THE ROLE OF GLYCOSAMINOGLYCANS IN FATIGUE INJURED TENDON

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This dissertation reviews how tendon overuse injuries disrupt the extracellular matrix (ECM) and its interactions with cells at multiple scales, influencing repair after injury and suggesting that therapeutics for tendinopathy should be assessed with consideration of their effects on the ECM and its role in mechanotransduction. Utilizing our fatigue loading model of early-onset, subrupture tendinopathy, we characterized bulk and location specific increases in glycosaminoglycans (GAGs), including increased decorin-associated dermatan sulfate in the midsubstance ECM and increased chondroitin sulfate and hyaluronic acid in the pericellular matrix after fatigue injury. We hypothesized that the increase in GAGs with fatigue injury is a key contributor to tendon mechanical properties, mechanotransduction, and repair in response to exercise. When we removed the increased GAGs from fatigue injured tendons by *ex vivo* enzymatic treatment, we observed increased microscale strain, reduced dynamic modulus, and increased loss tangent relative to naïve control tendons. When we continuously reduced GAGs *in vivo* after fatigue injury, we observed an increase in tenomodulin, decreased loss tangent in the toe region, and increased loss tangent in the linear region, consistent with *ex vivo* GAG removal. These findings demonstrate a role for post-injury GAGs in directly and indirectly modulating multiscale mechanics and viscoelasticity as well as limiting tenogenic phenotype. Clinically, GAGs could serve as a diagnostic or therapeutic target to modulate mechanotransduction and enable reparative exercise after fatigue injury and tendinopathy.

BIOGRAPHICAL SKETCH

Patrick Muljadi was born in 1993 and grew up in Golden, CO. He attended the University of Colorado Boulder, earning a B.S. degree in Mechanical Engineering in 2015. In 2016, he began his Ph.D. degree at Cornell University in the Field of Biomedical Engineering under the guidance of Prof. Nelly Andarwais-Puri, specializing in the study of overuse tendon injury, biomechanics, and repair.

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INTRODUCTION

Tendons play a crucial role in the human body, transmitting load from muscle to bone and enabling locomotion, joint positioning, and stability. Every motion we make from performing household chores, working most professions, or engaging in our favorite hobbies and sports is enabled by our tendons. Tendinopathies are common and debilitating conditions resulting from everyday wear and tear and overuse, leading to significant socioeconomic costs and diminishing quality of life. Many of the most common therapeutic strategies for tendinopathy are limited due to their focus on chronic and late-stage disease. Diagnosis and intervention only occur after significant loss of function, and pain management strategies such as NSAIDs and immobilization simply address symptoms and not the underlying disease. Surgical repair can address post-rupture cases but suffer from high retear rates due to persisting damage. None of these later stage options return tendons to their original function or reliably prevent further disease progression or reinjury. A better understanding of early-stage progression and pathogenesis of tendinopathy may improve strategies to prevent or repair damage accumulation leading to chronic disease, disability, and high rates of reinjury.

Background

The unique structure of tendons is what enables their function, consisting primarily of highly aligned type I collagen that forms densely packed fiber bundles capable of withstanding high tensile loads. In between fibers are glycosaminoglycans or GAGs, which are attached to proteins to form proteoglycans. These highly charged polysaccharides attract water, thereby regulating tissue swelling, fluid motion, and fibril interactions. Resident tendon cells or tenocytes secrete and maintain the extracellular matrix in response to mechanical and biochemical stimulation. This

hierarchical structure also enables tendon mechanotransduction, where the transfer of macroscale loads to microscale matrix deformations are sensed by tendon cells and regulate gene and protein expression. Optimized loads, such as exercise, can signal an anabolic response and adaptation to greater loading demands. In contrast, excessive load in the form of repetitive and strenuous activity can lead to matrix damage and cell injury. In the absence of treatment, this damage may accumulate and lead to severe and chronic tendinopathy. However, appropriate modified loading in the form of physical therapy can stimulate a tendon repair response and return tendon function.

To enable investigation of the progression from early-stage overuse to late-stage tendinopathy, we have established an *in vivo* fatigue loading model in rat patellar tendon. Compared with clinical observations in human patients, this model induces well-characterized, consistent amounts of sub-rupture damage while enabling measurements of cellular responses and remodeling or repair at earlier timepoints during disease progression. Fatigue loading induced damage results in an increase in apoptosis, decreased upregulation of remodeling genes, and impaired collagen organization and macroscale mechanical properties that do not recover with normal activity. These *in vivo* fatigue loading findings are consistent with clinical observations of tendon overuse, wherein any attempt of tendons to repair is outpaced by accumulation of further damage.

