20 MPa, PT: 19.17 MPa) (Figure 1D, Appendix Table 1). The similar UTS between AT and PT at P28 is completely consistent with the patellar tendon having a 1.3-fold greater MTL and 1.4-fold greater CSA at P28 than the AT (Figure 1C, B). The Young's modulus (tissue stiffness measured as the slope of

the stress:strain curve) of the AT increased more (simple linear regression; tendon modulus vs age slopes; p= 0.0049) than the PT (Figure 1E). Since strain is equal to the length of the tissue during the test minus the starting length, the strain on a longer tendon is inherently lower at any given point of the test relative to a shorter tendon. This would decrease the denominator of the slope of the stress:strain curve and increase modulus. Therefore, the greater increase in modulus of the Achilles is likely due to the increase in length (R^2 = 0.94) of the tissue (Figure 1A, Appendix Table 1).

Achilles and patellar tendon collagen content increase with age in parallel with MTL

Throughout the early post-natal period, total collagen content increased with age in both the Achilles and patellar tendon (tissue that attaches patella to tibia) (Figure 1F). There was a strong association (R^2 = 0.96) between age and collagen concentration (% dry mass) of the Achilles tendon, which increased from 14% to nearly 90% (Appendix Figure 1). Collagen concentration was not reported for the patellar tendon because it was not possible to get an accurate dry mass of the patellar tendons at P7 and P14.

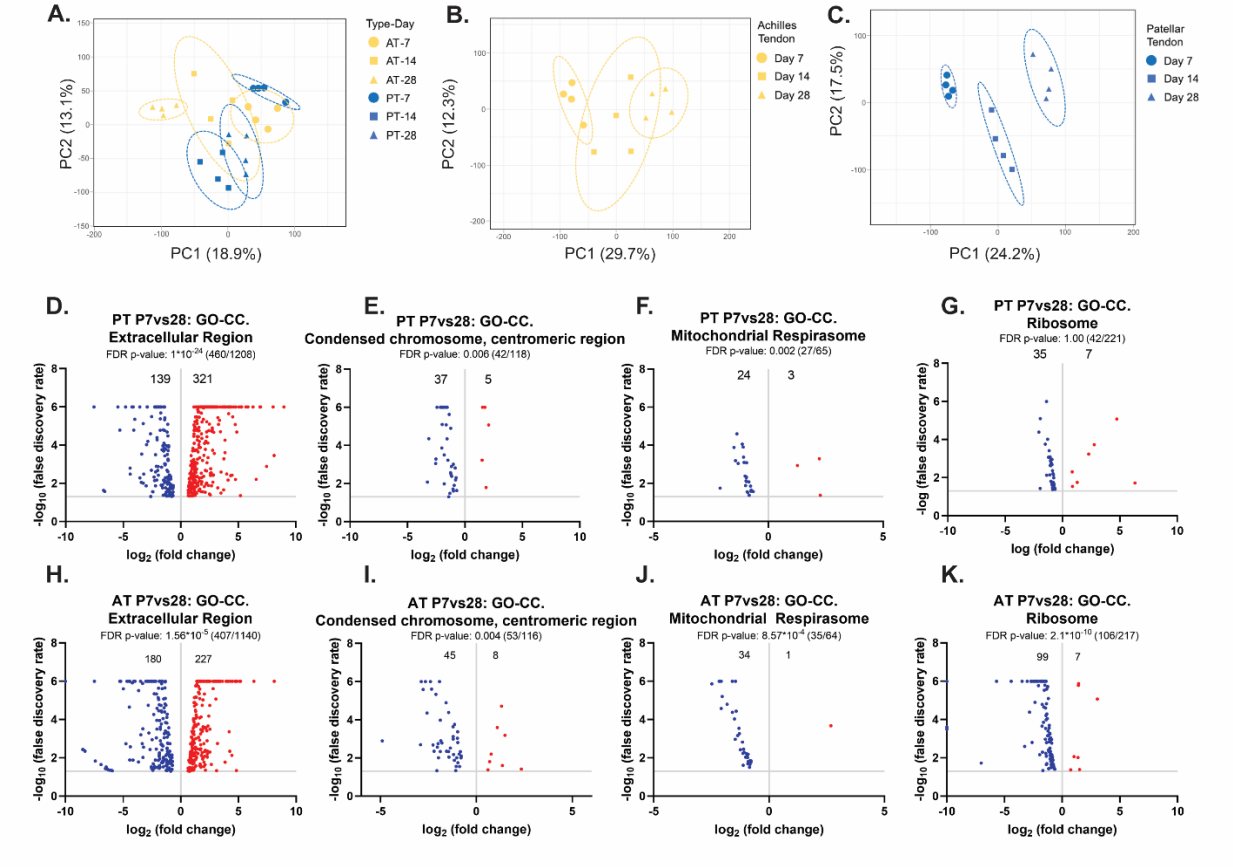

Figure 2. PCA and gene ontology analysis of Achilles and patellar tendon at P7, P14, and P28.

(A-C) Principal component analysis of Achilles (yellow) and patellar (blue) tendons at P7 (circle), P14 (square), and P28 (diamond). (A) The Achilles and patellar tendon cluster together at P7 & P14 and distinctly at P28. (B) Achilles tendon P7 and P28 form distinct clusters, yet P14 Achilles overlaps with P7 and P28. (C) Patellar tendons are transcriptionally distinct at P7, P14, and P28. (D-K) Selected gene ontology - cell compartments with FDR p-value and number of DEG relative to gene set size in Over-Representation Analysis. Each dot in the volcano plots represents a gene, with blue dots representing down regulated transcripts and red dots representing upregulated transcripts. (D,H) Transcripts encoding for the extracellular region were upregulated in the Achilles and patellar tendon from P7 to P28. (E,I) Condensed chromosome, centromeric region and (F,J) mitochondrial respirasome transcripts were downregulated from P7 to P28 in

both tendons. Ribosomal genes (G) did not decrease expression in the patellar tendon and (J) significantly decreased in the Achilles tendon. (A-K) n = 4 biological replicates per timepoint.

Transcriptional groupings and regulation of selected cell compartments

The Achilles and patellar tendon are transcriptionally similar at P7 and P14, but diverge at P28

To understand how patterns of gene expression compare between the Achilles and patellar tendons throughout early post-natal development, we performed 3' Tag RNA-seq (Lohman *et al.*, 2016). There were between four to six million reads per sample with the percent mapped ranging from 70-90% (Appendix Table 2). Most of the variability in the percent mapping can be attributed to differences in RNA Quality Score (RQS). The P28 Achilles tendons have a considerably lower RQS score around 5, while the other groups range from 7-9 RQS. We chose to use the 3' Tag Seq method because it is more resistant to transcript degradation and is more accurate than traditional RNA seq with low abundance transcripts (Lohman *et al.*, 2016). The sequencing quality metric, mean quality score, suggests that the quality of the sequencing data was excellent (Appendix Table 2). The number of detected genes for each sample is included in Appendix Table 2 and there was no difference in the detected genes at P28 between the Achilles and patellar tendon.

Principal component analysis (PCA) was used for dimension reduction and to classify tendons into transcriptionally similar groups. PCA could not distinguish between the Achilles and patellar tendons at P7 or P14. However, at P28, the AT and PT formed separate clusters, suggesting that the tendons transiently lose transcriptional similarity when growth begins to slow, and movement patterns reach maturity (Figure 2A). In the Achilles tendon, the P14 samples have the most variance and this cluster overlaps with two distinct P7 and P28 clusters (Figure 2B). The patellar tendon has distinct clusters at P7, P14, and P28 with each timepoint having little PC1 variance between samples within a group (Figure 2C).

Volcano plots of Gene Ontology (GO) – Cell Compartments

We next chose to consider how each individual tendon developed over time during the early post-natal period. To do this, we considered the GO – cell compartments that were regulated from P7 to P28 in each tendon. The most regulated (lowest FDR p value) GO – cell compartment term for the patellar and Achilles and patellar tendon, respectively, were extracellular region (FDR p-value: $5.172*10^{-24}$) and ribosomal subunit (FDR p-value: $2.814*10^{-9}$), respectively (Figure 2D, K). In the P7 vs P28 patellar tendon comparison, the myofibril compartment was downregulated regulated (FDR p-value: $5.172*10^{-6}$) while lipid biosynthetic processes were upregulated (FDR p-value: $2.514*10^{-6}$) (data not shown). This suggests that P7 patellar tendon could have muscle tissue contamination, while the P28 patellar tendon sample may include part of the fat pad.

Interestingly, no GO terms related to ribosome were regulated in the patellar tendon while the top four cell compartments in the Achilles were all ribosomal related (ribosomal subunit, cytosolic ribosome, ribosome, and cytosolic large ribosomal subunit) (data not shown). We chose to present the volcano plots for the GO term ribosome because it encompasses the largest gene set out of any ribosomal related GO terms. In the Achilles tendon, 106/217 ribosome genes were differentially expressed with all but 7 of the regulated transcripts being downregulated (Figure 2K). Even though ribosomes were not significantly regulated in the patellar tendon (FDR = 1.00), there were 42 regulated genes and the majority of them were also downregulated (35 downregulated, 7 upregulated) (Figure 2G).

More extracellular region genes were upregulated than downregulated throughout development in the Achilles (407 DEG/ 1140 terms. FDR p-value: $1.56*10^{-5}$) and patellar tendon (460 /1208. FDR p-value: $1*10^{-24}$) (Figure 2D, H). In contrast, most genes involved in chromosome condensation and the mitochondrial respirasome decreased from P7 to P28 in both the patellar and Achilles tendon (Figure 2 E,F,I,J). This suggests that early in post-natal development (P7), cellular resources are allocated towards cell division, energy production, and protein synthesis.

Later in development, cell division decreases, and cellular resources shift towards producing a robust extracellular matrix capable of transmitting the forces required for movement.

Next, we considered major signaling pathways involved in development and growth that were regulated in the patellar and Achilles tendon from P7 to P28 (Appendix Table 3). PPAR signaling pathway was significantly upregulated in the patellar tendon (FDR p = 0.004), but not the Achilles (FDR p = 0.990), presumably due to the development of the infrapatellar (Hoffa's) fat pad (Appendix Table 3, Appendix Figure 2). PI3K-AKT signaling was also significantly upregulated in the patellar tendon from P7 to P28 (Appendix Table 3, Appendix Figure 6). Despite TGF-β signaling being a necessary for tendon formation and maintenance of tendon cell fate (Pryce *et al.*, 2009; Havis *et al.*, 2016; Tan *et al.*, 2020), we did not identify TGF-β signaling as significantly regulated in the patellar (Appendix Figure 4) or Achilles tendon (Appendix Figure 5) from P7 to P28 (Appendix Table 3). TGF-β signaling tended to be different between the tendons at P28 (Appendix Table 3).

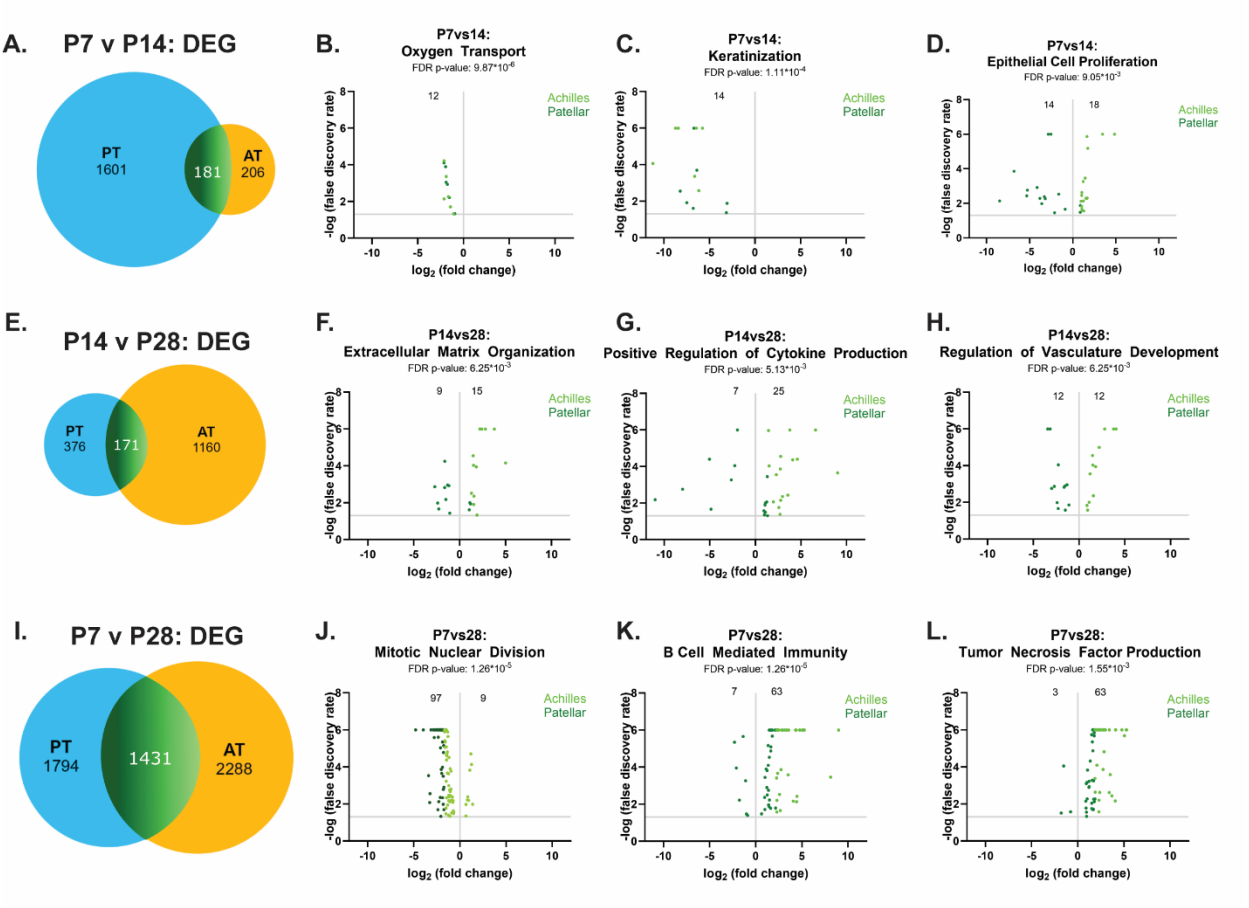

Figure 3. Over-enrichment analysis of shared DEG throughout early post-natal development.

(A, E, I) Number of differentially expressed genes (log_2 fold change |>0.6| & FDR p-value <0.05) that are unique or shared between the patellar (blue) and Achilles (yellow) tendon. To determine processes common to tendon development at each time range, DEG that were shared (green gradient) were used for an ORA. (B-D, F-H, J-L) Selected significant GO terms identified in ORA displayed in volcano plots. Each dot represents a gene with Achilles DEG in light green and patellar DEG in dark green. (A-L) n = 4 biological replicates per tendon, per timepoint.

Coordinated regulation of gene expression in the patellar and Achilles tendon from P7 to P14, P14 to P28, and P7 to P28

To understand gene regulation in tendon development, we identified differentially expressed genes (log FC ≥ |0.6|, FDR p-value <0.05) that were common to the patellar and Achilles tendon, or uniquely differentially expressed in either tendon from P7 to P14, P14 to P28, and P7 to P28. The greatest transcriptional shift in the patellar tendon occurred between P7 and P14, whereas there was a larger shift in gene expression in the Achilles from P14 and P28 (Figure 3A, E). Approximately 10% of differentially expressed transcripts were shared in both tendons from P7 to P14 (181/1998) and P14 to P28 (171/1,707). However, when comparing gene expression from an immature tendon (P7) to a more mature (P28) tendon, 25% of DEG are shared between the Achilles and patellar tendon suggesting that there is a core set of genes that are regulated comparably during postnatal tendon development (Figure 3L).

To investigate the shared processes common to tendon development, we conducted an over-representation analysis with the shared differentially expressed genes at each timepoint (Figure 3). From P7 to P14, there were three major regulatory themes: oxygen transport, keratin-based processes, and epithelium development (Figure 3B-D, Appendix Figure 6). Oxygen transport, which included primarily hemoglobin genes, was downregulated in both tendons (Figure 3B). It has been suggested that tendon is surrounded by a keratin containing epithelial layer that decreases thickness in the post-natal period (Taylor *et al.*, 2011). Consistent with this notion, we observed a large decrease in keratin-related gene expression and significant regulation of epithelial cell proliferation (Figure 3 C,D).

Following the onset of mechanical loading from P14 to P28, the major regulatory themes were extracellular matrix structure, cytokine production, and vasculature development (Figure 3F-H, Appendix Figure 7). During this time period, genes related to cell division decreased in expression (data not shown) while genes involved in cytokine production increased expression in the Achilles and patellar tendon (Figure 3G).

Processes regulated from P7 to P28 in the patellar and Achilles tendon were largely similar to those regulated from P14 to P28 (Appendix Figure 8). Cell division was transcriptionally downregulated (Figure 3J), while immune cell gene expression increased (3K,L).

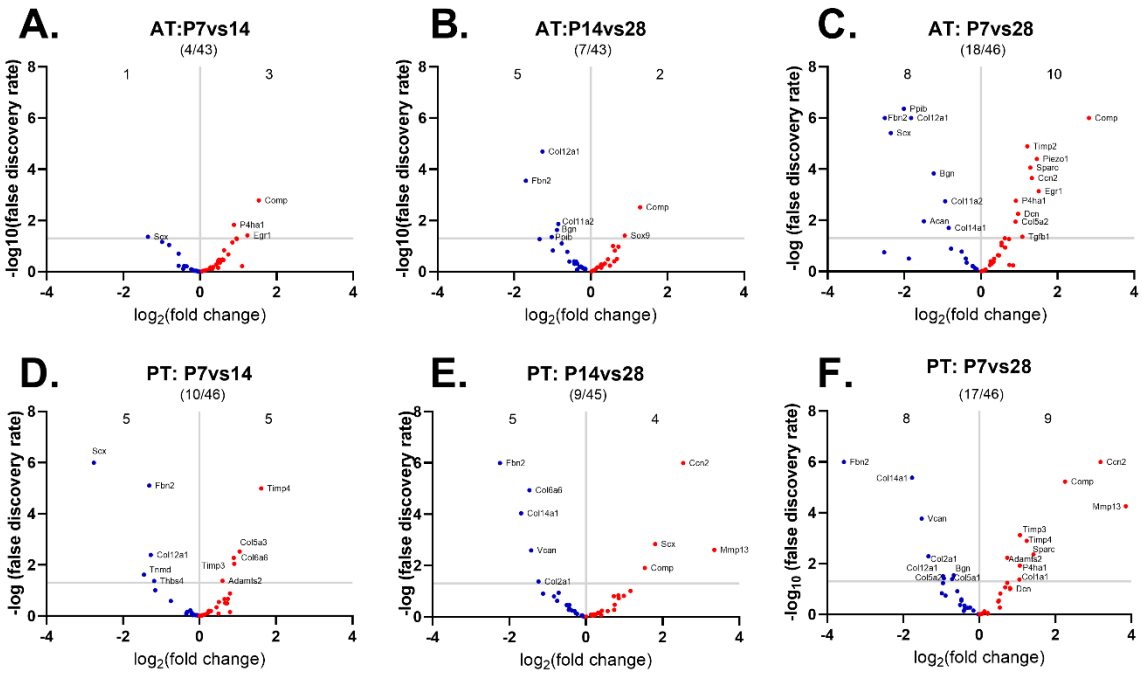

Figure 4. Expression of tendon associated genes throughout post-natal development.

(A-F) Each dot in the volcano plots represents a gene, with blue dots representing down regulated transcripts and red dots representing upregulated transcripts. The P7 to P28 comparison in the (C) Achilles and (F) patellar tendon had more DEG identified than the (A,D) P7 to P14 and (B, E) P14 to P28 comparisons. (A-F) n= 4 biological replicates per tendon, per timepoint.

Volcano plots of selected tendon genes

To better understand gene expression related to tendon biology, we curated a list of 53 known tendon associated genes (Appendix Table 4) based on the review by Huang, Lu, and Schweitzer (Huang *et al.*, 2015*a*). In both the patellar and Achilles tendons, expression of nearly 40% of

these genes were significantly regulated from P7 vs 28 (Figure 4C, F). There were similar numbers of up and downregulated genes in each tendon. Expression of Col1a1 was upregulated (patellar) or non-significantly upregulated (Achilles; FDR p-value = 0.054) from P7 to P28. Col3a1 expression was not regulated. Expression of collagen V (Col5a1/2/3) was decreased in the patellar and increased in the Achilles tendon. Collagens XII (Col12a1) and XIV (Col14a1) are minor tendon collagens also known as fibril-associated collagens with interrupted triple helices (FACIT collagens) that are essential for tendon development and their expression decreased between P7 and P28 (Figure 4C,F)(Ansorge *et al.*, 2009; Izu *et al.*, 2021).

Transcription factors relevant to tendon development include scleraxis (Scx), mohawk (Mkx), and early growth response factor 1 (Egr1). Expression of scleraxis, a tendon/ligament specific TF (Schweitzer *et al.*, 2001), decreased in the Achilles from P7 to P28 (Figure 4C). In the patellar tendon, scleraxis expression decreased from P7 to P14 and then increased from P14 to P28 (Figure 4D, E). Egr1, a load-dependent transcription factor for type I collagen (Lejard *et al.*, 2011), was significantly (Achilles) or non-significantly increased (patellar) from P7 to P28 (Figure 4 C,F)

Expression of specific glycoproteins and proteoglycans were also regulated throughout post-natal development. Cartilage oligomeric matrix protein (Comp), a glycoprotein that binds fibrillar collagens to assist with fibrillogenesis (Sodersten *et al.*, 2005; Halász *et al.*, 2007) and whose levels correlate with stiffness and UTS (Smith *et al.*, 2002), was one of the genes that increased expression most in both tendons. Decorin (Dcn) and biglycan (Bgn) are two small leucine-rich proteoglycans (SLRPs) that control fibril morphology (Zhang *et al.*, 2006; Robinson *et al.*, 2017). Here, we find a similar pattern of expression in both tendons: biglycan expression decreases concomitant with increased decorin expression (significant in AT; trend in PT) from P7 to P28. Fibrillin 2 (Fbn2), a glycoprotein that forms microfibrils (Calve *et al.*, 2018) and encapsulates

tendon cells (Ritty *et al.*, 2003), expression decreased in both tendons as cell number declined (Figure 4C,F)

Expression of Sparc and Ccn2, matricellular proteins located within the extracellular matrix that have primarily signaling rather than structural roles, increased between P7 and P28 (Figure 4C, F). Matrix turnover genes, metalloproteinases and tissue inhibitors of metalloproteinases, were not altered (Mmp) in either tendon or increased (Timp) in both tendons (Figure 4C, F).

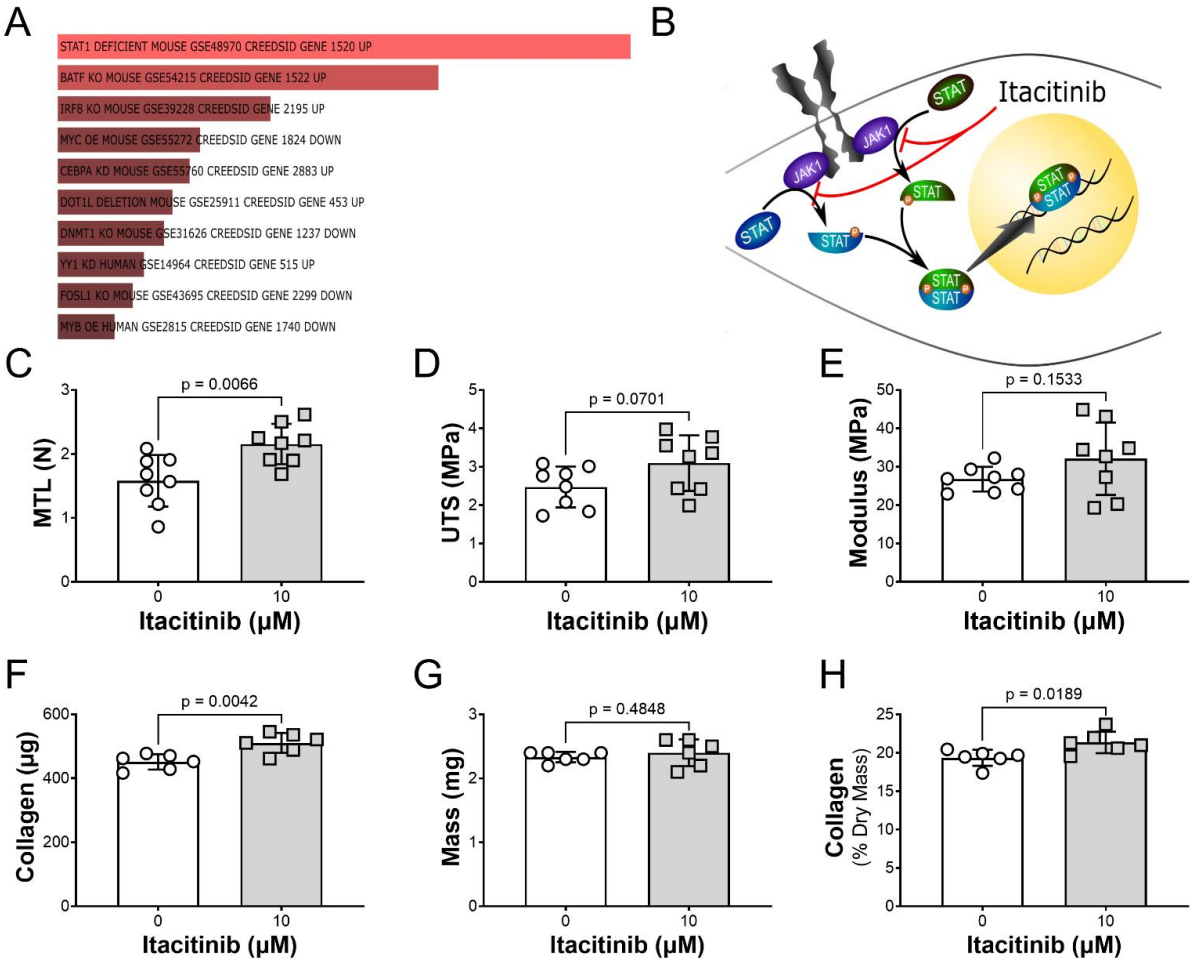

Figure 5. JAK1 inhibition improves engineered ligament collagen content and mechanics.

(A) Enrichr transcription factor perturbation results for the top 200 genes upregulated in the PT at P28. Sorted by p-value. (B) Diagram of Itacitinib mechanism of action as a selective JAK1

inhibitor to repress JAK/STAT signaling. (C-H) Engineered ligaments treated with 10 μM Itacitinib for six days after construct formation. JAK1 inhibition increased the (C) MTL, (F) collagen content, and (H) percent collagen of engineered ligament constructs. (C-H) n = 8 for mechanics, n = 6 for mass and collagen data.

Engineered ligament collagen content and mechanics with STAT inhibition

To understand how the gene regulatory networks identified in this screen may play a role in tendon mechanics, we selected the 200 genes that had the greatest $\log_2$ fold change in the PT relative to the AT at P28, reasoning that these genes may have contributed to the better mechanical properties in the PT. Enrichr transcription factor perturbation followed by expression analysis (Xie *et al.*, 2021) revealed that the transcriptional landscape following *Stat1* deficiency (Semper *et al.*, 2014) was most similar to the top 200 genes with expression higher in the patellar tendon than Achilles tendon at P28, suggesting that decreasing STAT activity may improve mechanics (Figure 5A, Appendix Table 5). To test this hypothesis, we identified a pharmaceutical repressor of JAK1/STAT signaling, itacitinib; a selective JAK1 inhibitor (Kavanagh *et al.*, 2022) and determined its effect on an engineered ligament model developed by our laboratory (Paxton *et al.*, 2010) (Figure 5B). Following treatment for 6 days with 10 μM itacitinib, the mechanical (MTL) properties and collagen content of human engineered ligaments increased significantly. The material properties (UTS and modulus) were not significantly improved, suggesting the improved mechanics were the result of the increased collagen content in the engineered tissues (Figure 5C,F), consistent with the increased MTL due to greater tissue CSA seen in the PT at P28.

Discussion

This study identified the morphological, mechanical, and transcriptional changes that occur during the early-post natal period in rat Achilles and patellar tendons. These tendons were selected because they are two commonly injured tendons (Taunton *et al.*, 2002; Janssen *et al.*, 2018) and understanding the processes that contribute to their development may help guide rehabilitation strategies. We found that the mechanical and material properties of the Achilles and patellar tendons are strongly associated with collagen content and increase linearly with age. At the transcriptomic level, both tendons show a decrease in cell cycle, mitochondrial, and ribosomal genes, and an increase in extracellular matrix expression between P7 and P28. These changes in gene expression either reflect or underlie the increase in collagen content and mechanical strength in early post-natal development. Lastly, we identified JAK/STAT signaling as a potential negative regulator of patellar tendon development and then used an *in vitro* engineered ligament model to demonstrate that JAK1 inhibition can increase collagen content and mechanics of an engineered ligament.

In both the Achilles and patellar tendons, increases in collagen content mirrored the increases in tendon strength (MTL). In the patellar tendon, collagen content and MTL both increased 9-fold from P7 to P28. The collagen content of the Achilles tendon increased 11.6-fold from P7 to P28 while MTL increased approximately 11-fold. Prior work by Theodossiou and colleagues also observed a 10-fold increase in rat Achilles MTL within the first 10 days after birth. Further, extrapolating our Achilles MTL data predicts an MTL of 2.3 N at P10 which is completely consistent with the 2.65 N measured in their work (Theodossiou *et al.*, 2019). We also observe a near identical increase in UTS of Achilles and patellar tendon, with no difference in the rate of increase, from P7 to P28 which suggests that these tendons increase strength in proportion to calculated CSA throughout development. As a limitation, we did not directly measure CSA, only length and width, and assumed as thickness of 0.5 mm for all tendons.

One of the interesting differences between these tendons is that the Achilles increased in length, while the patellar tendon increased in CSA between P7 and P28. Although not significant, the rate of increase in MTL is greater in the patellar tendon and the PT tends to have a higher MTL at P28 than the Achilles. This is consistent with the suggestion that both tendons increase in material strength (UTS) throughout development, yet the patellar tendon develops a slightly higher absolute strength (MTL) because it has a larger CSA. By contrast, the Achilles tendon gets ~3-fold longer between P7 and P28 and, since Young's modulus is linearly related to the initial length of the tissue, this would result in an ~3-fold greater modulus even without an improvement in material properties between P7 and P28. This likely explains why the Achilles tendon shows a significantly greater improvement in modulus when compared with the patellar tendon.

While our sequencing data were of high quality, there is the possibility of bias introduced in the analysis from lower RNA quality scores in the P28 Achilles tendon group. It is possible that the P28 patellar tendon group and not the others had more transcript degradation. RNA degradation occurs fastest with pseudo and non-coding genes, which means that during the transcript normalization process, expression of more stable transcripts such as ECM genes become inflated relative to less stable transcripts (Reiman et al., 2017).

The AT and PT cluster together early in development (P7 and P14), becoming transcriptionally divergent by P28 on a PCA plot. This separation of clusters could be driven by the differences in RIN scores or real biological differences within the tissues. Since only the AT is affected by poorer RNA quality scores, and ECM gene expression goes up with age in the AT and PT we suggest that the transcriptional differences at P28 are biologically relevant and not driven by the differences in RNA quality. This separation of clusters was in contrast to our hypothesis that the two tissue would be transcriptionally equivalent. Our initial hypothesis was based on the fact that in the adult rat only 4% of total transcripts are differentially regulated (1.65-

fold) (Disser *et al.*, 2020). The discrepancy between our P28 data (25% DEG) and that of Disser in the adult (4% DEG) suggests that there could be larger transcriptional variation between the Achilles and patellar tendon in early post-natal development, but when both tendons are mature, they become transcriptionally similar. The most likely biological explanation is that the difference we observed at P28 is due to the need for different genes to increase the length of the Achilles between P7 and P28. Once bone growth stops, the two tendons once again become transcriptionally similar.

In both the patellar and Achilles tendon, there were four main cell compartments transcriptionally regulated throughout early post-natal development: ECM, mitochondria, ribosomes, and cell cycle. Transcripts annotated to the extracellular region were almost twice as abundant at P28 than P7 in both the Achilles and patellar tendon. In contrast, transcripts annotated to cell division, condensed chromosome/centromeric region, mitochondrial respirasome, and ribosome were all most abundant at P7. The most likely explanation for these data is that there is a decrease in proliferation, which requires the centrosome, large metabolic expenditure, and high levels of protein synthesis. As far as the protein synthesis findings, Moore and De Beaux found that the rough ER fraction increased slightly from birth to two weeks before decreasing at six and twelve weeks in both the rat extensor digitorum longus (EDL) and tail tendons (Moore & De Beaux, 1987*a*). Therefore, the decrease in protein synthesis appears to be a developmental change that occurs independent of mechanical load since the relatively unloaded tail tendon demonstrated an effect similar to the loaded EDL tendon. The decrease in tendon cellularity and proliferation that occurs during post-natal development is well established (Grinstein *et al.*, 2019) and a microarray analysis of cells isolated and cultured from post-natal rat Achilles tendon also identified cell cycle genes as the top regulated KEGG pathway (Liu *et al.*, 2012; Chen *et al.*, 2016). Lastly, the decrease in mitochondria is interesting because mitochondrial genes are also downregulated in tendinopathy (Steffen *et al.*, 2022) yet

upregulated in tendon regeneration (Qin *et al.*, 2021), suggesting that the P7 timepoint may be the ideal developmental comparison for tendon regeneration.

In an effort to find pathways that could help augment tendon development/repair, we screened the top 200 genes that were higher (using $\log_2$ fold change) in the patellar tendon reasoning that this reflected the greater radial growth and MTL. Interestingly, using Enrichr we found that our gene set was similar to STAT1 knockout mice (Semper *et al.*, 2014). In myoblasts, JAK1-STAT1/STAT3 signaling contributes to myoblasts proliferation and represses differentiation (Sun *et al.*, 2007). Our transcriptional data suggests that proliferation subsides during early post-natal development, which is consistent with the Enrichr analysis which reported transcriptional similarity with knockout of STAT1. Despite reports of STAT inhibition decreasing collagen synthesis (Chakraborty *et al.*, 2017; Papaioannou *et al.*, 2018), pharmaceutically inhibiting JAK1 in our engineered ligaments increased collagen content and the mechanical strength of the tissues. It is possible that inhibiting JAK1 increased the differentiation of the resident cells towards a mature phenotype, i.e. more resources allocated to extracellular matrix gene expression, thus increasing the collagen content and mechanics of the engineered ligaments.

In conclusion, we demonstrate that during the development of the musculoskeletal system the patellar and Achilles tendon increase mechanical and material properties, and collagen content in a linear manner. Not surprisingly, this increase occurs concomitant with a transcriptional shift to produce more ECM. Our data identify a transcriptional signature of a developing tendon: higher transcript levels for cell proliferation, mitochondrial respirasome, and greater protein synthesis. These findings are relevant to both understand the basic processes that occur during tendon development and may provide hints to guide tendon regeneration. Lastly, using the transcriptomic data we have identified a novel mechanism to improve tendon/ligament mechanics (inhibiting JAK1/STAT signaling) that may be useful in improving tendon repair following injury. Further studies should investigate how these transcriptional changes relate to

protein abundance/activity and determine whether more of the transcriptional regulators identified can promote tendon development and regeneration.

Data Availability

RNA seq files were submitted to NCBI Sequence Read Archive. The patellar tendon files are listed under accession PRJNA926122. The Achilles tendon samples are under accession PRJNA926134.

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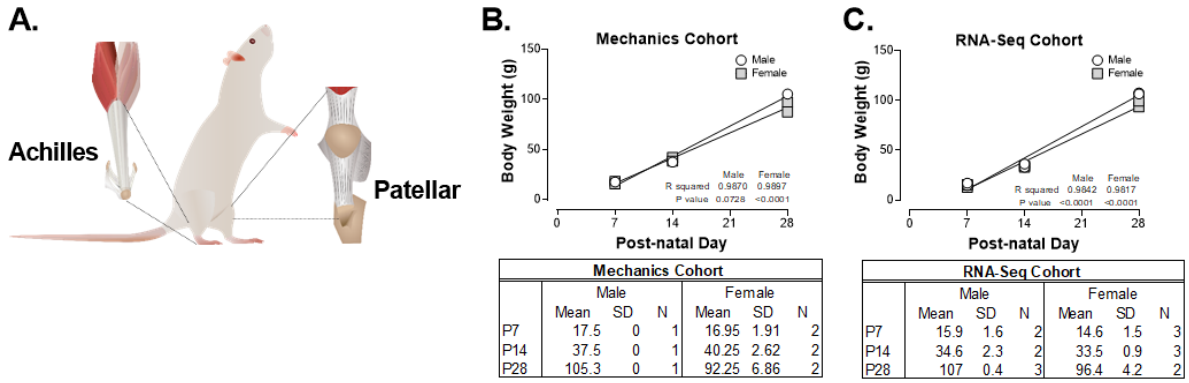
Author Contributions

DS and KB conceived the experiments, collected the tissues, and performed the mechanical tests. DS and MM performed the bioinformatic analyses. DS and KB wrote the manuscript, and all authors approved the final text.

Competing Interests

The authors declare no conflict of interest in relation to the data presented.

Supplemental Information



Mechanics Cohort

	Male			Female		
	Mean	SD	N	Mean	SD	N
P7	17.5	0	1	16.95	1.91	2
P14	37.5	0	1	40.25	2.62	2
P28	105.3	0	1	92.25	6.86	2

RNA-Seq Cohort

	Male			Female		
	Mean	SD	N	Mean	SD	N
P7	15.9	1.6	2	14.6	1.5	3
P14	34.6	2.3	2	33.5	0.9	3
P28	107	0.4	3	96.4	4.2	2

Supplemental Figure 1. Experiment Design and Body Weights. (A) The Achilles and patellar tendon were collected at P7, P14, and P28 to establish tendon mechanical properties and compare gene expression throughout the early post-natal period. (B,C) There was a linear increase in body weight ($R^2 > 0.95$) in males and females from P7 to P28 in both the mechanics and RNA-seq cohorts.

Supplemental Table 1

MTL (N)							
	Achilles			Patellar			AT vs PT
	Mean	SD	N	Mean	SD	N	p-value
P7	1.13	0.33	3	1.85	0.43	3	0.9848
P14	3.86	0.08	3	5.98	1.38	3	0.444
P28	12.33	1.84	3	16.04	2.37	3	0.0524
UTS (MPa)							
	Achilles			Patellar			AT vs PT
	Mean	SD	N	Mean	SD	N	p-value
P7	1.63	0.47	3	3.28	0.93	3	0.8825
P14	8.24	0.46	3	8.74	2.22	3	0.9994
P28	21.20	2.64	3	19.17	2.84	3	0.7688
Modulus (MPa)							
	Achilles			Patellar			AT vs PT
	Mean	SD	N	Mean	SD	N	p-value
P7	8.73	1.24	3	23.47	5.70	3	0.5973
P14	27.50	12.86	3	36.11	11.30	3	0.9247
P28	103.75	11.95	3	76.73	16.35	3	0.0926
Length (mm)							
	Achilles			Patellar			AT vs PT
	Mean	SD	N	Mean	SD	N	p-value
P7	3.59	0.37	3	5.66	1.78	3	0.12
P14	6.61	0.68	3	4.92	0.57	3	0.27
P28	9.59	0.10	3	6.11	0.86	3	0.0049
CSA (mm^2)							
	Achilles			Patellar			AT vs PT
	Mean	SD	N	Mean	SD	N	p-value
P7	0.69	0.01	3	0.57	0.03	3	0.0005
P14	0.47	0.03	3	0.68	0.01	3	<0.0001
P28	0.59	0.03	3	0.82	0.02	3	<0.0001
Collagen Content (mg)							
	Achilles			Patellar			AT vs PT
	Mean	SD	N	Mean	SD	N	p-value
P7	0.22	0.04	3	0.08	0.05	3	0.9967
P14	0.54	0.24	3	0.31	0.13	3	0.9686
P28	2.56	0.86	3	0.73	0.27	3	0.0007
Collagen Concentration (%)							
	Achilles			Patellar			AT vs PT
	Mean	SD	N	Mean	SD	N	p-value
P7	13.79	4.79	3	---	---	---	---
P14	30.54	6.40	3	---	---	---	---
P28	88.21	7.52	3	---	---	---	---
Dry Mass (mg)							
	Achilles			Patellar			AT vs PT
	Mean	SD	N	Mean	SD	N	p-value
P7	1.77	0.86	3	---	---	---	---
P14	1.83	0.49	3	---	---	---	---
P28	2.97	1.21	3	---	---	---	---

2way ANOVA. Tukey's multiple comparisons.

MTL: Age p<0.0001 (****). Tendon p=0.0053 (**). Interaction p=0.2065 (NS).

UTS: Age p<0.0001 (****). Tendon p=0.9638 (NS). Interaction p=0.2616 (NS).

Modulus: Age p<0.0001 (****). Tendon p=0.8183 (NS). Interaction p=0.0141 (*)

Length (mm): Age p<0.0002 (***). Tendon p<0.0316 (*). Interaction p<0.0006 (***).

CSA (mm^2): Age p<0.0001 (****). Tendon p<0.0001 (****). Interaction p<0.0001 (****).

Collagen (ug): Age p<0.0001 (****). Tendon p<0.0013 (**). Interaction p<0.0031 (**).

Unpaired t Test with Welch's correction.

Collagen (%): p=0.9224 (NS)

Mass: p=0.0917 (NS)

Supplemental Table 2

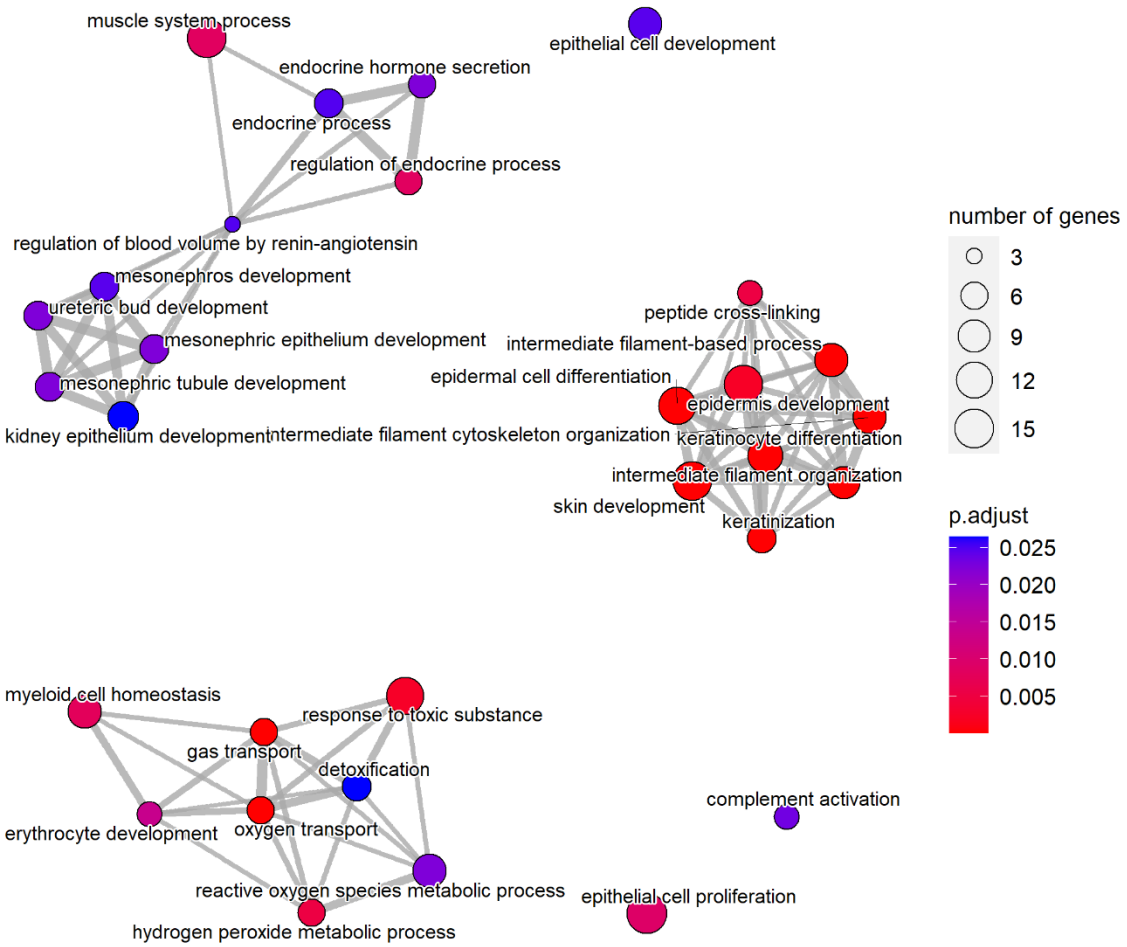
ID	Age	BW (g)	Sex	Tendon	RQS (RIN)	Total Reads	Reads Mapped	% Mapped	% ≥ Q30 bases	Mean Quality Score
1	7	14.8	M	AT	8.4	5,663,952	5,061,325	89.36	88.02	36.91
2	7	12.9	F	AT	8.6	4,698,176	4,180,628	88.98	87.66	36.85
3	7	15.4	F	AT	8.2	5,468,810	4,874,486	89.13	87.73	36.86
5	7	17	M	AT	8.3	7,441,362	6,424,804	86.34	87.51	36.82
7	14	33	M	AT	7.6	5,287,272	3,966,053	75.01	85.71	36.38
8	14	36.2	M	AT	7.9	5,465,732	4,881,144	89.3	88.35	37.01
9	14	34.5	F	AT	7.5	5,355,378	4,532,020	84.63	86.44	36.56
10	14	32.9	F	AT	7.5	5,233,663	4,456,249	85.15	86.71	36.63
11	28	93.4	F	AT	4.1	6,242,492	4,535,065	72.65	85.46	36.36
13	28	107.3	M	AT	5.3	5,931,719	4,444,534	74.93	85.95	36.48
14	28	99.4	F	AT	5.2	5,750,550	4,444,512	77.29	86.26	36.51
15	28	106.5	M	AT	5.6	5,924,374	4,410,805	74.45	86.33	36.52
1	7	14.8	M	PT	8.9	6,933,974	5,960,110	85.96	87.55	36.81
2	7	12.9	F	PT	8.8	5,934,565	5,425,035	91.41	88.56	37.07
3	7	15.4	F	PT	8.8	5,911,251	5,212,583	88.18	87.53	36.80
4	7	15.5	F	PT	8.9	6,883,645	6,136,951	89.15	88.09	36.93
6	14	33.1	F	PT	8.6	5,174,971	4,589,195	88.68	86.68	36.59
7	14	33	M	PT	8	5,666,334	5,093,618	89.89	87.65	36.83
8	14	36.2	M	PT	8.5	5,706,146	5,181,485	90.81	88.23	36.98
9	14	34.5	F	PT	8.6	5,902,536	4,879,339	82.67	87.39	36.81
11	28	93.4	F	PT	8	6,139,965	5,057,353	82.37	85.38	36.28
13	28	107.3	M	PT	7.3	5,178,907	4,734,823	91.43	87.96	36.91
14	28	99.4	F	PT	6.9	4,575,372	4,067,260	88.89	86.78	36.63
15	28	106.5	M	PT	7	4,294,986	3,811,240	88.74	88.02	36.95

Expression Profiling of Rat Patellar Tendon

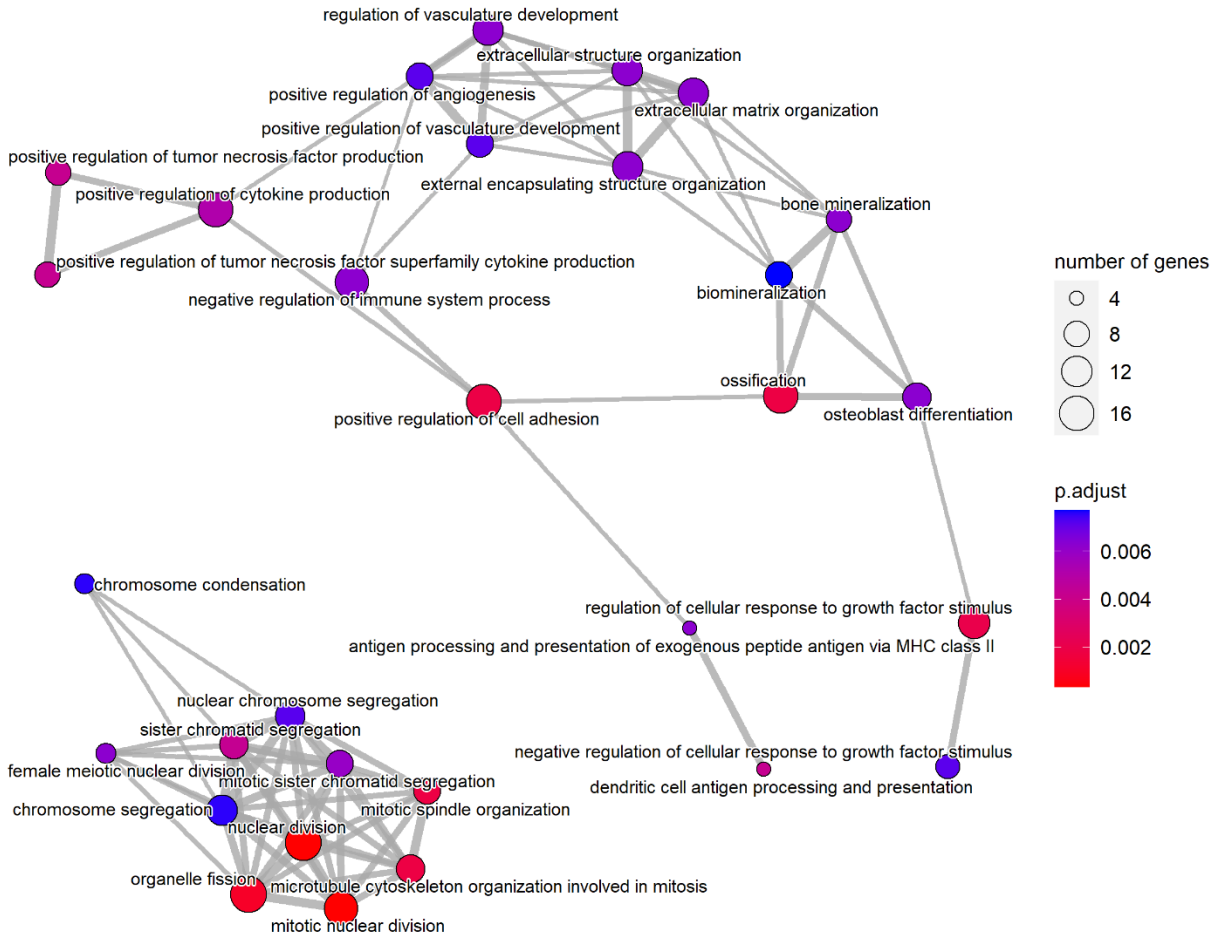
3' Tag-Seq Analysis Using CLC Genomics Workbench v21.0.3

First 12 bp trimmed from each read

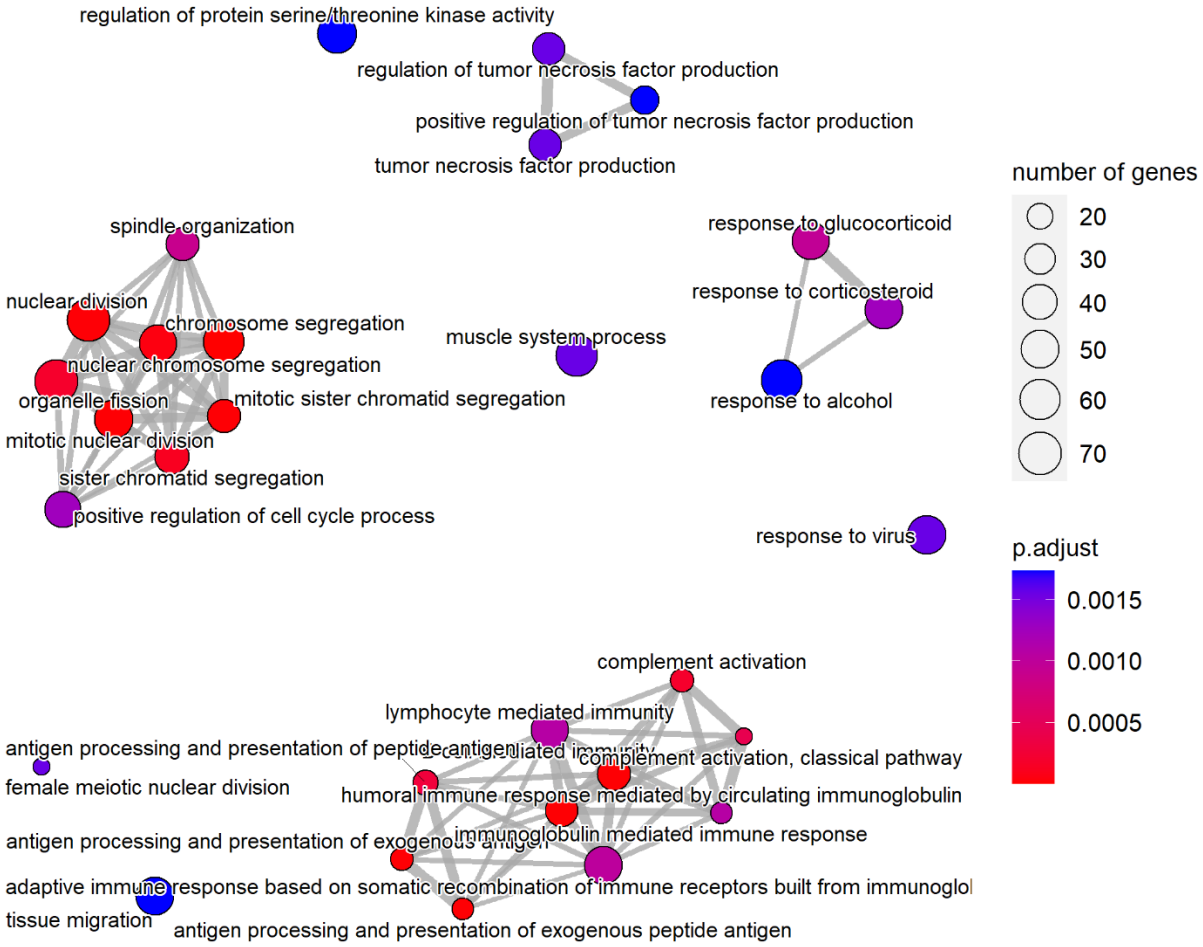
Reads with a minimum length of 20 bp of quality read kept.



Supplemental Figure 2. P7 to P14 shared processes in the patellar and Achilles tendon. Over-representation analysis using genes that are differentially expressed from P7 to P14 in the Achilles and patellar tendon. The top 30 terms, with the lowest FDR p-value, are displayed in the enrichment map.



Supplemental Figure 3. P14 to P28 shared processes in the patellar and Achilles tendon.



Supplemental Figure 4. P7 to P28 shared processes in the patellar and Achilles tendon.

Supplemental Table 3

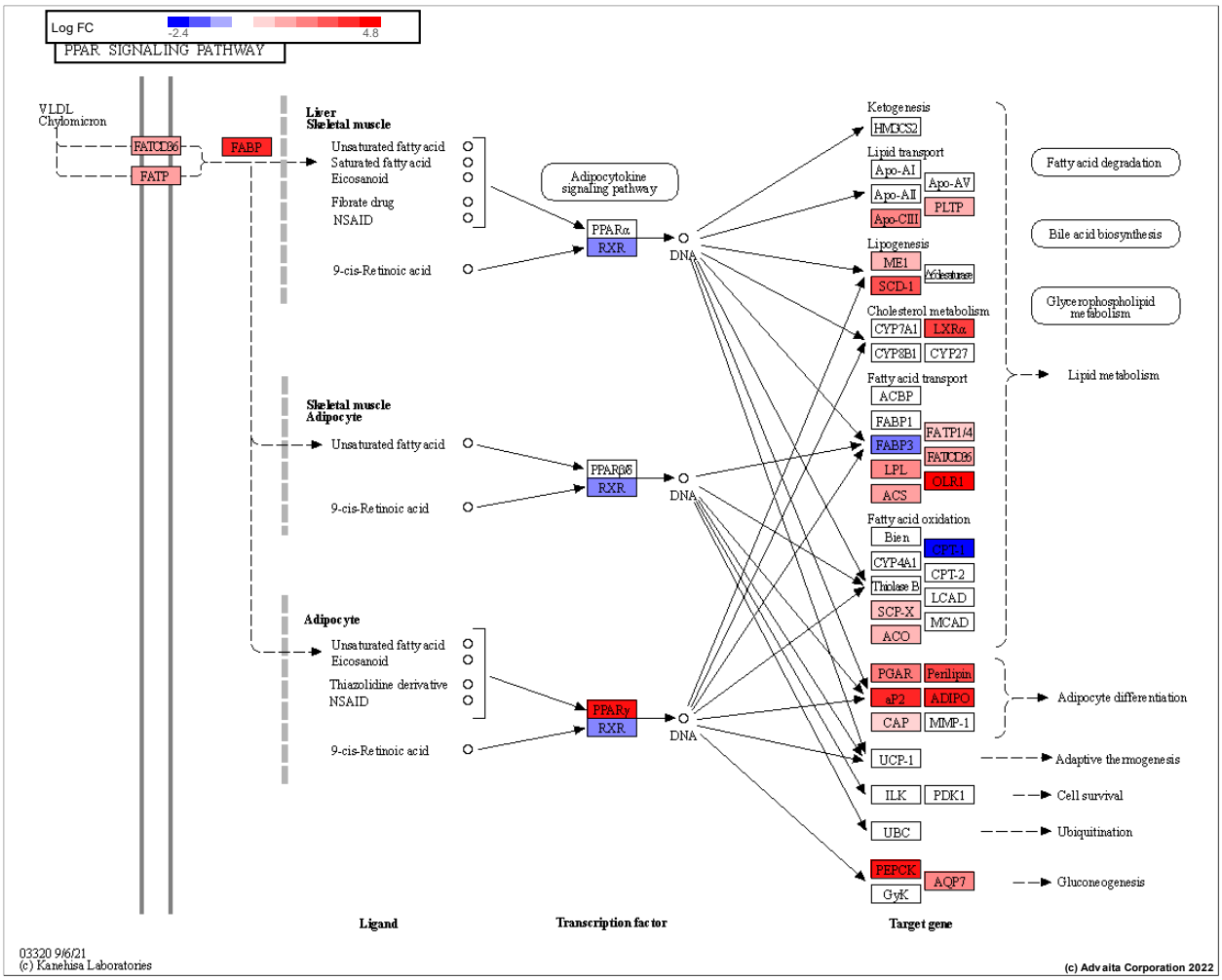
Major Signaling Pathways - Advaita

	PT: 7 v 28		AT: 7 v 28		P28: AT vs PT	
Pathway	**DEG/total**	**FDR**	**DEG/total**	**FDR**	**DEG/total**	**FDR**
Hedgehog	5/45	0.977	12/46	0.902	9/42	0.932
Hippo	26/128	0.457	34/125	0.950	34/115	0.752
JAK-STAT	28/95	0.609	31/91	0.785	27/91	0.756
MAPK	71/238	0.155	68/233	0.785	63/229	0.754
Notch	10/50	0.965	15/48	0.851	13/48	0.833
PI3K-AKT	84/266	0.037 *	90/253	0.264	75/249	0.488
PPAR	29/60	0.004 **	12/57	0.990	18/57	0.649
TGFβ	17/77	0.936	23/77	0.603	32/75	0.053 ^
Wnt	33/133	0.619	41/125	0.820	32/122	0.752

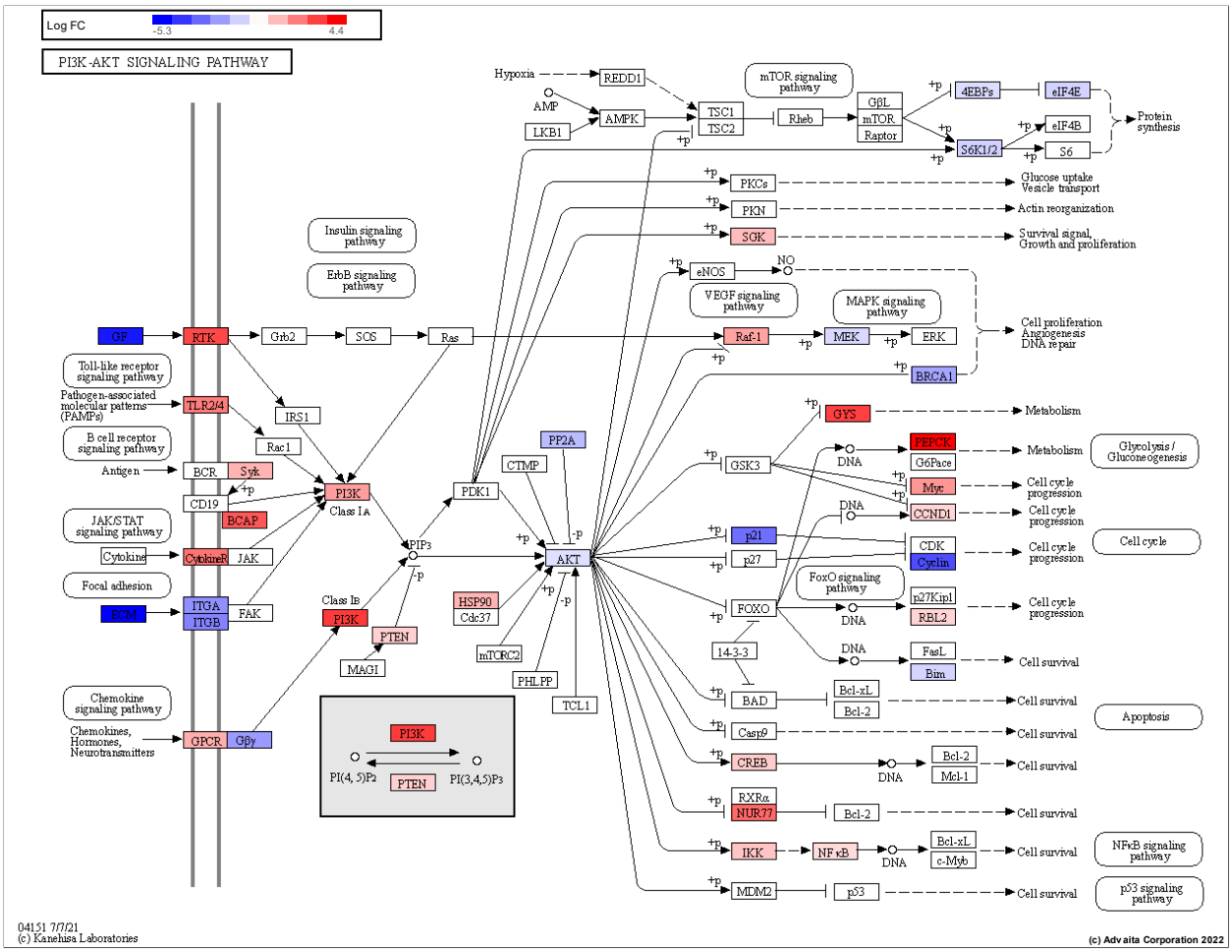
** = P< 0.01

* = P<0.05

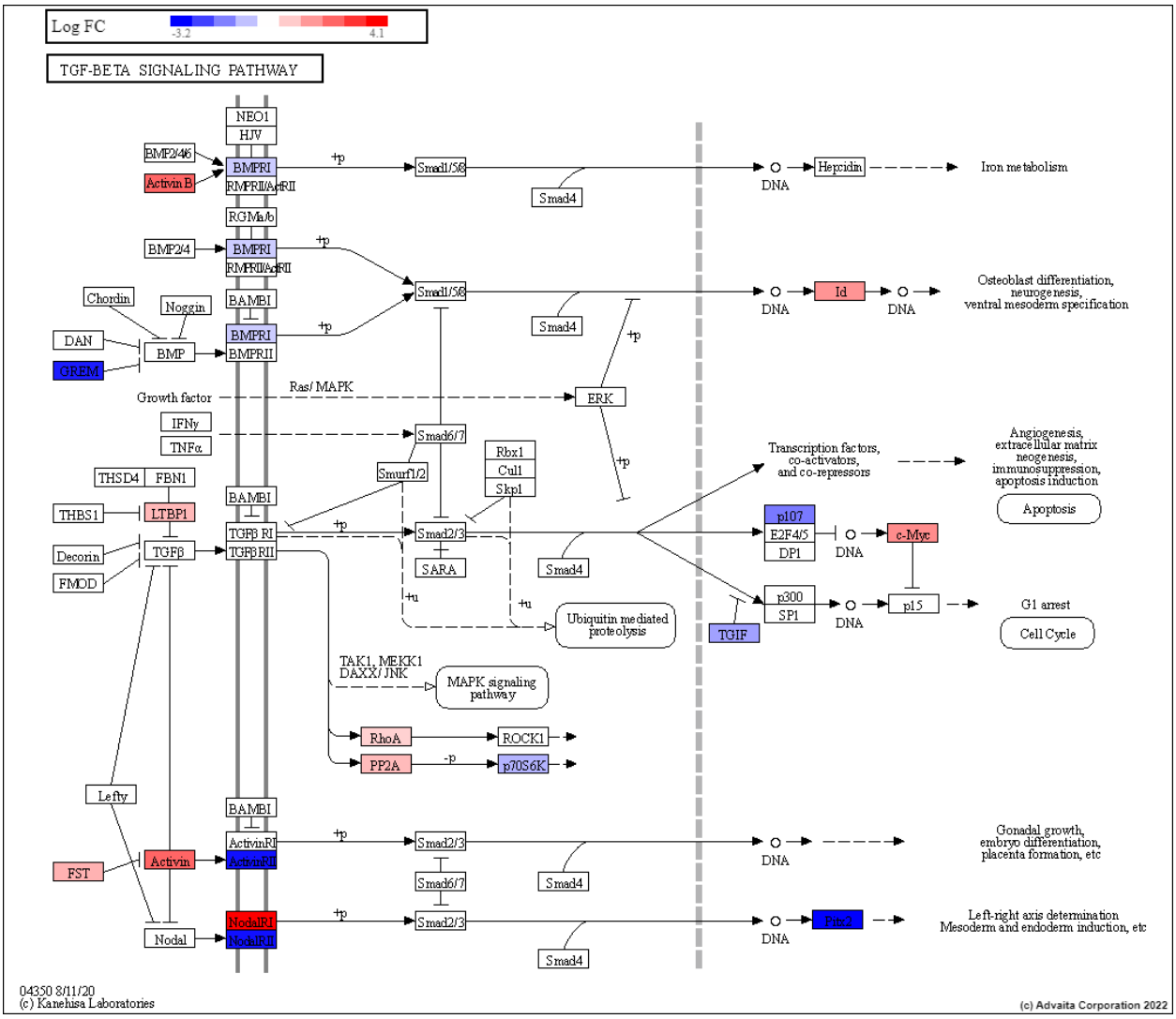
^ = P <0.1



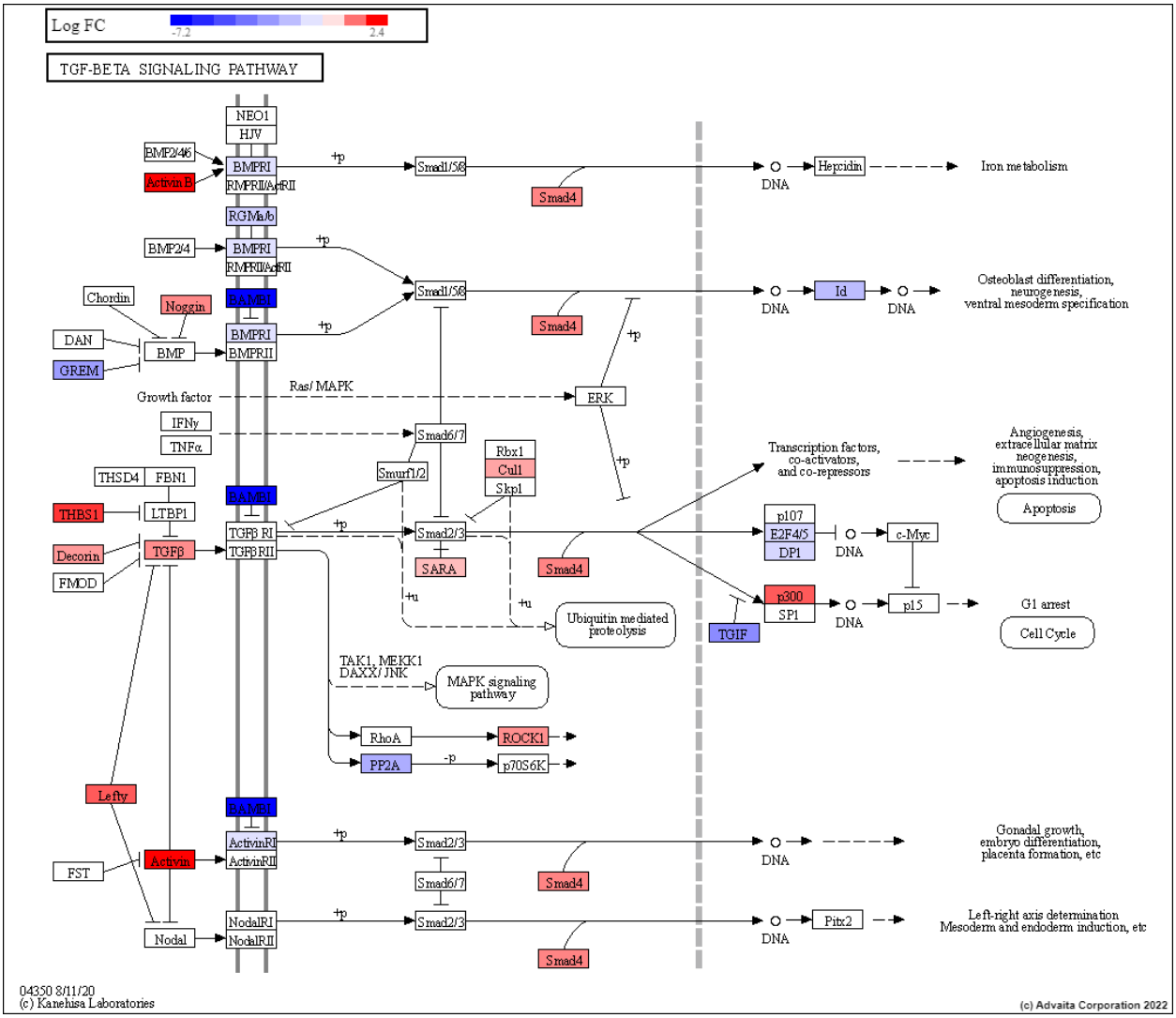
Supplemental Figure 5. PPAR Signaling in the Patellar Tendon from P7 to P28. This pathway diagram was generated using Advaita iPathwayGuide.



Supplemental Figure 6. PI3K-AKT Signaling in the Patellar Tendon from P7 to P28. This pathway diagram was generated using Advaita iPathwayGuide.



Supplemental Figure 7. TGFβ Signaling from P7 to P28 in the Patellar Tendon. This pathway diagram was generated using Advaita iPathwayGuide.



Supplemental Figure 8. TGFβ Signaling from P7 to P28 in the Achilles tendon. This pathway diagram was generated using Advaita iPathwayGuide.

Supplemental Table 4

Tendon Collagens	Collagen modification/ export	Turnover	SLRPs	Cytokines/ growth factors	Matrix Regulation	TFs	Tenocyte Surface	Mechano-sensitive	Cartilage
Col1a1	Adamts2	Mmp1	Dcn	Tgfb1	Lox	Scx	Tnmd	Sparc	Comp
Col3a1	Adamts3	Mmp8	Bgn	Tgfb2	Larp6	Mkx	Thbs4	Piezo1	Col2a1
Col5a1	Adamts14	Mmp13	Fbn1	Tgfb3		Egr1			Sox9
Col5a2	P4ha1	Timp1	Fbn2	Gdf5					Acan
Col5a3	Ppib	Timp2	Vcan	Gdf6					Col10a1
Col6a1		Timp3	Fmod	Ccn2					
Col6a2		Timp4	Lum						
Col6a4									
Col6a5									
Col6a6									
Col11a1									
Col11a2									
Col12a1									
Col14a1									

Chapter 3. Scleraxis and Collagen I Expression Increase Following Pilot Isometric Loading Experiments in a Rodent Model of Patellar Tendinopathy

This chapter was previously published as:

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Abstract

The effect of mechanical load on tendinopathic tissue is usually studied in the context of identifying mechanisms responsible for tendon degradation. However, loading is also one of the most common treatments for tendinopathy. It is therefore possible that different loads result in different cellular responses within a tendon. To test this hypothesis, we first established a rodent model of tendinopathy that has a transcriptional signature similar to human tendinopathy. Tendinopathy was modeled in the rat by producing a lesion in the central core of the patellar tendon using a biopsy punch, followed by two weeks to allow scar formation. We performed 3' Tag RNA-Seq to identify genes that were differentially expressed between the native and scarred rat patellar tendon. Genes involved in extracellular matrix (ECM) structure and turnover were increased, mitochondrial genes were decreased, and there was no inflammatory signature in the tendinopathic tissue. These transcriptional changes phenocopy previously published whole transcriptome analysis in human tendinopathy. After validating the model, the initial response to injury and loading was determined. Two weeks after creation of the patellar tendon lesion, the tendon was loaded using either 4 x 30s isometric or a time-under-tension matched (360 x 0.33s) dynamic protocol. Injured +/- loading and contralateral control tendons were collected eighteen hours after loading, RNA was extracted, and gene expression was quantified using qRT-PCR of the scar with or without loading. The expression of scleraxis and type I collagen increased following isometric loading relative to those loaded dynamically. By contrast, the expression of type II collagen increased in the dynamic samples relative to those loaded isometrically. These data suggest that dynamic loading of a central core tendon injury increases fibrocartilage markers, whereas long isometric loads stimulate markers of tendon regeneration.

Key Words: Tendinopathy, Transcriptome, Extracellular Matrix, Tendon, Injury, Collagen

Introduction

Persistent patellar tendon pain and loss of function is diagnosed as patellar tendinopathy (Scott *et al.*, 2020). The incidence of anterior knee pain, which includes patellar tendinopathy, in the general population has been reported at 23%, and in adolescents this number rises to 29% (Smith *et al.*, 2018). The prevalence of patellar tendinopathy is highest in elite volleyball and basketball players, with well over one-third of players affected (Lian *et al.*, 2005). In fact, structural abnormalities in the patellar tendon are present in over 60% of elite basketball players (Benítez-Martínez *et al.*, 2019). Increased training load correlates with disruption of the tendon structure (Maciel Rabello *et al.*, 2019), strongly supporting the idea that overuse contributes to the development of tendinopathy. Even though injury resulting from excessive chronic loading, or an acute overload are thought to initiate the clinical condition, loading though rehabilitation exercises is also thought to be required for healing an injured adult tendon. However, the molecular basis of this contradiction remains poorly understood.

One reason why the molecular basis of tendinopathy and the response to subsequent loading is poorly understood is the lack of a validated rodent model of tendinopathy. There are a small number of rodent physiological patellar tendon injury models (Glazebrook *et al.*, 2008) and animal models for other tendinopathies (Soslowsky *et al.*, 2000; Beason *et al.*, 2012); however, the repeatability and similarity of these models to the human condition has yet to be determined. Given that overuse is thought to contribute to tendinopathy, an uphill running protocol has been successfully used to damage Achilles tendon structure in some (Glazebrook *et al.*, 2008; Abraham *et al.*, 2011) but not all instances, highlighting the variability in many of the existing "physiological" models of tendinopathy. In contrast, Beason and colleagues (Beason *et al.*, 2012) have demonstrated that a biopsy punch creates a highly reproducible tendon injury, but whether the resulting tendon lesion is similar to human tendinopathy has yet to be determined.

Further, while pain is a defining feature of human tendinopathy, tendons can have structural damage without being painful (Lee *et al.*, 2020) and this structural damage precedes rupture

(Kannus & Jozsa, 1991). Chronic human tendinopathy lacks an inflammatory signature (Jelinsky *et al.*, 2011; Pingel *et al.*, 2012; Tran *et al.*, 2020), shows increased matrix turnover (Samiric *et al.*, 2009; Parkinson *et al.*, 2010; Jelinsky *et al.*, 2011; Pingel *et al.*, 2012; Heinemeier *et al.*, 2018), a larger population of smaller collagen fibrils (Kongsgaard *et al.*, 2010; Pingel *et al.*, 2012, 2014), and increased intratendinous vascularity (Jelinsky *et al.*, 2011; Tran *et al.*, 2020; Risch *et al.*, 2021). More specifically, the increased matrix turnover seems to result from increased expression of the mRNA for type I and III collagen (Jelinsky *et al.*, 2011; Pingel *et al.*, 2012) and either no change (Parkinson *et al.*, 2010) or increased (Pingel *et al.*, 2012) expression of certain matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), the proteins that regulate the resorption of tendon matrix. There are also more large proteoglycans (Samiric *et al.*, 2009; Parkinson *et al.*, 2010) with negatively charged glycosaminoglycan (GAG) side chains that increase the water content of the tendinopathic tissue (Samiric *et al.*, 2009) and the presence of these molecules correlates with pain (Attia *et al.*, 2014).

Cook and colleagues state that in degenerative tendinopathy, interventions influencing tendon structure are less critical, as the pathology [of tendinopathy] appears to have limited reversibility (Cook *et al.*, 2016). We hypothesize that the “limited reversibility” of tendinopathy is due to stress shielding of the injured region. The rationale underlying this hypothesis is as follows: 1) Injury to a tendon weakens the material properties of the damaged region (Eliasson *et al.*, 2009); 2) Since the damaged area is mechanically inferior, stress preferentially passes through neighboring regions of the tendon that are stiffer (shielding the injured area from stress); 3) Experimentally, stress shielding the entire healthy patellar tendon produces a tissue with the hallmarks of a scar (Sakabe *et al.*, 2018): increased cellularity, collagen disorganization, and reduced mechanics (Yamamoto *et al.*, 1993, 1999); and 4) Injured tendons with lower material properties, such as tendons from fetuses (Beredjiklian *et al.*, 2003; Howell *et al.*, 2017) and β-aminopropionitrile (BAPN) treated adult tendons (Alves *et al.*, 2001), heal with relatively good

collagen alignment since stress shielding is not possible. These data suggest that stress shielding in injured adult tendons results in scar formation and tendinopathy.

If tendon scar formation is the result of stress shielding and lack of tensile load, applying directional load through the scar should increase genes enriched in developmental or mature tendons and accelerate tendon regeneration. One way to apply a tensile load to a viscoelastic material like a tendon (Atkinson *et al.*, 1999) is to hold it at a constant length under tension. Isometric contractions have been used in the past to treat tendinopathy and provide immediate pain reduction (Rio *et al.*, 2017; Baar, 2019). During a long (30+ sec) isometric hold, stress relaxation occurs and the stress within the tissue decreases with time. As the stiffness of the stronger/healthy part of the tendon decreases over time, it dips below that of the of the injured region resulting in directional load passing through the scar. Given that tenocytes deposit collagen along the line of force (Kapacee *et al.*, 2008), we hypothesize that this tensile load signal is essential for repair. However, whether isometric loading provides a sufficient molecular signal to promote recovery has yet to be tested. The current study was designed to test the hypothesis that isometric loading a central core tendinopathy would increase the expression of tendon markers (*COLIα1, SCX, MKX, TNMD, FMOD*), whereas an equal time-under-tension using dynamic loading would increase the expression of cartilage markers (*ACAN, COMP, COLIIα1*).

Materials and Methods

Animals

All experiments were approved by the University of California Davis Institutional Animal Care and Use Committee under Protocol #21306. Wistar rats were obtained from Charles River Laboratories and housed in 12-hour light and dark cycle with two to three per cage and *ad libitum* access to food and water throughout the experiment. For the sequencing experiments, patellar tendons were collected from three adult and three injured adult (4.5 months) rats. For the injury and loading experiments, eighteen adult (4 month) male rats had a patellar tendon injury induced using a 2mm biopsy punch (described in detail below) and recovered for 14 days with normal cage activity. The biopsy punch caused a complete tendon rupture in one rat, so seventeen rats were assigned to the experimental groups: scar (5), isometric (6), and dynamic (6).

Patellar Tendon Injury

Rats were anesthetized with isoflurane (2.5%). The right distal hindlimb was shaved and skin prepared aseptically. The patellar tendon was exposed using blunt dissection though a small longitudinal incision on the lateral aspect of the knee. To induce injury, a flat stainless-steel probe was placed underneath the tendon and a 2 mm biopsy punch was inserted into the central core of the patellar tendon distal to the patella (Figure 1). Following injury, the wound was closed with sterile sutures and an intraperitoneal (IP) injection of buprenorphine (0.05-0.1 mg/kg) was administered as analgesic. Rats resumed normal walking within 15 minutes and were monitored daily for fourteen days post-op.

Loading Protocol

Fourteen days post-injury, the rats in the isometric and dynamic groups were loaded using separate protocols that were matched for duration and time-under-tension. The “scar” only group was not loaded and only received the injury to the right patellar tendon. Exercised rats were anesthetized with isoflurane (2.5%) and placed in a 3D printed device with the right knee secured at 120˚. The quadriceps muscle was stimulated using a Dual-Mode Muscle Level System (Aurora

Scientific) to place a load across the patellar tendon. Both protocols used a pulse frequency of 100 Hz to elicit a near maximal contractile force and a pulse width of 0.2 ms. The isometric loading protocol consisted of 4 x 30 second isometric contractions each separated by two minutes of rest because most stress relaxation within a rat PT occurs in the first 30 seconds (Figure 6). The dynamic loading protocol was 360 x 0.333 second contractions separated by one second of rest between contractions. Even though the contraction duration is much shorter in the dynamic group, ground contact time during running is ~0.24 seconds. Therefore, the dynamic contraction protocol is similar to what an average runner would experience on an 8-minute run. The loading protocols totaled 8 min under tension because tendon cells are maximally activated within 10 min of loading (Paxton *et al.*, 2012). Following exercise, rats were once again injected with buprenorphine for analgesia and allowed to recover before being returned to their cage.

Tissue Collection

Patellar tendon tissue was collected eighteen hours after loading, on day fifteen after injury. Rats were anesthetized and patellar tendon tissue surgically removed. The patellar tendon was trimmed to exclude enthesis regions. Patellar tendon tissue was weighed and then immediately snap-frozen in liquid nitrogen. Tendons were stored at -80˚C until RNA extraction.

3' Tag RNA-Seq and DGE Analysis

RNA was isolated using an Animal Tissue RNA Extraction Kit (Norgen, Ontario CA). Sequencing libraries were generated using a 3′-Tag sequencing method (QuantSeq 3′ mRNA-Seq Library Prep Kit FWD for Illumina; Lexogen, Greenland, NH) and sequenced using a HiSeq 4000 (Illumina, San Diego, CA) with single end 90 bp reads at the UC Davis Genome Center similarly to previous techniques described (Meyer *et al.*, 2011; Lohman *et al.*, 2016). For each sample, approximately 10M single end reads were generated. Sequencing data were analyzed using CLC Genomics Workbench 21.0.3 (QIAGEN) similarly to a previous analysis(Mienaltowski *et al.*, 2019). Within Workbench, the quality of raw reads were checked with a Workbench program QC for Sequencing Reads 0.2 based upon FastQC and then trimmed with Trim Reads 2.5 within

Workbench. Reads were mapped to the *Rattus novegicus* (Ensembl Rnor_6.0) annotated reference genome using Workbench's RNA-Seq Analysis 2.3 program. The Differential Expression for RNA-Seq 2.5 program within Workbench was used for evaluating gene expression differences between uninjured and scarred tendon tissue. Statistical significance in expression differences was determined by student's t test with false discovery rate multiple test correction $q < 0.05$. To generate a dendritic heat map of expression levels amongst all 8 samples, all differentially expressed genes ($q < 0.05$) were applied. Gene enrichment analysis based upon cell compartment gene ontology categories and $\log_2$ fold change of expression >0.6 and $q < 0.05$ was performed using Workbench's Gene Set Test 1.2 program. Moreover, pathway analysis was performed by applying the same list of genes, $\log_2$ fold changes, and q values to Advaita's iPathwayGuide (Advaita, Ann Arbor, MI) (Draghici *et al.*, 2007; Nguyen *et al.*, 2019). Relationships between the rat tendinopathy model and human tendinopathy were made by comparing gene symbols, fold changes, and q-values to a previously published human tendinopathy microarray expression study by Jelinsky and colleagues (Jelinsky *et al.*, 2011) (relative to matched normal human tendon) to scarred patellar tendon versus uninjured patellar tendon rat expression data.

Total RNA and Gene Expression- Injury and Loading

RNA was isolated using guanidinium thiocyanate-chloroform-isopropanol method. Prior to homogenization, patellar tendon tissues were finely minced on a glass plate on dry ice. Samples were homogenized in TRIzol (Ambion, ThermoFisher, Waltham, MA). Phase separation was performed with chloroform (Millipore, ThermoFisher, Waltham, MA). The RNA containing aqueous phase was isolated. Glycogen (RNA grade, ThermoFisher, Waltham, MA) was added (0.4 µg/µL) to improve RNA precipitation and 100% isopropanol added to aid in the precipitation of RNA. RNA was washed twice with 70% ethanol, dried, and resuspended in water. RNA concentration was measured by spectrophotometry (Epoch Microplate Spectrophotometer,

BioTek Instruments Inc., Winooski, VT). cDNA was synthesized by combining one microgram of RNA and a cDNA synthesis mix (MultiScribe RT, 10x RT buffer, 10x Primers, dNTPs, RNase inhibitor; Applied Biosystems, Foster City, CA) and running an RT reaction using the settings provided by the manufacturer. cDNA was diluted (1 µg/ 100 µL) prior to qPCR. Each sample was amplified in triplicate with 3 µL cDNA, 5 µL SYBR green (BioRad, Hercules, CA; PCRbio.com, Wayne, Pennsylvania), and 2 µL of 10 uM primer (Invitrogen, ThermoFisher, Waltham, MA) (Supplemental Table). qPCR reactions were performed on a CFX384 Touch Real-Time PCR Detection System (BidRad, Hercules, CA). GAPDH was used as housekeeping control. Absolute C_T for GAPDH was not different between groups (Supplemental Fig 1). Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.(Livak & Schmittgen, 2001)

Statistical Analysis- Injury and Loading Experiments

RNA in the injured and contralateral control tissues was compared using a paired t test since the analysis compared the uninjured and injured leg from the same animal. These fold comparisons could not be made for three rats because RNA of adequate quality could not be isolated from contralateral control leg. Outlier tests were performed with ROUT with Q = 1%. Gene expression relative to contralateral control tissue in the scar, isometric, and dynamic groups was performed as follows: 2^(-ΔΔCt) values were log transformed, fold change comparisons made where applicable (injured vs contralateral control), and then statistical tests performed. Gene expression in the scar and control legs for each loading group (no load, isometric, and dynamic) was compared using mixed model analysis, due to the data missing from the excluded callouses. Multiple comparisons were made using Tukey's multiple comparisons test. Within animal changes due to the injury were determined between the injured and contralateral control leg raw data and then log-transformed. Following log transformation, the groups were analyzed using one-way ANOVA. All statistical analysis was performed with GraphPad Prism software (version 8.3.1 for Windows).

Results

Rat Model of Patellar Tendinopathy

Two weeks following removal of the central third of the rat patellar tendon using a biopsy punch (Figure 1B), the gross appearance of the patellar tendon to the surgeon was that vascularity increased (Figure 1C). Fifteen days after injury, there was a visible scar that did not differ between groups at the injured site in the patellar tendon (Figure 1C). Hematoxylin and Eosin (H&E) stains of the uninjured patellar tendon showed collagen fibers aligned with the direction of force transfer and elongated fibroblasts between collagen fibers (Figure 1D). Two weeks after injury, there was a notable collagen disorganization with the fibers wrapping around the injured site. There was also an increase in cellularity and loose connective tissue that formed distal to the injury (Figure 1E&F). The loss of collagen organization, increased cellularity and vascularity are hallmarks of tendinopathy (Aström & Rausing, 1995; Rui *et al.*, 2012).

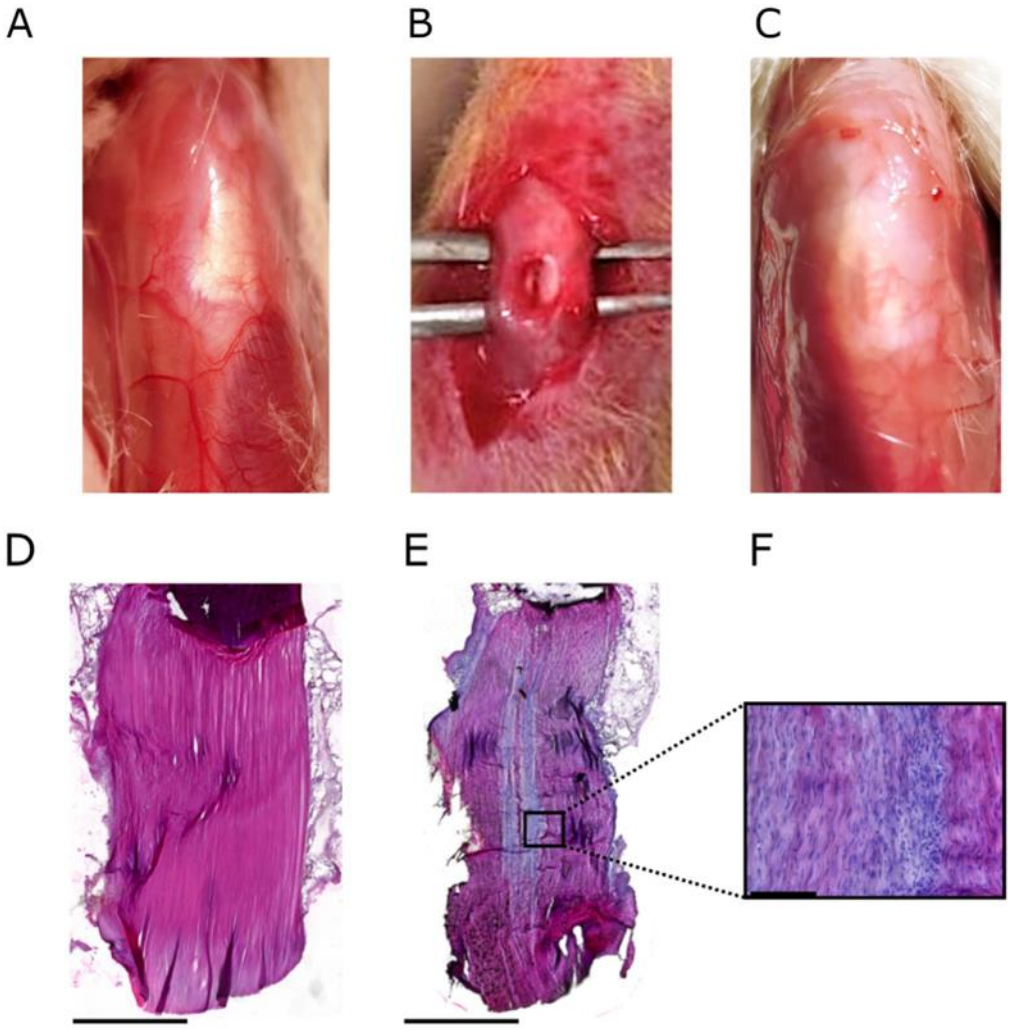

Figure 1: Model of patellar tendinopathy. (A) Intact patellar tendon with synovial sheath. (B) Injury was induced to the central core of the patellar tendon with a 2 mm biopsy punch. (C) Fifteen days after injury there was noticeable vasculari- zation and enlargement of the patellar tendon. H&E stained patellar tendon at 10X: (D) healthy and (E) injured scale bar is 1.8mm; and (F) 20X injured, scale bar is 150mm.

Transcriptomics in Healthy and Injured Rat PTs

We performed 3'TAG-seq of adult healthy and injured tissue to assess which genes change expression in response to injury. Approximately 6% of genes were differentially regulated with 12,041 identified and 707 classified as differentially expressed (0.6 log fold change/ 1.5-fold change and $p < 0.05$).

Heat Map (rat scar vs healthy): We created a heat map to describe the relationship of all genes with significant (no minimum absolute fold change, $q < 0.05$) differences in expression between the healthy adult (A) and scar tendon (S). The dendritic branches at the top of the figure describe the relatedness of the samples based on the expression of these genes. Each sample is a column, while each row is a gene. Gene set 1 has higher expression in the scar group than the healthy adult. Gene set 2 has lower expression in all the scar samples, yet a higher expression in two but not all four healthy adult samples (Figure 2).

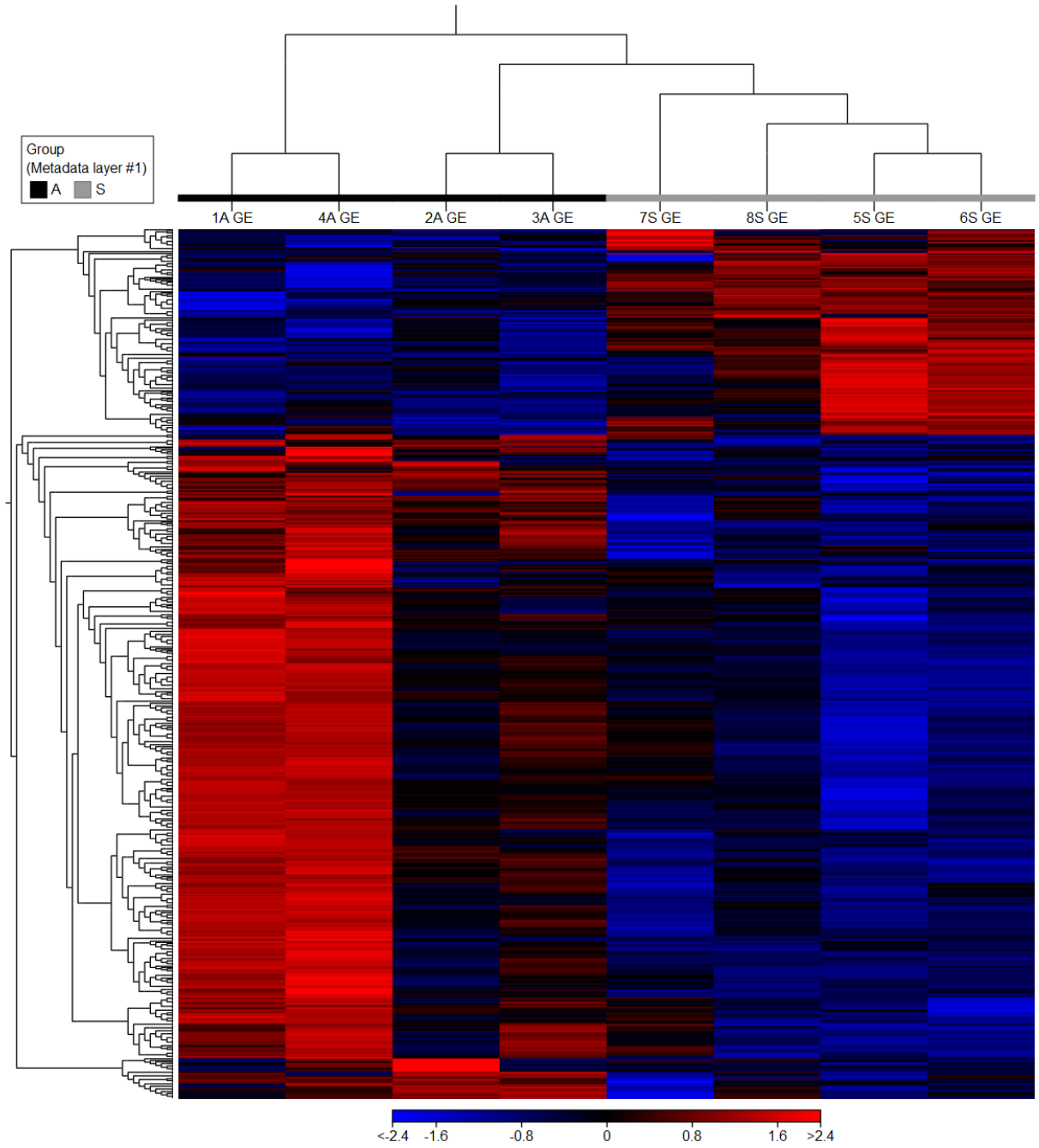

Figure 2: Heat Map of 3' TagSeq transcriptome analysis for all significant (FDR p<0.05) differences in expression between the healthy and adult scar rat patellar tendon. The uninjured adult (A) samples are the left four columns, while the injured scar adult (S) are to the right. The colors indicate levels of expression with red representing higher expression and blue lower expression of a particular gene.

Gene Ontology- Cellular Compartment (rat scar vs healthy): Differentially regulated transcripts in the rat patellar tendon tendinopathic tissue can be classified into gene ontology categories based on the cellular compartment in which proteins encoded by those transcripts are located. In our analysis, we decided to rank cellular compartments that contained a minimum of 200 detected genes by the ratio of differentially expressed genes to total genes. In this case, the largest regulated gene set relative to gene set size belonged to the mitochondrial membrane (Table 1). Most genes belonging to the mitochondrial membrane were downregulated in expression in the injured patellar tendon (Supplemental Table 3). Extracellular matrix genes were the second most regulated group (Table 1) and expression of most of these genes were upregulated in the injured tendon (Supplemental Table 4). Membrane protein complexes were among the most regulated groups, yet 12/50 DEGs were shared between membrane protein complex and mitochondrial membrane (Supplemental Tables 3, 5, & 6).

Table 1. Top selected cellular compartments regulated in rat scar vs healthy

Description	DEG/ Detected Genes	Detected Genes	DEG	FDR p-value
Mitochondrial membrane	0.11	289	32	1.34E-05
Extracellular matrix	0.09	206	19	0.022921
Membrane protein complex	0.09	573	50	1.21E-05
Adherens junction	0.08	260	22	0.027486
Anchoring junction	0.08	269	22	0.0422
Cytoskeleton	0.07	601	45	0.001951
Mitochondrion	0.07	1132	84	1.36E-06
Extracellular space	0.07	962	65	0.001063
Cytoplasmic part	0.05	6894	327	6.87E-05

Descriptions of cellular compartments with >200 detected genes and FDR<0.05 were selected. Cellular comparments displayed in decending order of DEG/ Detected Genes.

Rat vs Human Tendinopathy: We next compared our RNAseq findings to microarray data of human tendinopathy because we were interested in the transcriptional similarity between the rodent model of tendinopathy and the human condition. The human tendinopathy data consisted of male (n=15, age: 32-66) and female (n=8, age: 46-65) samples from supraspinatus (n=15), extensor carpi radilis brevis (n=3), flexor/pronator (n=2), patellar (n=2), and distal biceps (n=1)

tendons. Twelve of the patients also had a history of at least one corticosteroid injection. The microarray of human tendinopathy identified that 5% of genes were differentially regulated (paired t-test; $p<0.01$, fold change ≥1.5).

The genes presented in Table 2 were determined to have an orthologous relationship between human and Norway rat species using NCBI Orthologs. The top regulated transcripts shared between the human and rat tendinopathy data sets were: *BNC2*, *CTHRC1*, and *MARCKS*. Increased matrix turnover and a lack of inflammation were characteristic of both human tendinopathy and the rat model. Expression of *COL1A1*, *COL5A1*, *COL5A2*, *COL8A2*, and *COL12A1* were significantly increased in both groups. *COL3A1* was only significantly increased in human tendinopathy, yet was elevated (FC=2.1, p=.209) in the injured rat patellar tendon. Expression of *MMP2* and *MMP14*, but not *TIMP1* or *TIMP2*, increased in human tendinopathy and the rat model. The kinases *JAK3* and *DCLK1* were also both expressed at greater levels in scar tissue. Interestingly, *JAK3* transcripts were only detected in the scar tissue in the rat tendinopathy model (Table 2). Notably, inflammatory pathways were not regulated in human tendinopathy (Jelinsky *et al.*, 2011) or the rat patellar tendinopathy model (Supplemental Table 7).

Table 2. Comparison of expression patterns of rat central core tendinopathy model with human tendinopathy

Gene Symbol	Gene Name	Human q	Human FC	Rat q	Rat FC
Top regulated transcripts (Table 3)					
BNC2	Basonuclin 2	0.000315	2.9	0.0205	2.6
CTHRC1	Collagen triple helix repeat containing 1	0.0011	4.4	6.72E-13	24.4
MARCKS	Myristoylated alanine-rich kinase C substrate	0.0045	2.8	0.0216	2.8
Matrix metallopeptidase and TIMP genes (Table 6)					
TIMP1	Tissue inhibitor of metalloproteinase 1	0.3	1.6	0.94	1.1
TIMP2	Tissue inhibitor of metalloproteinase 2	0.5	1.1	0.16	1.9
MMP2	Matrix metalloproteinase 2	0.0482	1.8	0.00421	2.9
MMP14	Matrix metalloproteinase 14	0.0166	1.9	0.000505	3.8
Collagen genes (Table 7)					
COL1A1	Collagen, type I, alpha 1	0.0396	1.7	0.00218	4.1
COL3A1	Collagen, type III, alpha 1	0.0428	2	0.209	2.1
COL5A1	Collagen, type V, alpha 1	0.0428	2	0.0019	2.8
COL5A2	Collagen, type V, alpha 2	0.0316	2.1	0.000836	3.6
COL8A2	Collagen, type VIII, alpha 2	0.0428	1.8	0.00686	2.9
COL12A1	Collagen, type XII, alpha 1	0.0316	2.1	5.06E-06	8.1
Kinases (Table 8)					
JAK3	Janus kinase	0.0783	1.8	N/A*	29.4
DCLK1	Doublecortin-like kinase 1	0.00783	28	0.045	2

Table adapted from Tables 3, 6, 7, and 8 of: Jelinsky et al. *Regulation of gene expression in human tendinopathy*. BMC Musculoskeletal Disorders 2011, 12:86. Microarray data compared to RNAseq findings for rat patellar tendinopathy model. * Not detected in control tissue.

Gene Expression and Loading

Patellar tendons from the isometric and dynamic groups were loaded fourteen days after injury and all tissues collected eighteen hours later. The unexercised contralateral control tendons were collected at the same time. There were no group differences in baseline body weight, patellar tendon wet weights, or RNA content. There was a significant increase in the amount of RNA per tissue wet weight of the injured patellar tendons in comparison to contralateral control tissue (Table 3).

Table 3. Baseline body weight, patellar tendon weight, total patellar tendon RNA.

	Body Weight (g)	PT control (mg)	RNA control (ng)	RNA/ PT (ng/mg)	PT scar (mg)	RNA scar (ng) *	RNA/ PT scar (ng/mg) *
Scar	438 ± 8.66 (n=5)	65 ± 5.61 (n=5)	17398 ± 4856 (n=4)	263.7 ± 83.00 (n=4)	72.6 ±15.42 (n=5)	31644 ± 20633 (n=5)	455.9 ± 326.9 (n=5)
Dynamic	440 ± 8.30 (n=6)	66.5 ± 14.43 (n=6)	22636 ± 5874 (n=5)	341.5 ± 95.74 (n=5)	59.6 ±16.94 (n=5)	37272 ± 14880 (n=5)	615.3 ± 140.0 (n=5)
Isometric	426 ± 16.83 (n=6)	51.33 ± 13.79 (n=6)	18489 ± 6463 (n=5)	353.8 ± 65.76 (n=5)	57 ±13.47 (n=5)	30652 ± 14089 (n=5)	531.5 ± 209 (n=5)

Values are mean ± standard deviation (number of measures). Patellar tendon weight is the wet weight.
*Significant scar vs control (mixed-effects analysis). RNA (ng) p = 0.0215. RNA/ PT (ng/mg) p= .0042

Expression of genes enriched in developing tendons: Isometric loading increases scleraxis expression

The expression of tendon associated genes, SCX, TNMD, or FMOD was greater in the injured leg regardless of group (Figure 3A-D). Following isometric exercise, there was an increase in SCX expression relative to contralateral control leg in the isometric group (Figure 3E). There were no statistically significant differences in expression between loading groups for MKX, TNMD, or FMOD. However, for each of those genes the isometric group tended to show a greater relative increase (Figure 3F-H).

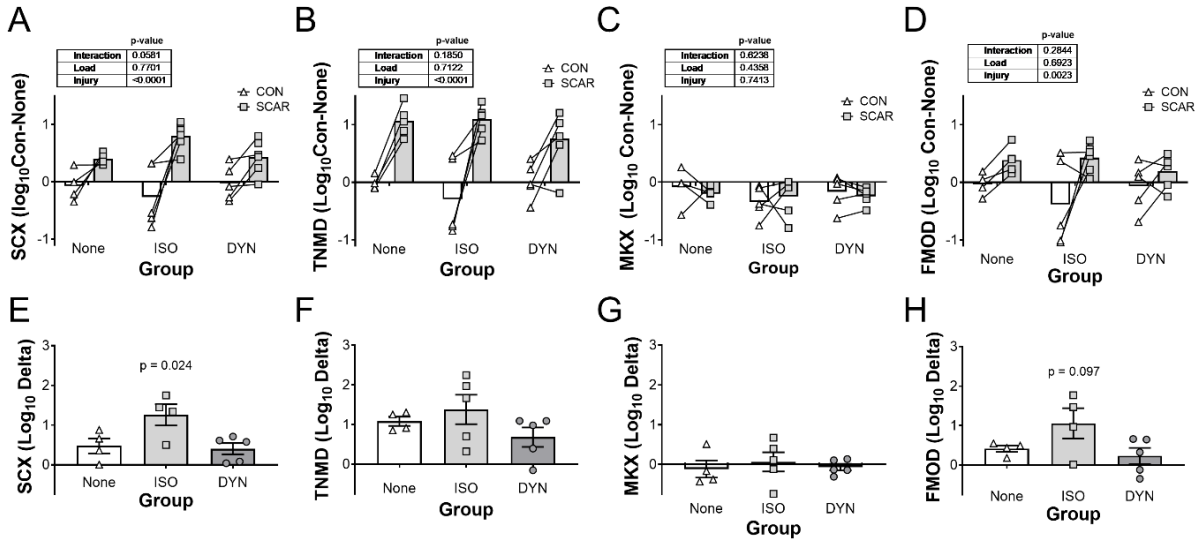

Figure 3: Expression of tendon enriched markers following injury and no load (none), isometric (ISO: 4 x 30 s), or dynamic (DYN: 360 x 0.33 s) loading. (A-D) Expression levels relative to contralateral uninjured control patellar tendon. (E-H) Gene expression in control (white bars) and

injured (grey bars) legs for each loading group: none, isometric (ISO), and dynamic (DYN), relative to the no loading control expression. (A,E) The expression of scleraxis increased in the tendon scar and further increased with an isometric load (Mixed effects model: exercise x scar p=.0279. Tukey's multiple comparisons test: none scar vs isometric scar, p= .0018; scar isometric vs scar dynamic, p=.0207). (B,F) Mohawk expression was unaffected by injury or load. (C,G) Tenomodulin expression increased in the tendon scar. (D,H) Fibromodulin expression increased in the tendon scar.

Collagen gene expression: collagen Ia1 increases with isometric, whereas collagen IIa1 expression levels increase with dynamic loading

The expression of collagen Ia1, IIa1, and IIIa1 all increased in the in the injured patellar tendon (Figure 4A-C). There was a significant increase in the expression relative to contralateral control leg of collagen Ia1 in the isometrically loaded group (Figure 4D). The expression of collagen IIa1 increased in the dynamic group relative to the isometric group (t-test), whereas type III collagen tended to be higher in the isometric group; however, this was not statistically significant (Figure 4D-F).

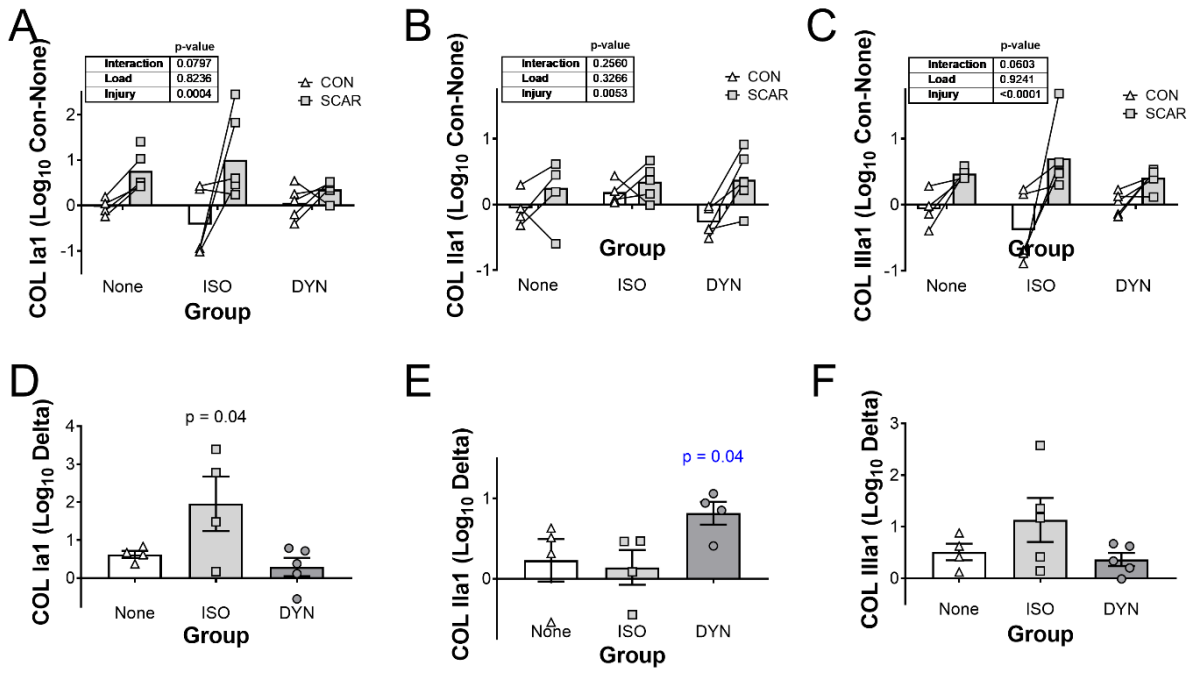

Figure 4: Expression of collagens Iα1, IIα1, and IIIα1. (A,D) There were no differences in collagen Iα1 expression, but isometric loading tended to increase the expression (D) relative to the no load control and (A) relative to the contralateral patellar tendon. (B,E) Collagen IIα1 (E) expression increased in the tendon scar and the (B) expression was significantly elevated in the dynamic load group relative to the control limb compared to the isometric group and substantially increased compared to the scar (Ordinary one-way ANOVA, p=.0298; Tukey's multiple comparisons: isometric vs dynamic, p= .0405; none vs dynamic, p= .0615). (C,F) Expression of collagen IIIα1 was increased in the tendon scar.

Expression of cartilage associated genes: ACAN and COMP increase following injury

The expression of aggrecan and cartilage oligomeric matrix protein was increased in the injured patellar tendon regardless of group (Figure 5A, B). Expression of the SOX9 was not changed with injury (Figure 5C). There were no differences in expression of these genes relative to contralateral control following loading (Figure 5D-F).

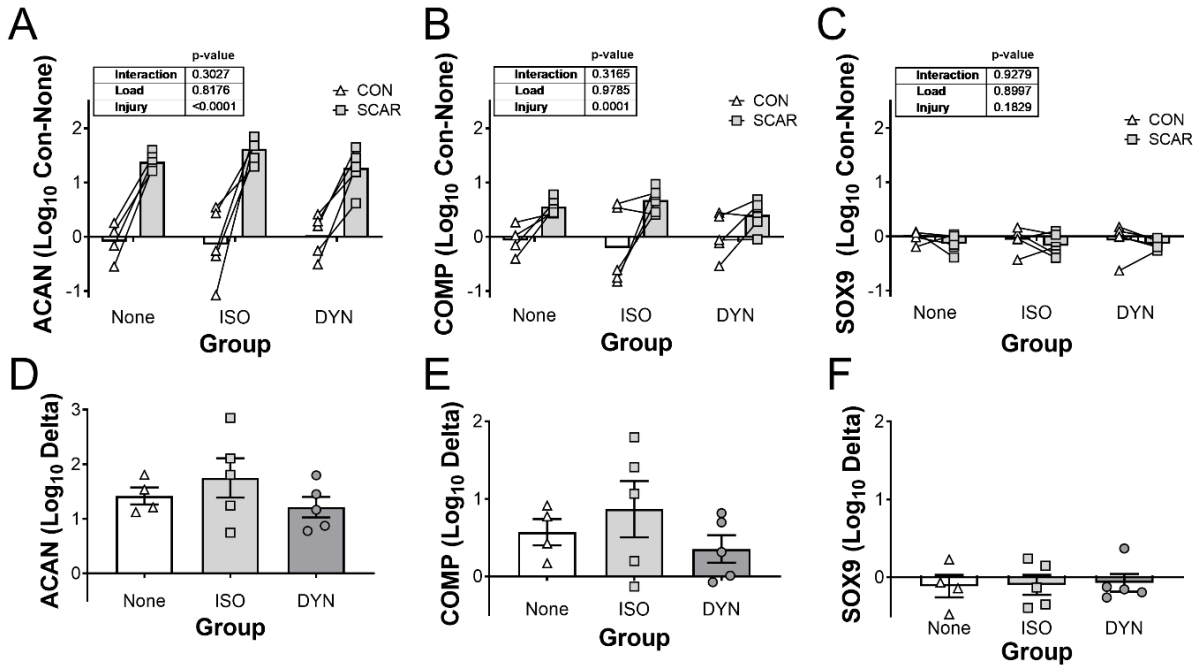

Figure 5: Expression of selected fibrocartilage markers. (A,D) Expression of aggrecan increased in the patellar tendon scar. (B,E) COMP expression increased in the patellar tendon scar and was unaffected by load. (C,F) SOX9 expression did not change.

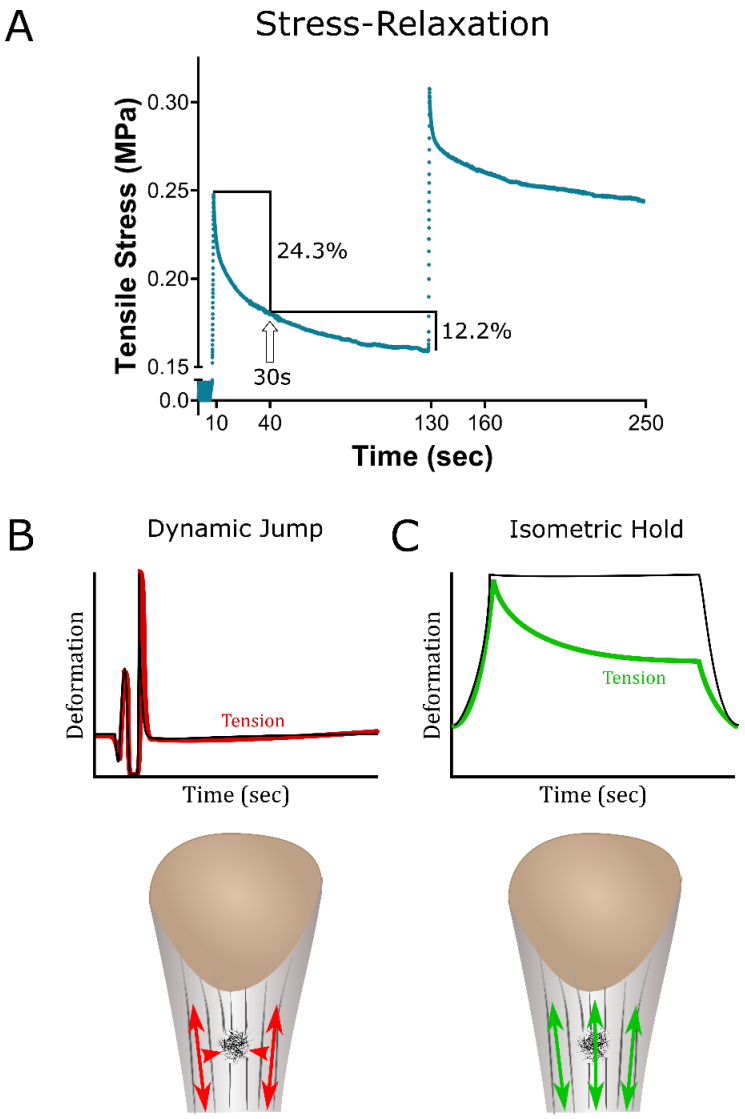

Figure 6: Measuring and modelling stress shielding and stress relaxation. (A) Following 10 pre-conditioning cycles, rat PT underwent a stress relaxation test (2 min hold following a 0.2MPa stress). Note that 75% of total relaxation occurs in the first 30 s. (B) Dynamic loading (such as during running or jumping) occurs quickly causing load to be shunted around the injured region.

With a central core injury, the injured region is loaded in radial compression as the tendon undergoes isovolumetric elongation. (C) Stress-relaxation decreases stiffness of the surrounding collagen below that of the injured region allowing load to pass though the injured region. Uniaxial load passing though the injured region provides a signal that increases the expression of genes enriched in mature and developing tendons.

Discussion

Tendinopathy is one of the most common musculoskeletal diseases without known treatment (Irby *et al.*, 2020). Here, we use transcriptomics to validate a rodent model to study tendinopathy and suggest that isometric loading improves the molecular response of a tendinopathic tissue. Two weeks of cage activity following removal of the middle third of the patellar tendon showed similar changes in gene expression, including increased matrix turnover, decreased mitochondrial gene expression, and a lack of inflammatory signature, as human tendinopathy. Isometric loading the injured patellar tendon increased the expression of tendon genes like scleraxis and collagen Ia1, whereas a time under tension matched dynamic loading protocol increased expression of collagen IIa1.

Fifteen days after injury to the central core of the patellar tendon, there was collagen disorganization with the fibers wrapping around the injured site, increased in cellularity, loose connective tissue, and increased vascularity within the injured region. These are all hallmarks of human tendinopathy (Aström & Rausing, 1995; Rui *et al.*, 2012). Similarly, the amount of RNA per gram of patellar tendon increased in the injured tendon. In humans, a similar increase in RNA/mg tissue has been observed in biopsies from patients with patellar tendinopathy resulting either from chronic overload (Olesen *et al.*, 2021) or an acute injury due to insertion of a biopsy punch (Heinemeier *et al.*, 2016). Since the ratio of RNA/DNA does not change (Heinemeier *et al.*, 2016), this suggests that the increase in RNA in the tendinopathic tissue reported here and in the work of Olesen is the result of an increase in cellularity. In support of this hypothesis, Figure 1 shows an increase in cellularity at the site of tendon injury. Both chronic tendinopathy and the biopsy punch also increased collagen Ia1 and IIIa1 expression in the injured human patellar tendon (Heinemeier *et al.*, 2016). Similarly, Corps and colleagues (Corps *et al.*, 2004) found that COLIa1 expression increased (8-fold) in painful human tendinopathy but decreased (on average) in samples from ruptured tendon. The 4.13-fold increase (FDR p-value = .002) in COLIa1

expression that we observe in the rat model of patellar tendinopathy correspond nicely with the human tendinopathy data.

Beyond the targeted analysis above, when comparing our transcriptomic data to the only published transcriptomic analysis of human tendinopathy (Jelinsky *et al.*, 2011) many important similarities were noted. First, the global pathway analysis performed by Jelinsky and colleagues identified genes encoding for the electron transport chain and oxidative phosphorylation as two of the most downregulated pathways in human tendinopathy. Our analysis likewise shows that the largest regulated gene set relative to gene set size was mitochondrial genes and like the human tendinopathy samples we found that most of the mitochondrial genes were downregulated in the injured patellar tendon. Second, Jelinsky and colleagues found that expression of extracellular matrix related (focal adhesion, integrin signaling, and collagen) and cell cycle progression genes were upregulated (Jelinsky *et al.*, 2011). Extracellular matrix genes were the second most regulated group in our transcriptomic analysis and as in human tendinopathy, we found that expression of these genes was increased in the injured tendon. Third, the top regulated transcripts shared between the human and rat tendinopathy data sets were: *BNC2*, *CTHRC1*, and *MARCKS*. Basonuclin 2 is one of the top regulated TFs in fibroblasts during early tendon development (E11 -> E13)(Liu *et al.*, 2015). CTHRC1 is located in the ECM and may negatively regulate collagen synthesis (Zhao *et al.*, 2018). The myristoylated alanine-rich C-kinase substrate (MARCKS) is important in fibroblast migration (Ott *et al.*, 2013). These data suggest that in both human tendinopathy and the rat patellar tendon injury model there is a signature of fibroblast migration into the tendinopathic region (MARCKS), early tendon development (BNC2), and the acute inhibition of collagen synthesis (CTHRC1). Fourth, the change in collagen gene expression was almost exactly the same in the rat patellar tendon model and human tendinopathy in general (Jelinsky *et al.*, 2011) and patellar tendinopathy more

specifically (Olesen *et al.*, 2021). Taken together, these data suggest that the rat patellar tendon injury model shows a change in gene expression that is characteristic of human tendinopathy.

Having established the relevance of the rodent model, we next addressed the effect of loading on tendinopathic tissue. The resulting data suggest that isometric loading (four 30-second contractions, each separated by two minutes of rest) is beneficial to treat tendinopathy because we found that expression of the tendon-enriched marker, scleraxis, increased 2.62-fold relative to an injured unexercised patellar tendon (6.8- versus 2.6-fold higher than their respective contralateral patellar tendon group) and the primary matrix gene, collagen Ia1, increased 3.12-fold relative to an injured unexercised patellar tendon 18 hours after isometric loading. By contrast, dynamic loading (360 contractions over eight minutes with each contraction lasting 333 milliseconds so that both the exercise duration and the time under tension were matched) tended to decrease scleraxis and collagen Ia1 expression and increased collagen IIa1 expression relative to the isometric group. These data suggest that different forms of loading can result in diametric changes in gene expression in the same tissue.

One possible explanation for the increase in scleraxis and collagen Ia1 expression following isometric loading is that this form of contraction resulted in a uniaxial strain across the cells within the injured region of the tendon. Following injury, the damaged region of a tendon can be shielded from further injury through a process called stress-shielding. While stress-shielding is widely appreciated in bone (Bobyn *et al.*, 1992), it is less well characterized in tendon. In tendon, stress-shielding occurs when the strong, aligned, healthy matrix shields a damaged region from load (stress). Preventing stress from passing through the damaged matrix may prevent catastrophic injury; however, without a uniaxial tensile force, proper matrix synthesis and orientation is not possible (Yamamoto *et al.*, 1993; Kapacee *et al.*, 2008). Isometric contractions can overcome stress-shielding through a process called stress-relaxation. Stress-relaxation is a property of viscoelastic materials, such as a tendon, whereby a material undergoes a time-

dependent decrease in stress at a constant strain (Atkinson *et al.*, 1999). In large human tendons, stress decreases by ~60% in the first 30 seconds (Atkinson *et al.*, 1999). Similarly, in the rat patellar tendon we have seen that within the first 30 seconds stress in the patellar tendon approaches a nadir after decreasing ~25% (Figure 6). We hypothesize that as the strong portions of the tendon relax (become less stiff), the injured region becomes stiffer than the healthy tendon and a directional tensile strain passes through the scar and is sensed by the cells (Figure 6).

Collagen Ia1 expression following isometric loading increased significantly more relative to the contralateral control limb than seen in the dynamic group. In humans, one hour of leg extensions increased tendon collagen protein synthesis (measured by radiolabeled proline) that peaked at 24 hours post exercise and remained elevated 42 and 72 hours later (Miller *et al.*, 2005). This increase in collagen protein occurs in spite of an acute (4h) decrease in collagen Ia1 mRNA that returns to baseline levels 24 hours after resistance exercise regardless of sex (Sullivan *et al.*, 2009). In animals, a similar increase in collagen protein synthesis is seen with increased load. For example, two days of overload of the chicken anterior latissimus dorsi increased collagen synthesis rates 5-fold (Laurent *et al.*, 1985). These data suggest that loading increases collagen protein; however, whether this is the result of an increase in transcription as observed here or increased collagen translation remains unclear. It is possible that increases in collagen Ia1 mRNA are not the only way to increase collagen protein following loading.

Fibrocartilage forms as a musculoskeletal adaptation to compression. A tendon placed under compressive loads increases expression of markers of fibrocartilage such as the large proteoglycan aggrecan (Vogel *et al.*, 1994) and type II collagen (Benjamin & Ralphs, 1998) . The dynamic loading group showed a 6.6-fold increase in collagen IIa1 expression, whereas in the isometric group collagen IIa1 was unchanged. These data suggest that dynamic loading of a central core tendon injury may cause local compression of the cells within the scar. Increased

type II collagen expression is seen in mid-portion Achilles tendinopathy (De Mos *et al.*, 2009). Other markers of fibrocartilage, such as aggrecan were increased in the tendinopathic tissue regardless of loading. Aggrecan is a large negatively charged proteoglycan that pulls water into the tissue to help resist compression. Within a tendon, aggrecan is synthesized by cells in compressed regions (Vogel *et al.*, 1994; Ehlers & Vogel, 1998). In the current work, aggrecan expression increased more than 30-fold in the injured tendon, again suggesting that after a central core tendon injury compression increases within the injured region during normal cage activity. However, aggrecan expression did not increase further with acute loading. Interestingly, the cartilage-specific marker SOX9 was unchanged in the injured tendon even though it acts in an upstream activator for both collagen IIa1 (Bell *et al.*, 1997) and aggrecan (Sekiya *et al.*, 2000). This might reflect the timepoint of collection, 18 hours after loading where a load-induced increase may have subsided, and 15 days after injury, when a mature scar is expected to have already formed (Sakabe *et al.*, 2018).

There were significant limitations to the current study. First, even though the loading protocols were matched for duration and time under tendon, the dynamic loading (with repeated shortening contractions) was more metabolically costly, and more muscle fatigue was noted. This suggests that there may have been greater load across the tendon in the isometric group. Second, we measured only changes in gene expression. It is possible that if protein levels/phosphorylation had been analyzed a different molecular response would have been found. Third, the greater change in gene expression between the isometrically loaded and contralateral control limb was driven by an apparent decrease in expression in the control patellar tendon in 2-3 animals in the isometric group. It is possible that there was a systemic effect of exercise that decreased gene expression in the healthy limb; however, it is also possible that this reflects an issue with the quality of RNA from those subjects. Any future work should include an uninjured group to account for any potential systemic effect. Fourth, the data does not distinguish the type or

location of the cells that contributed to the expression data reported. Even though the tendons were trimmed to include the injured region, how much of the surrounding healthy tissue was taken likely varied between samples. Last, the timeline of collection was likely not ideal. A single time after loading is likely to have missed (either too early or too late) the peak expression of the genes measured. Despite these limitations, the data suggest that isometric loading promotes a tendon cell fate (Figures 3A/E and 4D), whereas dynamic loading promotes a more fibrocartilage expression (Figure 4E) pattern in central core patellar tendinopathy.

Perspective

Prior work by our lab used a combined nutrition and isometric loading protocol to effectuate complete regeneration of a central core patellar tendinopathy in an elite basketball player (Baar, 2019). The current work sought the molecular response to load in a rodent model of patellar tendinopathy. We propose that a scar forms within the core of the patellar tendon in part due to stress-shielding at the injured site. This stress shielding prevents the uniaxial stretch across the tenocytes within the scar that is necessary for aligned collagen deposition (Kapacee *et al.*, 2008) and scar revision. Data from the current work support the hypothesis that isometric loading decreases stress-shielding, providing a tensile load stimulus to the cells within the scar, and increases the expression of genes enriched in mature and developing tendons (COL Ia1 and SCX). However, it is essential to better understand the time course of the response, where changes in expression occur, and whether there are systemic effects of loading before isometric loading can be used as a tool for resolving tendinopathy.

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Author Contributions

D.S. and K.B. conceived the study design. D.S. and K.B. performed tendon injury and loading experiments. D.S. and M.M. conducted bioinformatic analysis of RNA-sequencing data. D.S. performed the RNA extractions, qPCR, histology, and drafted the original version of the manuscript. All authors analyzed and interpreted results and revised the final version of the manuscript.

Competing Interests

KB has received an honorarium, support for travel, and a research contract from PepsiCo on a nutritional intervention tangentially related to the current work. KB is paid by professional sports and Olympic teams to consult on tendon issues. DS and MM have no competing interests.

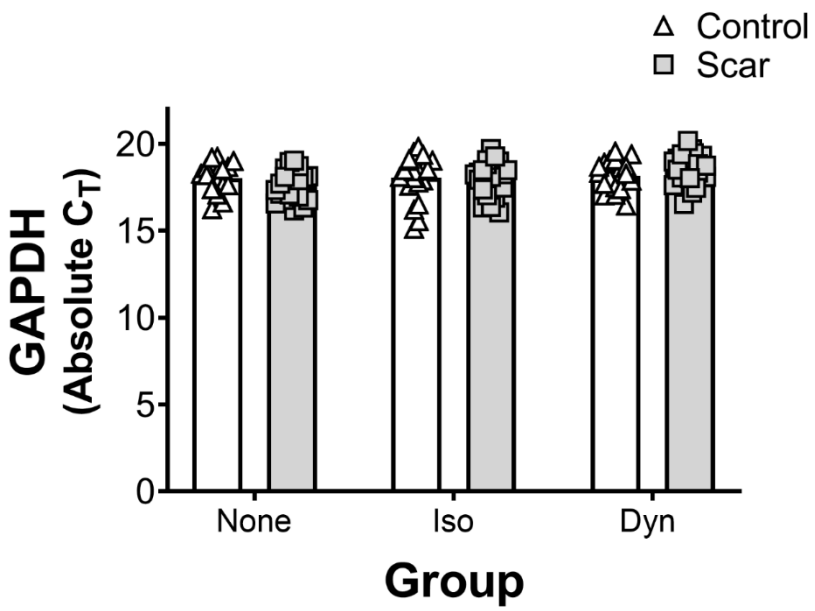
Data and Materials Availability

The 3’ TAG Seq data raw read files have been deposited to the NCBI BioProject under the accession # PRJNA936858. Metadata will be maintained to characterize each sample included in genomic analysis; these will include study design details, sample details (e.g., rodent age at time of experimentation, samples collected with anatomical information), and any relevant sample processing details as required and recommended by GEO, SRA, and NIH in general. All other data is available in the main text or supplementary materials.

Supplemental Information

Supplemental Table 1. Rat age, RNA characteristics, and reads mapped to the *rattus norvegicus* genome.

Sample ID	Group	Age	RNA (ng)	RNA Quality Score	Total Reads	Reads Mapped	% Mapped
1	Adult	4.5 mo	1368	4.5	9573798	8865627	92.6
2	Adult	4.5 mo	1045	3.2	10180538	9407400	92.41
3	Adult	4.5 mo	555	4.7	10642166	9934868	93.35
4	Adult	4.5 mo	1495	6.3	11700939	11019941	94.18
5	Scar	4.5 mo	1410	5.6	10150502	9241915	91.05
6	Scar	4.5 mo	2200	5.5	12897513	11897684	92.25
7	Scar	4.5 mo	570	3.1	9333008	8194132	87.8
8	Scar	4.5 mo	2025	5	8,525,644	7,903,247	92.7



Supplemental Figure #1. Neither patellar tendon injury nor exercise altered GAPDH the absolute cycle threshold (C_T) values. GAPDH C_T values are pooled from three qPCR experiments with each sample run in triplicate.

Supplemental Table 2. Primer Sequences for injury and loading experiments

Gene	Efficiency	Forward	Reverse
SCX	0.9693	5' CTG GCC TCC AGC TAC ATC TC 3'	5' GCA GAA GGT GCA GAT CTG TTT 3'
TNMD	0.9925	5' TGG GGG AGC AAA CAC TTC TG 3'	5' AAG TAG ATG CCA GTG TAT CCG TTT 3'
MKX	0.9746	5' TGC TAA GAG CGT ATC TGG ACC C 3'	5' ATG GTA AGG GCT GAC AGG GA 3'
FMOD	0.993	5' CAA CCC AAG GGA CAA CAT GC 3'	5' GGG GTC GTA GTA GGT GGA CT 3'
COL1A1	0.9927	5' GTC TCA AAG ATG GTG GCC GTT 3'	5' TCT CCG CTC TTC CAG TCA GA 3'
COL2A1	0.9824	5' GGC TCC CAG AAC ATC ACC TA 3'	5' ATC CTT CAG GGC AGT GTA CG 3'
COL3A1	0.9586	5' TGA ACC TGG TGA ACG TGG CT 3'	5' CGA GCC CTC AGA TCC TCT TTC 3'
ACAN	0.9619	5' CAG ATG GCA CCC TCC GAT AC 3'	5' CCA CTG ACA CAC CTC GGA AG 3'
COMP	0.9681	5' ACA GAT GCT TCG AGA ACT CCA G 3'	5' GCG TTG CAC TCA TTA ACG TC 3'
SOX9	0.9763	5' CTC CTA CTA CAG CCA CGC AG 3'	5'GCT GTG TGT AGA CGG GTT GT 3'
GAPDH	0.9602	5' GTC ATC CCA GAG CTG AAC GG 3'	5' GCC TGC TTC ACC ACC TTC T 3'

Supplement Table 3

Cellular Compartment- Mitochondrial Membrane. DEGs for rat healthy vs scar patellar tendon.														
Name	Chromosome	Region	Identifier	S vs. A - Fold change	S vs. A - Log fold change	S vs. A - FDR p-value	1A_S37 (GE) - TPM	2A_S38 (GE) - TPM	3A_S39 (GE) - TPM	4A_S40 (GE) - TPM	5S_S41 (GE) - TPM	6S_S42 (GE) - TPM	7S_S43 (GE) - TPM	8S_S44 (GE) - TPM
Wasf1	20	compleme	ENSRNOG	5.342585	2.417538	0.000143	1.7412	2.816866	4.155716	2.836303	32.6754	20.9576	3.365066	9.345372
Bcl2a1	8	96551245.	ENSRNOG	4.062849	2.022492	0.038406	0.69648	12.20642	19.22019	1.418152	38.7264	43.56975	9.253931	50.77652
Gja1	20	37876650.	ENSRNOG	4.003433	2.001238	0.000234	71.04098	117.8389	77.65994	120.5429	811.7416	564.2007	148.9042	180.3657
Fndc1	1	47605262.	ENSRNOG	3.682295	1.880605	0.007047	87.40826	399.2908	135.5802	143.706	1404.74	1049.81	139.6502	459.4808
Mt3	19	compleme	ENSRNOG	3.402878	1.766755	0.003761	5.571842	22.53493	9.090628	8.745269	13.61475	68.11221	25.23799	63.86004
Acad9	2	12278206(	ENSRNOG	-1.96028	-0.97106	0.043885	26.11801	29.81184	44.67394	41.59912	18.45555	16.82123	26.07926	20.55982
Dnajc15	15	compleme	ENSRNOG	-2.05332	-1.03796	0.04091	96.46251	77.69856	67.53038	75.16204	37.21365	36.6758	42.90459	51.39955
Dnajc19	2	12050413(	ENSRNOG	-2.06335	-1.04499	0.030698	125.3664	146.2423	99.21771	82.96188	52.0386	44.67278	63.09499	77.25508
Mfn1	2	11892973(	ENSRNOG	-2.08067	-1.05705	0.04368	52.58425	35.44557	44.93368	58.61694	24.50655	21.78488	33.65066	22.42889
Ndufaf4	5	39251337.	ENSRNOG	-2.23429	-1.15982	0.048296	37.26169	27.46445	20.77858	37.81738	20.27085	9.100012	16.82533	14.32957
Uqcc2	20	compleme	ENSRNOG	-2.24679	-1.16787	0.019721	83.57762	39.20139	40.77796	57.90786	25.11165	20.68184	26.07926	36.44695
Chchd3	4	compleme	ENSRNOG	-2.39772	-1.26167	0.040643	342.6683	149.0592	176.3582	347.6835	97.11854	104.2365	119.4598	147.9684
Hadhb	6	compleme	ENSRNOG	-2.54123	-1.34553	0.005674	182.8261	113.8483	130.6453	161.6693	43.86975	57.63341	48.79346	104.0451
Pink1	5	compleme	ENSRNOG	-2.55978	-1.35602	0.017212	267.1002	96.94715	107.7889	279.8486	52.94625	71.14555	119.4598	80.99323
Uqcrfs1	17	35677984.	ENSRNOG	-2.60834	-1.38313	0.032477	446.4438	158.6835	255.8362	301.83	72.612	96.79103	118.6186	203.4176
Sdhb	5	15948437(	ENSRNOG	-2.63617	-1.39844	0.006197	153.2256	66.19636	92.20494	169.4691	35.7009	41.63945	64.77752	59.18736
Atp5f1a	18	74156553.	ENSRNOG	-2.65518	-1.40881	0.037834	1239.387	576.7534	556.6062	862.9453	243.2502	283.2034	355.8557	460.1038
Cisd1	20	18493538.	ENSRNOG	-2.68938	-1.42727	0.010273	190.1391	74.41222	84.15324	250.3038	52.34115	68.11221	50.47599	79.12415
Acadl	9	compleme	ENSRNOG	-2.6901	-1.42766	0.003564	116.6604	62.67528	103.3734	143.4697	32.37285	40.53642	50.47599	50.77652
Ndufs3	3	compleme	ENSRNOG	-2.69044	-1.42784	0.012695	128.8488	36.61926	45.97261	85.32546	23.29635	28.67882	30.28559	40.80813
Cox5a	8	62298358.	ENSRNOG	-2.75257	-1.46078	0.019675	572.5067	216.664	295.0558	548.352	119.5072	149.185	154.793	235.1919
Timm10	3	72226613.	ENSRNOG	-2.77632	-1.47317	0.008683	23.33209	12.44116	15.06447	22.69043	5.74845	7.721222	1.682533	12.4605
Myoc	13	80517536.	ENSRNOG	-2.82797	-1.49977	0.044662	76.26458	220.8893	182.5918	154.3422	15.1275	30.60913	73.19018	116.8172
Acadm	2	compleme	ENSRNOG	-2.92111	-1.54652	0.007642	318.6397	148.355	174.2803	370.8467	69.28395	84.65768	105.9996	123.0474
Got2	19	9587653..(	ENSRNOG	-2.95988	-1.56554	0.005707	207.8993	73.00379	127.0091	264.958	60.20745	63.14857	42.06332	88.46952
Coq7	1	compleme	ENSRNOG	-3.0568	-1.61202	0.002852	16.71552	13.14538	14.545	36.63559	6.95865	6.893948	7.571398	8.099323
Ndufa9	4	compleme	ENSRNOG	-3.14734	-1.65413	0.000745	72.0857	32.15922	45.19341	91.9435	15.7326	19.85457	23.55546	26.16704
Nipsnap2	12	compleme	ENSRNOG	-3.2548	-1.70257	0.000938	186.3085	64.78793	79.21833	178.9235	35.39835	51.56673	26.92053	61.99097
Ndufaf6	5	compleme	ENSRNOG	-3.27833	-1.71296	0.002176	29.25217	36.854	14.02554	38.05374	4.2357	9.651527	6.730132	18.69074
Coq9	19	compleme	ENSRNOG	-3.74376	-1.90449	0.001908	76.96106	20.18754	21.55778	74.68932	7.56375	16.82123	10.93646	23.05192
Vdac1	10	37724915.	ENSRNOG	-4.15885	-2.05619	0.000965	597.2318	136.618	274.537	587.5875	85.62164	97.89406	81.60285	165.7246
Slc25a4	16	49266903.	ENSRNOG	-5.48302	-2.45497	0.00021	4982.271	961.9599	1757.608	6103.489	763.3336	675.0554	691.521	699.3454

Supplement Table 4

Cellular Compartment- Extracellular Matrix. DEGs for rat healthy vs scar patellar tendon.

Name	Chromosome	Region	Identifier	S vs. A - Fold change	S vs. A - Log fold change	S vs. A - FDR p-value	1A_S37 (GE) - TPM	2A_S38 (GE) - TPM	3A_S39 (GE) - TPM	4A_S40 (GE) - TPM	5S_S41 (GE) - TPM	6S_S42 (GE) - TPM	7S_S43 (GE) - TPM	8S_S44 (GE) - TPM
Cthrc1	7	77966722.	ENSRNOG	24.42473	4.610271	6.72E-13	25.42153	37.79296	16.36313	11.10886	1239.245	949.7103	84.96791	169.4628
Col11a1	2	21686342(	ENSRNOG	8.046479	3.008358	2.37E-05	227.0525	226.0535	68.56931	174.9054	3299.005	2264.8	356.697	368.8307
Tnn	13	compleme	ENSRNOG	7.330561	2.873924	0.024112	4.178881	0.938955	1.558393	9.927062	96.51344	41.63945	1.682533	0.623025
Thbs1	3	10986211	ENSRNOG	5.16428	2.368567	1.84E-05	4.178881	5.398994	5.973841	9.217986	69.88905	40.81217	15.98406	13.39503
Eln	12	24978483.	ENSRNOG	5.024433	2.328961	3E-06	18.80497	11.73694	8.571164	22.92679	67.1661	167.1093	25.23799	97.19187
Thbs2	1	compleme	ENSRNOG	4.167147	2.05906	0.000344	76.26458	119.2473	58.95922	72.79846	755.1648	485.8855	84.96791	172.5779
Col1a1	10	82745801.	ENSRNOG	4.138058	2.048954	0.002176	7839.233	14511.79	5923.194	4911.296	68951.44	48485.41	9915.167	22457.55
Col5a2	9	compleme	ENSRNOG	3.553762	1.829347	0.000836	60.59378	53.7552	61.03707	87.45269	458.9683	377.2368	77.39652	126.474
Lum	7	38819771.	ENSRNOG	3.427709	1.777245	0.026646	346.4989	876.9844	325.964	220.9953	3087.22	2364.348	210.3166	947.6207
Smoc2	1	56242346.	ENSRNOG	3.271625	1.710007	1.99E-05	29.60041	28.16866	32.72626	31.67206	158.2336	139.5335	53.84105	81.92776
Lox	18	compleme	ENSRNOG	3.24057	1.696248	0.010273	483.009	613.6074	223.6295	351.938	2821.279	1574.026	499.7123	1062.569
Col8a2	5	14430861:	ENSRNOG	2.943043	1.557309	0.00686	21.59089	34.74135	26.75242	48.21716	215.7181	109.2001	21.87293	77.8781
Col5a1	3	6430201..(	ENSRNOG	2.836814	1.504272	0.001901	10.4472	5.398994	14.02554	7.090759	23.5989	33.91823	35.33319	21.18284
Adamts15	8	compleme	ENSRNOG	2.674824	1.419444	0.011305	26.46625	13.14538	13.24634	4.490814	35.39835	52.66976	27.76179	51.08803
Loxl1	8	compleme	ENSRNOG	2.521235	1.334131	0.01897	14.62608	49.5299	33.76519	38.76281	121.3225	156.079	22.71419	78.18961
Nid1	17	compleme	ENSRNOG	2.247595	1.168382	0.025163	108.3027	105.163	127.5285	52.23526	264.7312	316.8459	180.8723	189.3995
Dmp1	14	compleme	ENSRNOG	-4.57109	-2.19254	0.004781	9.750723	19.71806	21.81751	6.381683	1.8153	1.654548	7.571398	3.426636
Hapln1	2	18354542.	ENSRNOG	-6.72365	-2.74924	0.00502	34.82401	4.460038	7.532235	77.28927	4.8408	5.515159	2.523799	8.099323
Ibsp	14	compleme	ENSRNOG	-9.25159	-3.2097	0.00028	110.7404	53.28572	17.66179	17.25418	2.4204	1.930305	9.253931	9.968397

Supplement Table 5

Cellular Compartment- Membrane Protein Complex. DEGs for rat healthy vs scar patellar tendon.														
Name	Chromosome	Region	Identifier	S vs. A - Fold change	S vs. A - Log fold change	S vs. A - FDR p-value	1A_S37 (GE) - TPM	2A_S38 (GE) - TPM	3A_S39 (GE) - TPM	4A_S40 (GE) - TPM	5S_S41 (GE) - TPM	6S_S42 (GE) - TPM	7S_S43 (GE) - TPM	8S_S44 (GE) - TPM
Cdh2	18	compleme	ENSRNOG	4.200434	2.070538	0.000236	20.89441	28.63814	15.84367	7.090759	188.7912	63.70008	41.22206	30.2167
Gja1	20	37876650.	ENSRNOG	4.003433	2.001238	0.000234	71.04098	117.8389	77.65994	120.5429	811.7416	564.2007	148.9042	180.3657
Itgbl1	15	11011414	ENSRNOG	2.704566	1.435397	0.013067	194.6662	301.17	188.3059	164.5056	1115.199	609.9765	392.0302	350.1399
RT1-Da	20	compleme	ENSRNOG	2.497651	1.320572	0.045337	39.69937	165.0214	198.1757	103.7614	321.6106	486.1612	120.3011	434.8713
Cdh11	19	2393059..	ENSRNOG	2.426031	1.278598	0.045405	28.55569	73.94274	54.8035	37.34466	234.4762	150.2881	34.49193	88.46952
Tmed3	8	compleme	ENSRNOG	1.98273	0.987488	0.048586	30.29689	48.82568	41.03769	18.19961	91.97519	86.31223	52.99979	61.05643
Dnajc15	15	compleme	ENSRNOG	-2.05332	-1.03796	0.04091	96.46251	77.69856	67.53038	75.16204	37.21365	36.6758	42.90459	51.39955
Dnajc19	2	12050413	ENSRNOG	-2.06335	-1.04499	0.030698	125.3664	146.2423	99.21771	82.96188	52.0386	44.67278	63.09499	77.25508
Mfn1	2	11892973	ENSRNOG	-2.08067	-1.05705	0.04368	52.58425	35.44557	44.93368	58.61694	24.50655	21.78488	33.65066	22.42889
Immp1l	3	95959703.	ENSRNOG	-2.1517	-1.10548	0.045298	62.33498	45.77408	36.88198	41.1264	24.50655	26.47276	12.619	29.59368
Ndufv1	1	compleme	ENSRNOG	-2.17242	-1.11931	0.045405	98.90019	40.37508	65.19279	66.88949	19.3632	23.71518	49.63472	44.54627
Ndufb7	19	24701049.	ENSRNOG	-2.17494	-1.12097	0.040643	104.8203	57.51102	67.79011	96.90703	34.4907	35.57277	47.95219	47.34989
Ndufa12	7	35125424.	ENSRNOG	-2.1794	-1.12393	0.04368	164.3693	94.36502	97.91905	151.5059	47.8029	54.60007	79.07905	74.45147
Sgcb	14	37113210.	ENSRNOG	-2.18029	-1.12452	0.022864	130.2418	152.1108	147.0084	204.4502	94.69814	85.76072	41.22206	93.76523
Uqcrc1	8	11767927	ENSRNOG	-2.18531	-1.12784	0.046731	181.4331	98.35558	105.9708	165.6874	43.5672	54.32431	84.96791	94.07675
Ndufs8	1	compleme	ENSRNOG	-2.21479	-1.14717	0.044954	69.64802	35.68031	37.66117	64.76226	20.5734	20.40609	30.28559	32.39729
Ndufs2	13	compleme	ENSRNOG	-2.31872	-1.21333	0.038431	270.5826	124.8811	118.6976	179.1598	55.6692	62.04553	92.53931	118.6862
Ndufs4	2	compleme	ENSRNOG	-2.36642	-1.2427	0.014038	175.1648	119.9516	119.4768	171.36	56.57685	62.87281	53.84105	98.43792
Chchd3	4	compleme	ENSRNOG	-2.39772	-1.26167	0.040643	342.6683	149.0592	176.3582	347.6835	97.11854	104.2365	119.4598	147.9684
Ndufb3	9	65478496.	ENSRNOG	-2.40816	-1.26793	0.040982	402.5656	244.3632	291.1598	419.5366	105.5899	138.4305	182.5548	189.7111
Sar1b	10	37215937.	ENSRNOG	-2.41495	-1.27199	0.017212	282.4227	119.4821	80.51699	203.5048	80.17575	89.34557	54.68232	94.38826
LOC100912599	1	31264755.	ENSRNOG	-2.42996	-1.28093	0.011617	212.7747	114.0831	130.6453	151.7422	49.31565	52.39401	86.65045	85.3544
Apoo	X	63520991.	ENSRNOG	-2.48416	-1.31276	0.02148	67.90682	42.253	25.45376	63.10775	24.50655	18.75154	19.34913	25.54402
LOC100362432	16	compleme	ENSRNOG	-2.51596	-1.33111	0.006291	138.5996	79.34174	67.53038	162.3784	46.89525	58.46068	41.22206	51.39955
Ndufb5	2	11913971	ENSRNOG	-2.57072	-1.36217	0.025273	569.3726	278.8698	319.2109	562.0608	156.1158	160.2154	195.1738	229.2731
Ndufb8	1	compleme	ENSRNOG	-2.57434	-1.3642	0.022256	379.9299	185.4437	197.916	293.0847	71.09925	103.1335	146.3804	129.9007
Uqcrfs1	17	35677984.	ENSRNOG	-2.60834	-1.38313	0.032477	446.4438	158.6835	255.8362	301.83	72.612	96.79103	118.6186	203.4176
Sdhb	5	15948437	ENSRNOG	-2.63617	-1.39844	0.006197	153.2256	66.19636	92.20494	169.4691	35.7009	41.63945	64.77752	59.18736
Atp5f1a	18	74156553.	ENSRNOG	-2.65518	-1.40881	0.037834	1239.387	576.7534	556.6062	862.9453	243.2502	283.2034	355.8557	460.1038
Cyc1	7	11740957	ENSRNOG	-2.66	-1.41143	0.044083	291.1287	93.42607	131.6842	288.3575	46.5927	67.56069	91.69805	130.2122
Ndufs3	3	compleme	ENSRNOG	-2.69044	-1.42784	0.012695	128.8488	36.61926	45.97261	85.32546	23.29635	28.67882	30.28559	40.80813
Cox5a	8	62298358.	ENSRNOG	-2.75257	-1.46078	0.019675	572.5067	216.664	295.0558	548.352	119.5072	149.185	154.793	235.1919
Ndufb9	7	98813040.	ENSRNOG	-2.78368	-1.47699	0.015018	369.4827	152.3455	188.0461	379.3556	73.51965	103.9607	127.0312	128.6546
Ndufab1	1	compleme	ENSRNOG	-2.9085	-1.54027	0.007813	323.8633	141.0781	177.9166	276.7759	47.50035	71.14555	95.90438	133.9503
Ndufs7	7	compleme	ENSRNOG	-2.92019	-1.54606	0.006549	130.9383	46.71303	37.14171	97.61611	16.3377	28.67882	33.65066	41.11964
Timm21	18	compleme	ENSRNOG	-2.93087	-1.55133	0.000663	56.06666	28.63814	39.73903	51.9989	17.5479	13.7879	15.98406	18.69074
Ndufs1	9	compleme	ENSRNOG	-3.00686	-1.58826	0.008622	35.52049	13.84959	18.96045	54.36248	10.58925	12.13335	7.571398	14.9526
Ndufa9	4	compleme	ENSRNOG	-3.14734	-1.65413	0.000745	72.0857	32.15922	45.19341	91.9435	15.7326	19.85457	23.55546	26.16704
Sgca	10	compleme	ENSRNOG	-3.39375	-1.76288	0.007047	28.20745	7.981121	10.12956	20.5632	3.0255	7.445464	2.523799	8.410835
Ndufv2	9	compleme	ENSRNOG	-3.49397	-1.80487	0.001634	363.2144	157.275	163.3716	283.1576	49.31565	54.04855	82.44411	119.3093
Pank1	1	compleme	ENSRNOG	-3.92628	-1.97316	0.012579	10.4472	2.582127	11.68795	35.92651	5.14335	5.515159	3.365066	3.115124
Vdac1	10	37724915.	ENSRNOG	-4.15885	-2.05619	0.000965	597.2318	136.618	274.537	587.5875	85.62164	97.89406	81.60285	165.7246
Atp5mc1	10	compleme	ENSRNOG	-5.10492	-2.35189	4.46E-06	266.7519	74.41222	72.98476	203.0321	27.2295	34.7455	28.60306	45.48081
Hjv	2	19865543	ENSRNOG	-5.72381	-2.51698	0.009034	546.3887	124.6463	156.3588	538.6613	1.51275	30.60913	150.5867	75.386
Chchd10	20	compleme	ENSRNOG	-6.54169	-2.70966	0.00062	510.52	59.62367	74.54315	403.2278	7.8663	28.12731	79.07905	63.54853
Kcnj11	1	compleme	ENSRNOG	-14.2959	-3.83753	1.56E-06	61.98674	5.164255	17.92152	82.72552	0	1.103032	8.412665	4.049661
Cox8b	1	compleme	ENSRNOG	-17.4765	-4.12735	1.28E-06	567.6314	46.24356	161.8132	422.1365	1.2102	4.412127	52.99979	15.26411
Cox6a2	1	compleme	ENSRNOG	-18.7857	-4.23157	2.69E-07	680.4612	38.73191	162.8521	741.457	2.4204	16.54548	52.15852	24.60948
Cav3	4	14438294	ENSRNOG	-25.1197	-4.65075	1.03E-09	33.08281	6.10321	12.72688	40.41732	0	1.930305	0.841266	1.24605
Itgb6	3	compleme	ENSRNOG	-35.5179	-5.15047	4.64E-09	27.85921	4.2253	12.20741	55.07156	0	0	3.365066	0.623025

Supplement Table 6

Cellular Compartment-12/50 DEG shared between membrane protein complex and mitochondrial membrane														
Name	Chromosome	Region	Identifier	S vs. A - Fold change	S vs. A - Log fold change	S vs. A - FDR p-value	1A_S37 (GE) - TPM	2A_S38 (GE) - TPM	3A_S39 (GE) - TPM	4A_S40 (GE) - TPM	5S_S41 (GE) - TPM	6S_S42 (GE) - TPM	7S_S43 (GE) - TPM	8S_S44 (GE) - TPM
Cdh2	18	compleme	ENSRNOG00000015602	4.200434	2.070538	0.000236	20.89441	28.63814	15.84367	7.090759	188.7912	63.70008	41.22206	30.2167
Gja1	20	37876650.	ENSRNOG00000000805	4.003433	2.001238	0.000234	71.04098	117.8389	77.65994	120.5429	811.7416	564.2007	148.9042	180.3657
Itgbl1	15	11011414	ENSRNOG00000004516	2.704566	1.435397	0.013067	194.6662	301.17	188.3059	164.5056	1115.199	609.9765	392.0302	350.1399
RT1-Da	20	compleme	ENSRNOG00000032844	2.497651	1.320572	0.045337	39.69937	165.0214	198.1757	103.7614	321.6106	486.1612	120.3011	434.8713
Cdh11	19	2393059..	ENSRNOG00000013481	2.426031	1.278598	0.045405	28.55569	73.94274	54.8035	37.34466	234.4762	150.2881	34.49193	88.46952
Tmed3	8	compleme	ENSRNOG00000013889	1.98273	0.987488	0.048586	30.29689	48.82568	41.03769	18.19961	91.97519	86.31223	52.99979	61.05643
Dnajc15	15	compleme	ENSRNOG00000009063	-2.05332	-1.03796	0.04091	96.46251	77.69856	67.53038	75.16204	37.21365	36.6758	42.90459	51.39955
Dnajc19	2	12050413	ENSRNOG00000049819	-2.06335	-1.04499	0.030698	125.3664	146.2423	99.21771	82.96188	52.0386	44.67278	63.09499	77.25508
Mfn1	2	11892973	ENSRNOG00000011057	-2.08067	-1.05705	0.04368	52.58425	35.44557	44.93368	58.61694	24.50655	21.78488	33.65066	22.42889
Immp1l	3	95959703.	ENSRNOG00000004829	-2.1517	-1.10548	0.045298	62.33498	45.77408	36.88198	41.1264	24.50655	26.47276	12.619	29.59368
Ndufv1	1	compleme	ENSRNOG00000018117	-2.17242	-1.11931	0.045405	98.90019	40.37508	65.19279	66.88949	19.3632	23.71518	49.63472	44.54627
Ndufb7	19	24701049.	ENSRNOG00000028717	-2.17494	-1.12097	0.040643	104.8203	57.51102	67.79011	96.90703	34.4907	35.57277	47.95219	47.34989
Ndufa12	7	35125424.	ENSRNOG00000007407	-2.1794	-1.12393	0.04368	164.3693	94.36502	97.91905	151.5059	47.8029	54.60007	79.07905	74.45147
Sgcb	14	37113210.	ENSRNOG00000002135	-2.18029	-1.12452	0.022864	130.2418	152.1108	147.0084	204.4502	94.69814	85.76072	41.22206	93.76523
Uqcrc1	8	11767927	ENSRNOG00000032134	-2.18531	-1.12784	0.046731	181.4331	98.35558	105.9708	165.6874	43.5672	54.32431	84.96791	94.07675
Ndufs8	1	compleme	ENSRNOG00000017446	-2.21479	-1.14717	0.044954	69.64802	35.68031	37.66117	64.76226	20.5734	20.40609	30.28559	32.39729
Ndufs2	13	compleme	ENSRNOG00000038372	-2.31872	-1.21333	0.038431	270.5826	124.8811	118.6976	179.1598	55.6692	62.04553	92.53931	118.6862
Ndufs4	2	compleme	ENSRNOG00000011383	-2.36642	-1.2427	0.014038	175.1648	119.9516	119.4768	171.36	56.57685	62.87281	53.84105	98.43792
Chchd3	4	compleme	ENSRNOG00000013211	-2.39772	-1.26167	0.040643	342.6683	149.0592	176.3582	347.6835	97.11854	104.2365	119.4598	147.9684
Ndufb3	9	65478496.	ENSRNOG00000011825	-2.40816	-1.26793	0.040982	402.5656	244.3632	291.1598	419.5366	105.5899	138.4305	182.5548	189.7111
Sar1b	10	37215937.	ENSRNOG00000004820	-2.41495	-1.27199	0.017212	282.4227	119.4821	80.51699	203.5048	80.17575	89.34557	54.68232	94.38826
LOC100912599	1	31264755.	ENSRNOG00000023387	-2.42996	-1.28093	0.011617	212.7747	114.0831	130.6453	151.7422	49.31565	52.39401	86.65045	85.3544
Apoo	X	63520991.	ENSRNOG00000046918	-2.48416	-1.31276	0.02148	67.90682	42.253	25.45376	63.10775	24.50655	18.75154	19.34913	25.54402
LOC100362432	16	compleme	ENSRNOG00000019811	-2.51596	-1.33111	0.006291	138.5996	79.34174	67.53038	162.3784	46.89525	58.46068	41.22206	51.39955
Ndufb5	2	11913971	ENSRNOG00000011949	-2.57072	-1.36217	0.025273	569.3726	278.8698	319.2109	562.0608	156.1158	160.2154	195.1738	229.2731
Ndufb8	1	compleme	ENSRNOG00000014078	-2.57434	-1.3642	0.022256	379.9299	185.4437	197.916	293.0847	71.09925	103.1335	146.3804	129.9007
Uqcrfs1	17	35677984.	ENSRNOG00000018281	-2.60834	-1.38313	0.032477	446.4438	158.6835	255.8362	301.83	72.612	96.79103	118.6186	203.4176
Sdhb	5	15948437	ENSRNOG00000007967	-2.63617	-1.39844	0.006197	153.2256	66.19636	92.20494	169.4691	35.7009	41.63945	64.77752	59.18736
Atp5f1a	18	74156553.	ENSRNOG00000017032	-2.65518	-1.40881	0.037834	1239.387	576.7534	556.6062	862.9453	243.2502	283.2034	355.8557	460.1038
Cyc1	7	11740957	ENSRNOG00000012457	-2.66	-1.41143	0.044083	291.1287	93.42607	131.6842	288.3575	46.5927	67.56069	91.69805	130.2122
Ndufs3	3	compleme	ENSRNOG00000009155	-2.69044	-1.42784	0.012695	128.8488	36.61926	45.97261	85.32546	23.29635	28.67882	30.28559	40.80813
Cox5a	8	62298358.	ENSRNOG00000018816	-2.75257	-1.46078	0.019675	572.5067	216.664	295.0558	548.352	119.5072	149.185	154.793	235.1919
Ndufb9	7	98813040.	ENSRNOG00000009364	-2.78368	-1.47699	0.015018	369.4827	152.3455	188.0461	379.3556	73.51965	103.9607	127.0312	128.6546
Ndufab1	1	compleme	ENSRNOG00000018129	-2.9085	-1.54027	0.007813	323.8633	141.0781	177.9166	276.7759	47.50035	71.14555	95.90438	133.9503
Ndufs7	7	compleme	ENSRNOG00000024568	-2.92019	-1.54606	0.006549	130.9383	46.71303	37.14171	97.61611	16.3377	28.67882	33.65066	41.11964
Timm21	18	compleme	ENSRNOG00000015142	-2.93087	-1.55133	0.000663	56.06666	28.63814	39.73903	51.9989	17.5479	13.7879	15.98406	18.69074
Ndufs1	9	compleme	ENSRNOG00000011849	-3.00686	-1.58826	0.008622	35.52049	13.84959	18.96045	54.36248	10.58925	12.13335	7.571398	14.9526
Ndufa9	4	compleme	ENSRNOG00000061684	-3.14734	-1.65413	0.000745	72.0857	32.15922	45.19341	91.9435	15.7326	19.85457	23.55546	26.16704
Sgca	10	compleme	ENSRNOG00000003998	-3.39375	-1.76288	0.007047	28.20745	7.981121	10.12956	20.5632	3.0255	7.445464	2.523799	8.410835
Ndufv2	9	compleme	ENSRNOG00000042503	-3.49397	-1.80487	0.001634	363.2144	157.275	163.3716	283.1576	49.31565	54.04855	82.44411	119.3093
Pank1	1	compleme	ENSRNOG00000018944	-3.92628	-1.97316	0.012579	10.4472	2.582127	11.68795	35.92651	5.14335	5.515159	3.365066	3.115124
Vdac1	10	37724915.	ENSRNOG00000006375	-4.15885	-2.05619	0.000965	597.2318	136.618	274.537	587.5875	85.62164	97.89406	81.60285	165.7246
Atp5mc1	10	compleme	ENSRNOG00000007235	-5.10492	-2.35189	4.46E-06	266.7519	74.41222	72.98476	203.0321	27.2295	34.7455	28.60306	45.48081
Hjv	2	19865543	ENSRNOG00000021200	-5.72381	-2.51698	0.009034	546.3887	124.6463	156.3588	538.6613	1.51275	30.60913	150.5867	75.386
Chchd10	20	compleme	ENSRNOG00000028356	-6.54169	-2.70966	0.00062	510.52	59.62367	74.54315	403.2278	7.8663	28.12731	79.07905	63.54853
Kcnj11	1	compleme	ENSRNOG00000021128	-14.2959	-3.83753	1.56E-06	61.98674	5.164255	17.92152	82.72552	0	1.103032	8.412665	4.049661
Cox8b	1	compleme	ENSRNOG00000014656	-17.4765	-4.12735	1.28E-06	567.6314	46.24356	161.8132	422.1365	1.2102	4.412127	52.99979	15.26411
Cox6a2	1	compleme	ENSRNOG00000019851	-18.7857	-4.23157	2.69E-07	680.4612	38.73191	162.8521	741.457	2.4204	16.54548	52.15852	24.60948
Cav3	4	14438294	ENSRNOG00000005798	-25.1197	-4.65075	1.03E-09	33.08281	6.10321	12.72688	40.41732	0	1.930305	0.841266	1.24605
Itgb6	3	compleme	ENSRNOG00000008346	-35.5179	-5.15047	4.64E-09	27.85921	4.2253	12.20741	55.07156	0	0	3.365066	0.623025

Supplement Table 7

Pathway Name	DE/ Detected Genes	DEG	Detected Genes	FDR p-value
Metabolic pathways	0.093023256	100	1075	0.000231
Pathways of neurodegeneration - multiple diseases	0.121546961	44	362	0.000226
Alzheimer disease	0.129251701	38	294	0.000158
Amyotrophic lateral sclerosis	0.13028169	37	284	0.000424
Huntington disease	0.150627615	36	239	0.000158
Prion disease	0.183856502	41	223	0.000226
Parkinson disease	0.193236715	40	207	0.000231
Thermogenesis	0.21761658	42	193	0.000197
Non-alcoholic fatty liver disease	0.230769231	30	130	0.000102
Calcium signaling pathway	0.120967742	15	124	0.023064
Adrenergic signaling in cardiomyocytes	0.18018018	20	111	0.000158
Oxidative phosphorylation	0.28440367	31	109	0.000197
Oxytocin signaling pathway	0.157407407	17	108	0.005439
Retrograde endocannabinoid signaling	0.2	19	95	0.000197
Carbon metabolism	0.206521739	19	92	0.000223
Glucagon signaling pathway	0.197368421	15	76	0.002793
Dilated cardiomyopathy	0.246376812	17	69	0.000158
Hypertrophic cardiomyopathy	0.303030303	20	66	0.000197
Protein digestion and absorption	0.1875	12	64	0.017772
Cardiac muscle contraction	0.365079365	23	63	0.000197
Arrhythmogenic right ventricular cardiomyopathy	0.272727273	15	55	0.000158
Biosynthesis of amino acids	0.236363636	13	55	0.001296
Fatty acid metabolism	0.2	10	50	0.027691
Citrate cycle (TCA cycle)	0.321428571	9	28	0.001821
Starch and sucrose metabolism	0.315789474	6	19	0.027691
2-Oxocarboxylic acid metabolism	0.375	6	16	0.012551

All pathways regualted (FDR <0.05) in rat scar vs healthy patellar tendon. Displayed in descending order of pathway size.

Chapter 4. Spatial Gene Expression in Adult Patellar Tendon

This chapter is currently under review at Matrix Biology Plus:

Steffen, D., Mienaltowski, M. J., & Baar, K. (2023). Spatial Gene Expression in Adult Patellar Tendon. *Matrix Biology Plus.*

Abstract

Tendons are dense connective tissues with relatively few cells which makes studying the molecular profile of the tissue challenging. There is not a consensus on the spatial location of various cell types within a tendon, nor the accompanying transcriptional profile. In the present study, we used spatial transcriptomics to determine the location and gene expression profile of cells within the adult rat patellar tendon. We found two populations of tendon fibroblasts, one located in the tendon midsubstance, while the other localized with red blood cells, pericytes, and immune cells to the tendon peripheral connective tissue. The top spatially expressed genes included multiple genes with known function in tendon: Col1a1, Dcn, Fmod, Sparc, and Comp. Further, a novel spatially regulated gene (AABR07000398.1) with no known function was identified. This dataset provides new spatial insights into gene expression in a healthy tendon.

Introduction

Tendons are dense connective tissues that contain a small population of cells that reside within an extracellular matrix primarily composed of collagen. Collagen molecules are secreted by these cells along the line of force (Canty *et al.*, 2006; Kapacee *et al.*, 2008). These molecules aggregate into fibrils, and fibrils aggregate into fibers. In the rat, fibers are the largest unit of collagen and tendon fibroblasts are located between fibers (Lee & Elliott, 2019). Less than 5% of the tendon volume is composed of cells (Moore & De Beaux, 1987*b*) which makes characterizing the molecular biology of this tissue challenging.

The most common cell type in tendon is thought to be tenocytes, a type of specialized fibroblast responsible for maintaining the structure of the extracellular matrix. Tenocytes are found in-between collagen fibers and have elongated projections that spread through the matrix. Tendon can also contain a small immune cell population that increase upon injury (Harvey *et al.*, 2019; De Micheli *et al.*, 2020; Noah *et al.*, 2020). Endothelial cells and pericytes, along with red blood cells, have been reported within tendon (Harvey *et al.*, 2019; de Micheli *et al.*, 2020) and are best characterized around blood vessels in the paratenon and outside the tendon mid-substance (Dyment *et al.*, 2013).

Few studies have attempted to characterize the transcriptome of the multiple cell populations found in a tendon. The best unbiased report on gene expression in tendon cell types have been generated using single cell-RNA sequencing (Harvey *et al.*, 2019; de Micheli *et al.*, 2020; Kendal *et al.*, 2020). These studies are limited by the lack of spatial resolution. Spatial transcriptomics has the capacity to address this gap by pairing histological and gene expression information. Here, we used spatial transcriptomics to provide the first thorough report of spatial gene expression in healthy, uninjured tendon.

Materials and Methods

Experimental Animals

All animal experiments were approved under UC Davis Institutional Animal Care and Use Committee (IACUC) protocol #22957. Male Sprague Dawley rats (3 months of age) were purchased from Charles River Laboratories (Wilmington, MA). Rats were housed under 12-hour light/dark cycle in pathogen-free conditions. Patellar tendons (PT) were dissected with patella bone and quadriceps muscle attached. Excess muscle was trimmed, the entire tissue rinsed in PBS, and placed in cryomold filled with optimal cutting temperature compound (OCT) (Tissue-Tek®, Finetek, USA). OCT embedded tendon was immediately frozen in isopentane-cooled liquid nitrogen and stored on dry ice until further storage at -80^{o}C.

Sample Preparation

Tissues were transferred to a Leica CM 3050S cryostat on dry ice and incubated to -20^{o}C at least 45 minutes prior to sectioning. Longitudinal sections (14 µM) were cut distal to proximal, starting at the anterior aspect of the patellar tendon. Excess OCT and tissue surrounding the PT was brushed away with cryostat brushes. PT sections were placed on top of spatial tissue optimization or gene expression slides (10× Genomics, Pleasanton, CA, USA) and adhered by touching the tissue to the back of the slide. The tissue optimization and gene expression slides contain oligonucleotides with poly(dT) designed for mRNA capture with additional oligonucleotide sequencing primer, spatial barcode, and UMI on the gene expression slides. Each 6.5 mm x 6.5 mm capture area has 5000 spots (55 µM diameter) with 100 µM center to center distance. Slides were stored in 50 mL tubes at -80^{o}C until analysis.

To determine the optimal duration of permeabilization for mRNA capture, tissue sections were methanol fixed, hematoxylin and eosin (H&E) stained, and then permeabilized with pepsin for varying amounts of time. cDNA was synthesized with fluorescent nucleotides, tendon tissue enzymatically removed, and the fluorescent cDNA footprint imaged using a Leica DMi8. The

strongest fluorescent signal was used to determine the optimal permeabilization time (21 minutes).

Library Preparation and Sequencing

Patellar tendon tissues on spatial gene expression slides were methanol fixed, H&E stained, then imaged under bright-field at 10X using a Lecia DMi8. Slides were placed in 50 mL tubes on ice and transported by bicycle within 10 minutes after imaging to the UC Davis Genome Center for permeabilization, library preparation, and sequencing. Library preparation was performed with 10X Visium Library Construction Kit (details). Libraries were paired end sequenced on a NovaSeq S4 200 and 87,840,443 reads (PF clusters) were generated.

Data Analysis

Space Ranger (version 1.3.1) was used to process raw FASTQC files and H&E stained brightfield images. Genome alignment was performed with STAR within the Space Ranger pipeline against a custom reference genome Rnor_6.0 (GCA_000001895.4). Space Ranger generated unfiltered feature-barcode matrix and spatial image files that were read into R (R version 4.3.1) for downstream analysis using Seurat (version 4.3.0) (Satija *et al.*, 2015; Butler *et al.*, 2018; Stuart *et al.*, 2019; Hao *et al.*, 2021). Individual spatial Seurat objects were created from each sample and with no filters applied for UMI counts, feature counts, or mitochondrial reads per spot. Samples were merged and spots with zero UMI counts were removed. Data were SCT transformed, and principal component analysis and UMAP performed with 50 reduction dimensions. Samples were integrated with Harmony (Korsunsky *et al.*, 2019). Differential expression analysis was performed using “FindAllMarkers” in Seurat on genes that were detected in at least 25% of the population and with a log-scale fold change threshold of 0.2. Cluster determination was performed using a shared nearest neighbor modularity at resolution 0.4. Cluster markers were determined considering upregulated genes only.

Cell type predictions were performed on the integrated Seurat object with analysis anchored against a mouse Achilles single cell reference (de Micheli *et al.*, 2020). Data transfer was performed using rat homologs identified by Ensembl. The top upregulated genes in each cell type are presented.

Identification of spatially variable features was performed individually on each sample. The "FindSpatiallyVariableFeatures" function in Seuat was applied to transformed data (SCT assay) and spatially variability calculated using the markvariogram method. The top expressed spatially variable genes were determined by multiplying the spatial metric (Moran's I) by the expression (Median Normalized Average Counts). Excel documents that contain the following information are available as supplemental files: Feature ID/ Name, Moran's I, P value, Adjusted p value, Feature Counts in Spots Under Tissue, Median Normalized Average Counts, Barcode, I Weighted Counts.

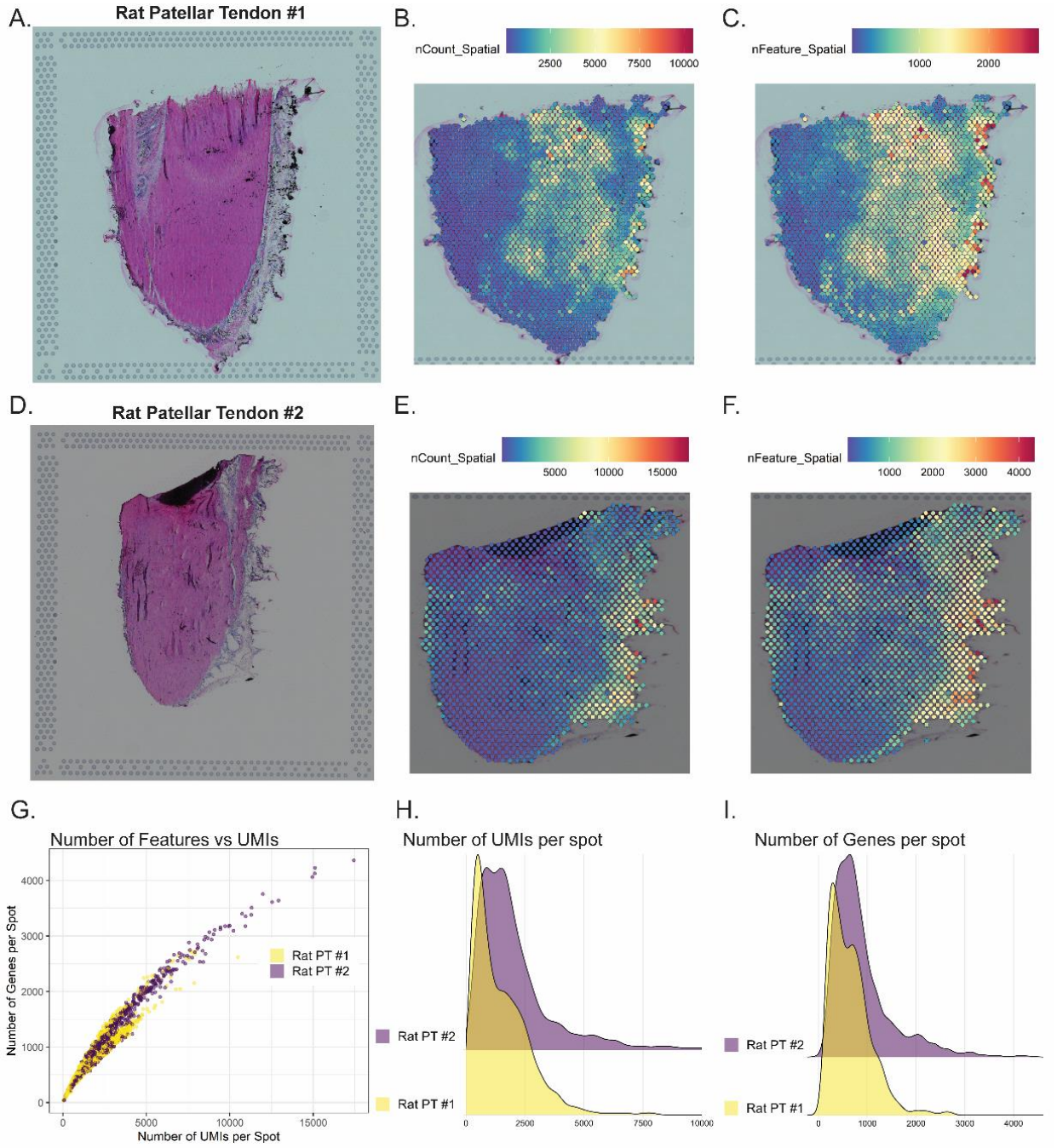


Figure 1. H&E Stains and QC Metrics. A&D) H&E stains of rat patellar tendon. B&E) Spatial projection of number of UMIs per spot. E&F) Spatial projection of number of genes per spot. G) Scatterplot of Number of genes per spot vs number of UMIs per spot. H) Ridge plot of Number of UMIs per spot for rat PT #1 and #2. I) Ridge plot of Number of Genes per spot for each sample.

Sample	Number of Spots Under Tissue	UMIs per Spot, Mean	Genes per Spot, Mean
Sample 1 (DS128_10LA)	2179	40,312	582
Sample 2 (DS129_1LA)	1624	64,438	716

Table 1. QC Metrics.

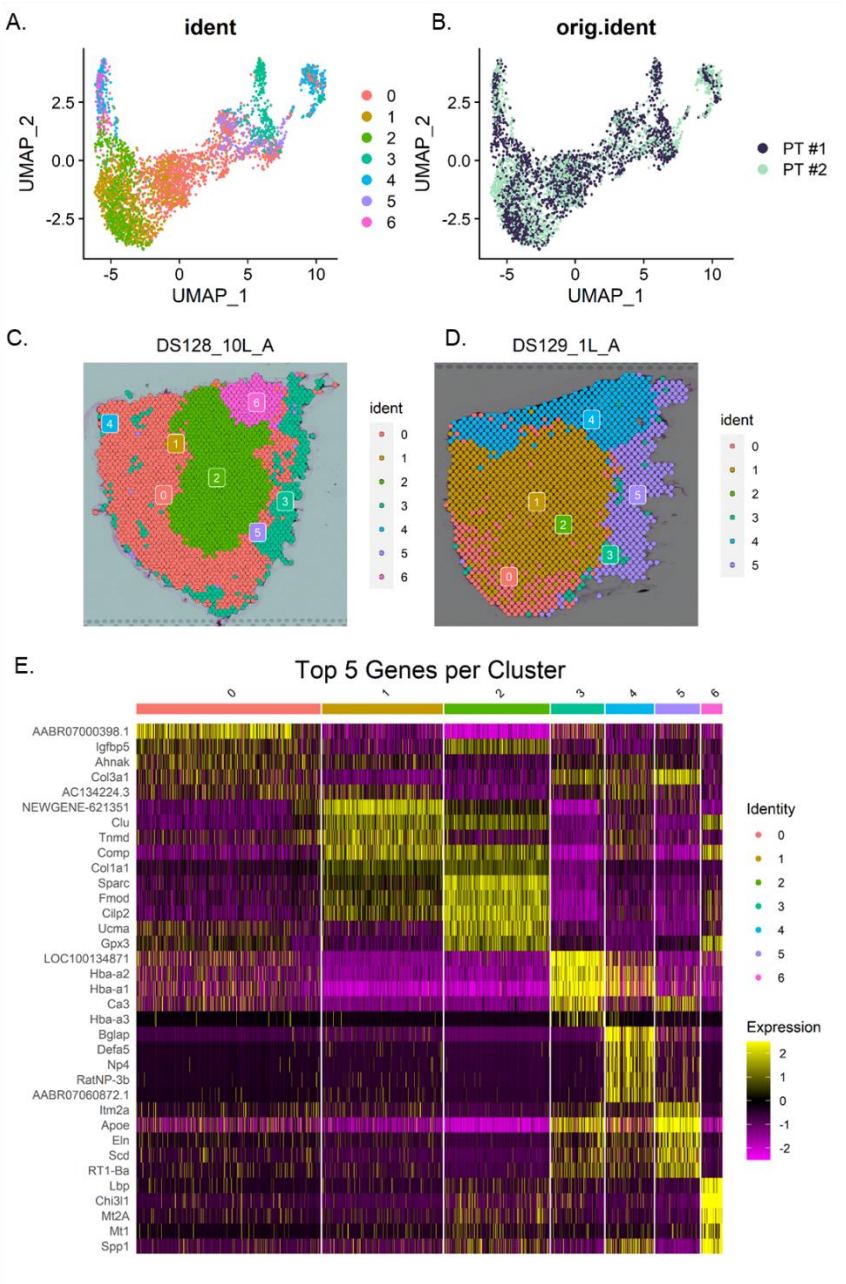


Figure 2. Dimensionality Reduction and Clustering. A) UMAP of integrated samples displaying 7 clusters identified in the merged and integrated PT #1 & PT #2 samples. B) UMAP of clustering indicating sample identity. C&D) Visualization of integrated clusters projected onto each sample identity. E) Heatmap of the top five upregulated (sorted by adjusted p value) genes per cluster. Expression is the $\log_2$ fold change of the cluster relative to all other clusters and is derived from the SCT normalized, scaled data. Note that NEWGENE-621315 is Col1a2.

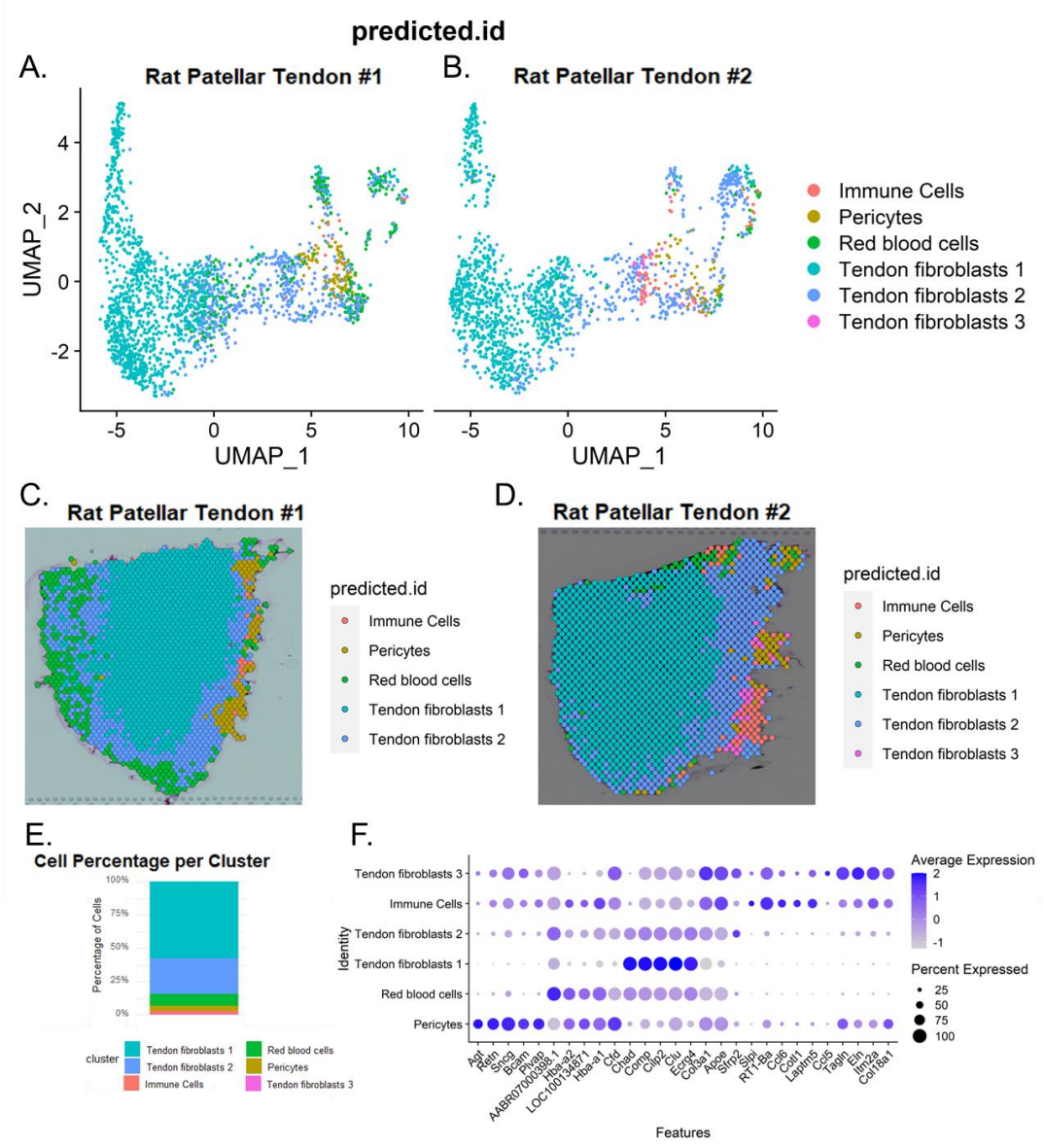


Figure 3. Cell Type Classifications. A&B) UMAP of predicted cell types in each tendon. C&D) Spatial projections of cell types. E) Bar graph of cell type proportion from combined samples. F) Dot plot of top 5 genes (ordered by adjusted p-value) expressed by each cell type. Average expression is derived from SCT normalized, scaled data and is the $\log_2$ fold change of the cluster relative to all other clusters. Percent expression is the proportion of spots in a cluster that express the gene.

	Immune Cells	Pericytes	Red Blood Cells	Tendon Fibroblasts 1	Tendon Fibroblasts 2	Tendon Fibroblasts 3
PT #1	14	98	294	1199	574	0
PT #2	61	58	43	985	449	28
Total (Counts)	75	156	337	2184	1023	28
Total (%)	1.97	4.10	8.86	57.43	26.90	0.74

Table 2. Cell type counts & percentages. Total counts is the number of spots assigned to particular cell type. Percentage is relative to total number of spots assigned to cell types.

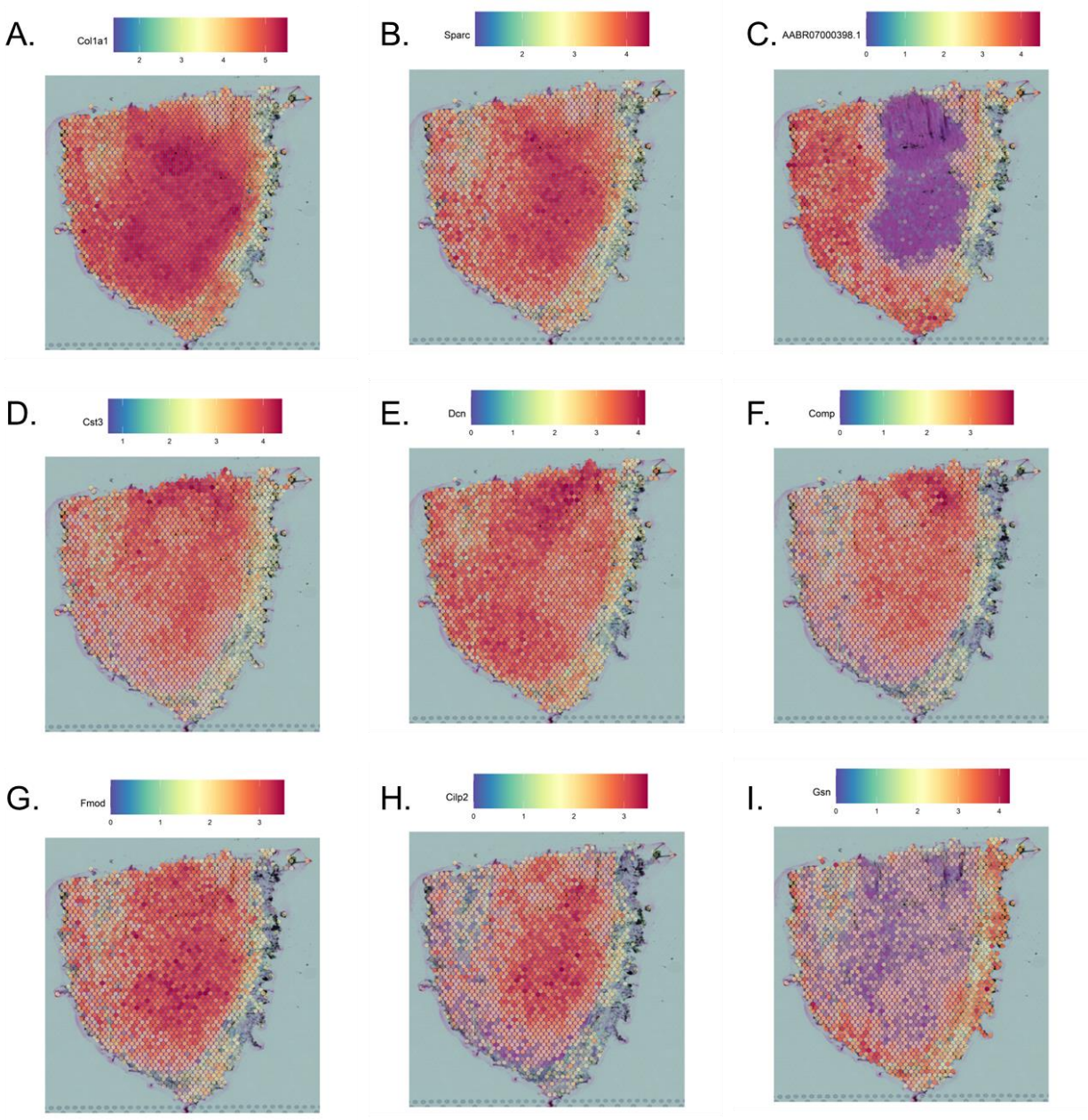


Figure 4. Top Expressed Spatially Variable Genes. The top expressed spatially variable genes listed in order of rank (Moran's I * mean normalized average counts) as represented in Patellar Tendon #1. A-I) Visualization of expression is displayed from SCT normalized data. A) Col1a1. B) Sparc. C) AABR07000398.1. D) Cst3. E) Dcn. F) Comp. G) Fmod. H) Cilp2. I) Gsn.

Feature ID	Feature Name	I	P value	Adjusted p value	Feature Counts in Spots Under	Median Normalized Average Counts	Barcodes Detected per Feature	I Weighted Counts
ENSRNOG00000003897	Col1a1	0.71527369	0	0	341200	119.342226	2179	85.3623541
ENSRNOG00000012840	Sparc	0.61957415	0	0	106461	34.1898364	2176	21.1831389
ENSRNOG00000047746	AABR07000398.1	0.88691529	0	0	22877	18.6978369	2003	16.5833974
ENSRNOG00000005195	Cst3	0.58877317	0	0	78775	25.7637236	2172	15.1689892
ENSRNOG00000004554	Dcn	0.55869705	0	0	63975	21.9593106	2170	12.268602
ENSRNOG00000048472	Comp	0.57837636	0	0	37768	11.8579461	2072	6.85835563
ENSRNOG00000003183	Fmod	0.52672321	0	0	34862	11.1207996	2070	5.85758327
ENSRNOG00000011292	NEWGENE_621351	0.44353899	0	0	29211	10.2482911	2115	4.54551668
ENSRNOG00000020622	Cilp2	0.58896235	0	0	26027	7.59677069	1879	4.47421195
ENSRNOG00000018991	Gsn	0.45189086	0	0	27830	9.66118374	2056	4.36580062

Table 3. Spatially variable genes in rat PT #1

Feature ID	Feature Name	I	P value	Adjusted p value	Feature Counts in Spots Under	Median Normalized Average Counts	Barcodes Detected per Feature	I Weighted Counts
ENSRNOG00000003897	Col1a1	0.77872775	0	0	313387	181.703927	1624	141.497889
ENSRNOG00000012840	Sparc	0.65726765	0	0	73798	41.072086	1624	26.9953534
ENSRNOG00000004554	Dcn	0.72746263	0	0	48188	30.7315186	1623	22.3560314
ENSRNOG00000005195	Cst3	0.69048372	0	0	51709	30.1193322	1622	20.7969087
ENSRNOG00000011292	NEWGENE_621351	0.67588844	0	0	47203	27.7045774	1622	18.7252034
ENSRNOG00000048472	Comp	0.72374572	0	0	35179	21.5583112	1612	15.6027356
ENSRNOG00000016460	Clu	0.69033478	0	0	25057	15.5229497	1612	10.7160321
ENSRNOG00000003183	Fmod	0.60170483	0	0	26427	15.2969393	1608	9.20424219
ENSRNOG00000003304	Chad	0.75767988	0	0	16459	11.0608636	1522	8.38059377
ENSRNOG00000047746	AABR07000398.1	0.63445968	0	0	22826	12.8344112	1546	8.1429164

Table 4. Spatially variable genes in rat PT #2

Results & Discussion

This is the first report of a complete spatial transcriptomics profile for tendon. To date, there has only been one other report of spatial gene expression in healthy tendon containing only the spatial expression pattern for four genes – no information was reported about the complete transcriptional profile or cell types present in tendon (Akbar *et al.*, 2021). The technique of spatial transcriptomics was pioneered in brain and cancer tissue and is rarely applied to very fibrous tissues such as tendon given that the method requires a permeabilization step to release mRNA onto a barcoded poly (T) capture slide (Ståhl *et al.*, 2016). Furthermore, the tissue sections must adhere onto the capture slide completely flat, and it can be challenging to obtain high quality longitudinal sections of tendon because it is relatively thin and collagen is notoriously difficult to section. Despite these challenges, we were able to obtain spatial transcriptomic information for two biological samples that were bioinformatically integrated to generate unbiased clusters and location of predicted cell types and identify the most spatially regulated genes.

The tissue morphology and QC metrics were similar for both samples. Each spot on the Visium slide contains a spatial barcode and every piece of RNA captured has a unique molecular identifier (UMI). The spots with the most UMIs (labeled in figure as nCount) have the most RNA. For each sample, the strip of collective tissue to the side of the tendon had the most RNA which is not surprising given the relatively hypocellular nature of the tendon mid-substance (Figure 1B,E,H). The average number of UMIs per spot was 40,312 for rat #1 and 64,438 for rat #2 (Table 1). The percentage of reads mapped to the genome was 93.8% and 83.7%, respectively, which is similar to mapping rates reported in other studies (Disser *et al.*, 2020, 2022). There was an excellent correlation ($R^2 = 0.97$) between the number of genes per spot and number of UMIs per spot (Figure 1G).

For cluster determination, the patellar tendon samples were merged and integrated together. We assumed that each patellar tendon should have similar cell populations; thus, integration should

increase the sample size and power of clustering. Clusters identified with the same number represent the same cell population in each sample. There were seven clusters identified by unsupervised clustering at a resolution of 0.4 (Figure 2A). Higher resolutions produced an increased number of clusters with a minimal number of spots that did not correspond to histologically distinct tissue areas. There appeared to be an equal contribution of each sample to each cluster on the integrated UMAP (Figure 2B). However, the spatial projections of the clusters revealed that only one cluster (cluster 0) contributed to a substantial number of spots in each tissue section (Figure 2C, D). This cluster was not characterized by robust expression of tendon-associated genes, but rather a general lack of strongly differentially expressed genes. Clusters 1 and 2, that morphologically correspond to the tendon mid-substance were characterized by expression of tendon-associated genes (*Col1a2*, *Tnmd*, *Comp*, *Col1a1*, *Sparc*, *Fmod*) and are likely tendon fibroblasts. The other clusters did not contain well-known markers for other cell types.

We proceeded to perform cell-type classifications by anchoring the data to a single-cell reference (de Micheli *et al.*, 2020). Interestingly, the single cell dataset identified 7 cell varieties in the mouse Achilles tendon, yet only 4 distinct cell types (immune cells, pericytes, red blood cells, and tendon fibroblasts), in our samples (Figure 3A, B). There were multiple populations of tendon fibroblasts that were distinguishable by spatial location and comprised 85% of the overall population (Table 2, Figure 3C-E). Tendon fibroblasts 1 comprised 57.4% of the cells and were located directly in the tendon mid-substance, whereas tendon fibroblasts 2 were less prevalent (26.9%) and located toward the periphery of the tendon (Figure 3C, D). UMAP visualization of the clusters showed a major cluster that was formed primarily of tendon fibroblasts 1 and 2 (Figure 3A, B). Tendon fibroblasts 1 were defined by expression of *Chad*, *Comp*, *Cilp2*, *Clu*, *Ecrg4*, *Mfge8*, *Fmod*, *Cst3*, *Cdn*, *Col1a1*, and *Sparc* (these genes all have equal p-values, only the first four are shown on the dot plot in Figure 3F). Tendon fibroblasts 2 were characterized by

expression of *Col3a1*, *Spo3*, *Strp2*, *Cfd*, and *AABR07000398.1*. Red blood cells also shared *Cfd* and *AABR07000398.1* as markers with tendon fibroblasts 2. Expression of hemoglobin genes *Hba-a1* and *Hba-2* were together expressed at higher levels in RBCs, but their expression was not restricted to RBCs (Figure 3F).

RBCs, immune cells, and pericytes were identified in the connective tissue located exterior to the tendon (Figure 3C, D). Despite the lack of endothelial cells identified, it is possible that vasculature still exists around the tendon since RBCs and pericytes were identified the peripheral connective tissue. One of the top markers in pericytes was *Bcam* which is generally expressed in vascular smooth muscle cells and pericytes (Muhl *et al.*, 2020) (Figure 3F). Immune cells made up only 2% of the total cell population and expressed secreted leukocyte peptidase inhibitor (*Slpi*) and *RT1-Ba*, a protein included in the MHC class II complex on antigen-presenting cells (Table 2, Figure 3 E,F).

To identify genes with spatially variable expression that are highly expressed in tendon, we ranked genes based on equally weighting the spatial variability score (Moran's I) and expression levels (Table 2 & 3). There were eight shared genes between the two samples: *Col1a1, Sparc, AABR07000398.1, Cst3, Dcn, Comp, Fmod, NEWGENE_621351 (Col1a2)* (Table 3, 4). The expression pattern for *Col1a2* could not be visualized in the dataset for unknown reasons. As expected, *Col1a1* had the highest expression levels and was robustly expressed throughout the tendon proper (Figure 4A). The small leucine rich proteoglycans *Fmod* and *Dcn* that regulate collagen fibrillogenesis and organization had similar expression patterns to *Col1a1* (Figure 4E, G). *Sparc* and *Cst3* also had expression similar to *Col1a1* (Figure 4B, D). *Sparc* is a matricellular protein that has previously been shown to be necessary to transduce the mechanical load signal for tendon growth (Wang *et al.*, 2021). *Sparc* and *Col1a1* expression suggests that in a healthy tendon load is passed predominantly through the middle of the tissue.

The fact that the two primary collagens in tendon were expressed in different locations by distinct tendon fibroblasts (Figure 2) is extremely interesting. Even in engineered tendons (Kapacee *et al.*, 2008), a small sub-population of tendon fibroblasts existed that oriented themselves perpendicular to the cells within the tendon proper. Our data suggest that the tendon fibroblast cell population 1 that form the central core of the tissue and express *Col1a1* are those cells that align with the line of force. In contrast, tendon fibroblast population 2 may align perpendicular to the line of force to form the sheaths that wrap around the core of the tissue. This could explain why *Col3a1* levels increase following injury when peritenon cells infiltrate the core of the tissue (Williams *et al.*, 1980). In support of this hypothesis, ligaments engineered from the tendon proper express higher levels of tendon genes (*Col1a1, Dcn, FMOD*) and lower CSPG4 (a perivascular marker) than constructs made from the peritenon (Pechanec *et al.*, 2020).

Interestingly, two genes named for their role in cartilage, cartilage oligomeric matrix protein (*Comp*) and cartilage intermediate layer protein (*Cilp2*), were also identified as top spatially variable genes in PT #1 (Figure 4F, H). They both had expression patterns similar to *Col1a1* (Figure 4A, F, H). *Comp* has been shown to be expressed in tendon (Smith *et al.*, 1997), while *Cilp2* is expressed in joint (articular and meniscal), but not growth plate cartilage (Bernardo *et al.*, 2011).

There were two genes, *AABR0700398.1* and *Gsn,* that had expression patterns opposite of *Col1a1* (Figure 4C, I). *AABR0700398.1* does not have a known function but was highly expressed in both PT samples (Figure 4C, Table 2 & 3). The gene *Gsn* encodes an actin binding protein that, because of its function, may play a role in cell shape and motility (Feldt *et al.*, 2019). It does not have a known function in tendon.

In conclusion, we report the first complete spatial transcriptomics dataset for tendon. In our analysis, the cell clusters determined by unsupervised clustering match tendon morphology but are not representative of obvious cell types. When anchored to a single cell reference, the

abundance and spatial location of the four major cell types identified in tendon match their expected location with tendon fibroblasts located throughout the tendon and immune cells, pericytes, and red blood cells located only in the tendon peripheral connective tissue. The gene expression visualization of the top spatially expressed genes included many genes that have a known function in tendon (*Col1a1*, *Sparc*, *Dcn*, *Fmod*, *Comp*) and their expression was throughout the tendon proper.

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Author Contributions

DS, MM, and KB conceived the study. DS & KB collected data. DS analyzed data with help from the UC Davis Bioinformatics Core. DS drafted the manuscript. DS, MM, and KB revised the manuscript and approved the final version.

Data Availability

The accession numbers for the RNA sequencing data will be made available upon publication.

Competing Interests

The authors declare no conflict of interest in relation to the data presented.

Chapter 5. Discussion

Motivation for Dissertation Studies

Movement is essential to the chronicles of human existence. First, movement in the form of hunting and gathering was essential to survival. In more recent decades, movement patterns have become increasingly demanding and repetitive in certain occupations and sports. The ability to withstand physical activity, without injury from overuse, is a balance of building tissue strength and allowing time to recover. It is unknown what tips the balance within a single person from positive adaptation towards overuse and injury. For most musculoskeletal tissues, the process to recovery is well defined. However, for overuse injuries in tendons, little is known about the optimal way to rehabilitate the injury. The original aim of this dissertation was to optimize an exercise protocol to treat tendinopathy. But instead, the work left me the question: what is optimal for a tendon?

The question “what is optimal for a tendon?” came at an interesting time in my graduate school career. I was studying plant root tip regeneration in developmental biology, a process with nothing obvious in common to the musculoskeletal system. However, we discussed an interesting paper in class (Efroni *et al.*, 2016) about a model for root regeneration that closely mimicked the embryonic sequence of root development. This concept about regeneration recapitulating development is a common theme throughout biology but that paper was my first introduction to the topic. Tendon researchers had already begun to study neonatal tendon regeneration in attempt to understand why the fetal tendons regenerate following injury, whereas adult tendons scar in response to the same injury (Dyment & Galloway, 2015; Howell *et al.*, 2017).

The regenerative tendon biology literature is sparse, and exclusively dedicated to studies conducted in fetal development. When I started my dissertation, conventional wisdom was that adult tendons could not regenerate. However, this changed shortly after starting graduate school. My mentor Keith Baar published a case study on professional basketball player that showed

regeneration within a damaged patellar tendon central core (Baar, 2019). This demonstrated what was previously thought impossible – regeneration of an aligned tendon collagen matrix in a previously tendinopathic patellar tendon. The question shifted from “Is tendon regeneration possible?” to “Is tendon regeneration a universal possibility? If so, what are the molecular mechanisms responsible for regeneration?”

To study adult tendon regeneration in a controlled scientific manor, we needed a model of tendinopathy and a basis of comparison for regeneration. Rats are a good model organism for tendon research because they have tendons large enough tendon to injure and analyze, without being so large that the animal husbandry costs are prohibitive, resulting in studies with few biological replicates. We decided to investigate the patellar tendon biopsy punch injury model as a candidate model of tendinopathy for largely two reasons. First, the model has been used previously in the literature and can produce a repeatable decrease in tendon strength (Lin *et al.*, 2006; Beason *et al.*, 2012). Secondly, the funding for my dissertation came from Clara Wu & Joe Tsai, the owners of the Brooklyn Nets, and many basketball players on their team (and ~75% of professional basketball players in general) suffered from patellar tendinopathy that limited performance. I chose to compare the process of tendon regeneration to post-natal tendon development. The departure from the classic fetal tendon regeneration comparison, to a post-natal tendon growth comparison, was based on the reasoning that walking rats and the rehabbing basketball player shared an exercise stimulus for tendon growth/ regeneration. I assumed that the molecular process of tendon regeneration in the basketball player elicited by rehabilitation exercises, would be more similar to tendon growth that occurs with early loading, than tendon growth that occurs without a strong mechanical load signal during the fetal period.

The other consideration prior to beginning these studies was the method(s) we should use to investigate the molecular mechanisms. Tendons are dense, hypocellular tissues, which makes studying the molecular profile of the tissue challenging. Given the dearth of knowledge around

tendon biology, I wanted an unbiased and broad molecular tool to characterize the changes that occur with development, injury, and exercise in a tendon. RNA sequencing seemed like an appropriate tool because it is an untargeted, global measure of gene expression. There are many variations of RNA sequencing and with the suggestion from my co-mentor Michael Mienaltowski, we decided on the method of spatial transcriptomics using the 10X Visium Platform such that we would capture morphological and transcriptional information at the same time in a single sample. This would allow us to define interesting regions in the tendon based on the histology, such as the scar tissue region, and compare gene expression to another region or sample. We also selected to use 3' Tag Seq, another RNA sequencing method, on adult and developmental samples because it is compatible with lower quality RNA and sequencing data obtained from spatial transcriptomics.

While applying for fellowships, I had the idea to use spatial transcriptomics to compare gene expression in a compressed tendon region versus a tensile tendon region. There was good evidence from Katherine Vogel's work that different types of loads (compressive versus tension) would elicit differences in collagen organization and proteoglycans in the adult tendon, and that there were potentially epigenetic differences between cells in the different regions that began *in utero* (Evanko & Vogel, 1990, 1993; Ehlers & Vogel, 1998; Perez-Castro & Vogel, 1999). I proposed this idea to Keith because there was value in characterizing the transcriptional profile underlying tendon cells in mechanically distinct niches. Tendon injures that occur in the central core of a tendon, such as the classic case of a central core patellar tendinopathy, are subjected to a compressive load during movement as the tendon undergoes an isovolumetric extension. This compression is potentially exaggerated with injury because of swelling related to the higher GAG and water content of the scar tissue. We decided that there was a better way to answer this basic question about how the type of loads alters gene expression in a tendon, and that was to model the process in engineered tendon constructs. The lab routinely makes engineered

ligament constructs from human ACL cells. These constructs would be a little different such that they would be made around a circular mold and form a rubber band that could be simultaneously loaded in tension and compression. We planned to strain these engineered tendons and measure changes in gene expression between the tensile and compressed region with qPCR.

Results / Future Directions of Dissertation Studies

The original plans for my dissertation were ambitious. We were planning to establish a rodent model of patellar tendinopathy, characterize post-natal tendon development, determine an optimal exercise protocol for tendinopathy by comparing the molecular signature of the tendon scar tissue (obtained from spatial transcriptomics) to that of tendon development, and understand how distinct types of mechanical load (tension and compression) altered gene expression in a tendon. Spatial transcriptomics was a new technique and had never been performed on tendon or any other dense connective tissue. There was little guidance on how to obtain tendon sections with high quality appearance and RNA. I began this process at the start of the COVID pandemic, without opportunity to visit another lab and learn how to section tendon. The protocol I developed for tendon preparation is described in detail in Chapter 4. It is worth noting that the most important steps in obtaining a high-quality tendon section is ensuring that the tendon is flat in the cryomold and to use a premium blade that can cut through a stiff tendon. Alternatively, there is now a protocol for spatial transcriptomics on formalin-fixed paraffin-embedded (FFPE) samples that was not available when we started data collection, but FFPE tendons would likely be much easier to section.

While troubleshooting the spatial transcriptomics tissue preparation protocol, we proceeded with experiments investigating a patellar tendinopathy model and tendon development. We first compared the transcriptional signature of the patellar tendon fourteen days after a biopsy punch to human tendinopathy. The transcriptional signatures were similar: increased extracellular matrix gene expression, decreased mitochondrial gene expression, and no inflammatory

signature (Jelinsky *et al.*, 2011; Steffen *et al.*, 2022). There was also a visible increase in cellularity and vascularity that mimicked the human condition. One question that remains from these experiments is: where do the cells located within the injury originate? This question could be answered using lineage tracing methods to label the most important cell types implicated in repair (tenocytes, immune cells, pericytes) with different genetic markers and tracing the fate of labeled cells and their progeny over time. However, given the cellular complexity of the intrafascicular matrix, and the lack of fascicles in the rat, this type of experiment is best conducted in a larger organism like a horse.

For the tendon developmental studies, we chose to do timepoints that corresponded to before walking (P7), shortly after onset of walking (P14), and at motor maturity (P28). These were chosen to understand the transcriptional changes that occurred with mechanical loading. In my first attempt at coding, I ran a few simple gene ontology analyses on the RNA-seq data and identified genes that were highest expressed in each tendon. The transcriptional changes were not surprising: increased ECM gene expression but decreased cell cycle and mitochondrial gene expression. From this dataset, I assumed that the regenerating adult tendon should undergo the same processes. During my qualifying exam and at the Orthopedic Research Society conference, my assumption about tendon development as the gold standard for regeneration was challenged, and both individuals suggested that it should be tested. There is minimal data on post-natal tendon regeneration, aside from a neonate tendon transection model. The transected neonate tendon heals with recruitment of scleraxis positive cells. However, it is not known how a smaller injury would affect healing, nor has the molecular mechanism of regeneration been determined. To test this hypothesis more thoroughly, the patellar tendon would be injured at day 7 with a biopsy punch and allowed to regenerate until day 28 when mechanics and histology would be compared with native patellar tendons. Next, a comparison(s) between the molecular profile of the regenerating neo-natal patellar tendon (day 14 and 28)

should be compared with our dataset on early-post natal tendon growth. This still would not address the question of whether a healing adult tendon is more similar to tendon regeneration or tendon development but would allow us to definitively determine whether a neonatal tendon regenerates and whether it does so by returning gene expression at day 14 towards that of day 7 in the native tissue.

Another interesting aspect of the developmental tendon studies were the processes that were unique to Achilles and patellar tendon growth. From P7 to P28, the Achilles tendon grew longitudinally while the patellar tendon grew radially. We took the top 200 genes expressed in the patellar tendon at P28, because these we argued were the genes contributing to radial growth and probed them in the Enrichr database. This analysis allowed us to identify downregulation of JAK/STAT signaling as the pathway that best matched the genes expressed in the radially growing patellar tendon. Using the engineered human ligaments, we showed that inhibiting JAK/STAT signaling resulted in more collagen (total and % dry weight) and better mechanics (MTL and UTS) (Steffen *et al.*, 2023).

The identification of JAK/STAT inhibition promoting patellar tendon growth is interesting and there are several things worth investigating in the future. I hypothesized that inhibition of JAK/STAT signaling improved collagen content and mechanics by shifting developmental tendon cells towards a mature phenotype (more ECM gene expression, like what we saw from P7 to P28). If this is the case, it is important to know how the cell populations in the engineered ligament constructs compare to that of an adult tendon. It is possible that the adult tendon contains more mature tendon cells and that inhibition of JAK/STAT signaling would not result in increased ECM gene expression. However, in the context of a tendon injury, pharmaceutical manipulation of JAK/STAT may be beneficial if the scar tissue contains cells that have not fully differentiated down the tendon phenotype.

The last data chapter of my dissertation presents a small fraction of the spatial transcriptomics data that we collected. There were 32 uninjured patellar tendon sections, plus 24 injured and exercised samples (not presented). The preliminary analysis by the UC Davis Bioinformatics core showed that many of the sections were composed almost entirely of red blood cells. In my own analysis, I noticed that samples that were sequenced on different machines did not integrate by bioinformatics and on a UMAP formed separate clusters. I do not know how to explain the poor results. It is possible that the tissue permeabilization did not work because sections were not adhered completely flat to the Visium gene expression slide or the tissue permeabilization time was inappropriate. I tested the permeabilization time on both healthy and injured tissue and had reasonable cDNA footprints. However, some injured samples had minimal detectable gene expression within the injury cluster which suggests the scar tissue was over permeabilized. The one interesting thing from the injury time course was the observation that previously injured tendons which looked histologically normal, still had distinct clusters from the healthy tendon.

I chose to focus my analysis of the spatial transcriptomics data on two healthy patellar tendon samples to determine basic information about spatial gene expression in tendon. I confirmed the location of 4 cell types that had been previously identified with single cell RNA-seq: tendon fibroblasts (with two prominent sub-populations), immune cells, pericytes, and red blood cells. The interesting part about the cell classification was the spatial location of the tendon fibroblast sub-populations. One subpopulation was located primarily in the mid-substance and the other was located around the tendon periphery. The other interesting part of the analysis was the visualization of the spatially expressed genes. There were many genes with a high spatial rank (Moran's I), but I decided to equally consider the expression level of a gene (mean normalized average counts) in addition to the spatial variability into order to gather a list of more highly expressed spatial genes. I was surprised that many of top expressed spatially variable genes were genes with a known function in tendon (*Col1a1, Sparc, Dcn, Comp, Fmod, Col1a2*). There

were two other genes identified in the top 10 (*AABR07000398.1, Cst3*) for both samples. The visualization of *AABR07000398.1* was most intriguing to me because it was expressed in an opposite pattern to most of the tendon proper genes, with highest expression around the tendon periphery and nearly no expression in the mid-substance. Hypothesizing that this gene was potentially a negative regulator of collagen expression, I began experiments to characterize its function. I designed primers for the gene and attempted to amplify it but was unsuccessful in obtaining a product. I still think that it would be interesting to clone this cDNA into a plasmid and express it in human ACL cells prior to making constructs to determine whether *AABR07000398.1* regulates collagen transcription.

The first and last parts of my dissertation investigated a loading protocol to treat tendinopathy. In a pilot study that was performed in tandem with the patellar tendinopathy model development experiments, I looked at changes in gene expression from a single isometric (4 x 30 s) or dynamic (360 x 0.33) load. The rationale was to compare two protocols that had the same time under tension but differed in the dynamics of the load. We know that continuing to train on an injured tendon can exacerbate a tendon injury, but doing isometric rehab exercises, like the professional basketball player described in the Baar case study, can heal the tendon. The difference between typical training and rehab is that most movements are performed quickly during training and slowly during rehab. We had hypothesized that stress relaxation experienced by a tendon during an isometric was exercise routine would be sufficient to simulate repair because tendon cells within the injured scar tissue would receive a tensile load signal that was necessary for repair. In contrast, during a dynamic movement the injury would be stress shielded, and compressed because it was a core injury, and potentially form fibrocartilage. This hypothesis was supported by results that showed an increase in *Col1a1* and *Scx* expression with an isometric load and *Col2a1* expression with a dynamic load.

The notion that tendon cells respond to a tensile load to synthesize collagen was also supported by unpublished engineered tendon experiments. The tendon constructs that were stretched in tension (2.5% or 5% strain) increased tendon-associated gene expression and decreased in *Col2a1* expression. This decrease in *Col2a1* expression is also something that we observed in the rodent injury + isometric experiment, although it was non-significant *in vivo*, likely due to the time point of collection. Interestingly, I observed an increase in *Col2a1* expression with compression at higher strains (10%). Unfortunately, due to contamination issues and engineering issues I did not get a complete dataset to publish. These experiments are being continued by another graduate student and his results are consistent with mine. These engineered tendon experiments support that isometric loads promote tendon regeneration by directing a tensile load and decreasing compressive load through the scar tissue.

The experiments for the original goal of my dissertation – optimizing an exercise protocol to treat tendinopathy – were the last experiments conducted. The factors I chose to optimize were joint angle, contraction number, contraction duration, and frequency. These factors were put into a Box Behnken model to generate an incomplete list of protocols to experimentally test. From those results, the model would be able to predict the outcomes of the protocols not tested. I originally wanted to compare transcriptomes from each exercise protocol to the developmental transcriptomes using Sourmash (Pierce *et al.*, 2019). The program would plot the relationships between the samples in a phylogenetic tree diagram, and the optimal exercise protocol would be the one closest related to the developmental transcriptome. However, because of an overwhelming amount of spatial transcriptomics sequencing data and lack of bioinformatic support, we chose to perform qPCR and build a Z-score based on changes in gene expression that we determined to be beneficial for tendon repair. The preliminary results from the model suggest that at a low force, a bent knee (45°) is best. The opposite is true at a high force; a straight knee had the best results. The validation of this experiment is still ongoing.

Future Areas of Study

After extensively studying tendon biology and tendinopathy throughout my dissertation, there are few areas of study which have not been discussed yet that are intriguing to me. Some of these topics already are experiments in progress or planned for the future. Others I have just thought about.

1. What are the consequences of tendon immobilization? Following three days of ankle joint immobilization in the rat, there was a 10% loss in gastrocnemius muscle mass and a 20% loss of tendon collagen. Collagen synthesis was also reduced by 55% in the muscle. There also appeared to be disruption to the tendon crimping on SHG images. However, SHG signal is generated by the polarization of light by collagen fibers and not all the tendon samples were placed at the same orientation on the slide. After this pilot, I do not think we cannot conclude that differences in SHG signal were due to sample orientation or immobilization. As follow up experiments. It would be interesting to compare the single leg immobilization to a fully unloaded (i.e., tail hang) rat to understand the role of loading versus mobility.

2. How does immobilization following tendon injury affect regeneration? Given that it is common practice for athletes with a tendon injury to wear a boot for a few days after an acute injury (i.e., biopsy punch equivalent to ~1/4 tendon width). It is possible that overloading the tendon in the early rehab is detrimental to repair. I think the interesting comparisons would be: A) injury + unloaded; B) injury + unloaded with optimal loading (10 min every 6 hours); C) injury + single leg immobilization; D) injury + single leg immobilization with optimal loading (10 min every 6 hours); and E) injury + cage activity. I hypothesize that optimal repair would occur with immobilization and optimal loading.

3. How is collagen synthesis, incorporation, and degradation in a tendon regulated? Laurent and colleagues were the first to show that nearly 50% of newly synthesized collagen is

degraded before it is incorporated into a tissue. They demonstrated that with overload, there was a five-fold increase in collagen synthesis and a two and half fold decrease in collagen degradation before incorporation (McAnulty & Laurent, 1987). Given that there is more collagen made than incorporated, and that all these processes are regulated, it would be interesting to identify other factors that can manipulate the amount of collagen synthesized, degraded, and incorporated into a tendon. Energy balance is the one factor that is most interesting. Collagen 1a2 expression drops massively with starvation (in this study the animals lost 20% of body weight over seven days while on an unpalatable ketogenic diet) (Nakao *et al.*, 2019). However, it is possible that with energy reduction there is a pressure to maintain the same amount of collagen incorporated into the tissue and as a result, there is less collagen degraded before incorporation. I suggest this because athletes with low energy availability have low bone mass and lower markers of collagen synthesis. Further, these athletes tend to have one successful competitive season and then get injured and are unable to compete the following year, suggesting poor collagen turnover.

4. Finally, how are collagen synthesis and fibril dynamics regulated in a circadian manner and does this contribute to tendinopathy? In healthy tendon, there is time-dependent expression of circadian clock genes, and the secretion of collagen is under circadian control (Chang *et al.*, 2020; Yeung *et al.*, 2023). In tendinopathy, much of this rhythmic expression is lost. It is also not known what entrains the circadian clock but given that mean fibril diameter is larger in the evening, it is possible that exercise throughout the day has a strong role (Yeung *et al.*, 2023). It would be interesting to know how fibril dynamics compare in habitual morning versus evening exercisers. If the fibril dynamics are the same, then intrinsic regulators of the circadian clock may have a stronger influence. In the context of tendinopathy, the time of day to perform rehab is an important

consideration for tendon repair. I hypothesize that rehab in the morning, to serve as a stimulus for fibril growth throughout the day, would result in a quicker restoration of tendon structure than rebab performed in the evening.

Conclusions

In conclusion, this dissertation developed a rodent model of tendinopathy, characterized Achilles and patellar tendon post-natal development, and demonstrated that tensile loads increase tendon associated gene expression.

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