As a mechanosensitive tissue, mechanical stimulation in the form of exercise has been investigated and utilized to repair these fatigue damaged, unresponsive tendons. While exercise initiated shortly after fatigue injury exacerbates damage, delayed exercise can induce repair, reducing collagen disorganization and improving mechanical properties. The fact that macroscale mechanical properties and collagen content are persistent across these timepoints suggests that microscale, non-collagenous matrix changes that occur during the first few weeks after injury might influence mechanotransduction and ultimately enable repair in response to load. A review of the role of the

tendon extracellular matrix (ECM) in mechanotransduction and its disruption and repair following overuse injury is presented in **Chapter 1**.

Perhaps most notably, GAGs are significantly increased with clinical late-stage tendinopathy and is a widely accepted clinical marker for tendon pathology. However, an increase in GAGs has also been observed in fatigue injury models of early-onset tendinopathy. This raises the question: Are increased GAGs in fatigue injured tendons a manifestation of disease or part of an attempt to repair early-onset injury? The fact that GAGs are increased during early-onset injury and are also present during reparative exercise suggests they may be imperative to the capacity of the tendon to repair in the weeks following fatigue injury. Therefore, our overarching hypothesis is that **GAGs enable a reparative response to load in fatigue injured tendons by modulating mechanotransduction and cell behavior.**

Focus and scope

The most common GAG type in the highly aligned tendon midsubstance is dermatan sulfate. The proteoglycan decorin and its single dermatan sulfate chain are attached to collagen fibrils, potentially mediating fibril load transfer and sliding. In contrast, the soft to hard tissue interface at the tendon insertion to bone has higher amounts of chondroitin sulfate and aggrecan. The many chondroitin sulfate chains of aggrecan regulate fluid motion and cause swelling, allowing tissues to resist compression. The tendon pericellular matrix, or the matrix directly around the cells, is also structurally distinct, mediating mechanical and biochemical interactions between cells and the greater extracellular matrix. The pericellular matrix of tendinopathic tendons is rich in proteoglycans and GAGs, suggesting that GAGs contribute to cell-matrix interactions. Given the location specific concentration and function of GAGs in healthy tendons, our first aim was to

characterize in what locations different GAG types are increased during early-onset tendinopathy. We hypothesized that the increase in GAGs post fatigue injury is location specific to restore the location dependent micromechanical environment disrupted by injury. Correspondingly, we expected an increase in dermatan sulfate and associated decorin in the tensile midsubstance and an increase in hyaluronan and chondroitin sulfate and associated aggrecan in the compressive insertion and pericellular matrix of tendon cells after fatigue injury. These experiments and findings are presented in **Chapter 2**.

Given the potential location specific, multiscale mechanical roles of post-injury GAG increases, we were subsequently interested in how these GAGs might affect a tendon's response to physical activity or load. The unique combination of the collagen and GAGs in tendons results in their viscoelastic properties, meaning their mechanical behavior is dependent on rate of strain and is comprised of both viscous and elastic components. We were also interested in assessing microscale contributions of GAGs to shear strain, as the translation of bulk loads to microscale cell strains is an important component of mechanotransduction. When loaded in bulk tension, tendons and their resident cells experience both shear and compression at the tissue and cell scales due to microscale behaviors like fibril uncrimping and sliding. This microscale shear and compression is potentially facilitated by GAGs and may also drive viscoelastic behavior. Based on this knowledge, our second aim was to assess the role of GAGs in modulating viscoelasticity and microscale strain in fatigue injured tendons. We hypothesized that the endogenous increase in GAGs following fatigue injury increases viscoelasticity and modulates microscale shear strain, influencing mechanotransduction and enabling a reparative response to exercise and loading. Experiments and findings addressing this hypothesis are presented in **Chapter 2**.

Finally, we were interested in investigating the biological role of GAGs in modulating repair after fatigue injury. GAGs may influence biological signals that enable repair through CD44, a principal cell surface receptor for hyaluronan. Hyaluronan interactions with CD44 influence collagen and tenomodulin expression, key markers of tenogenic phenotype, in addition to mediating apoptosis. Therefore, our final aim was to assess the role of GAGs in modulating biological response to fatigue injury. We hypothesized that the multiscale and viscoelastic mechanical changes resulting from the endogenous increase in GAGs following fatigue injury limits apoptosis and shifts cell phenotype, enabling repair and modulating long-term mechanical properties. Experiments and findings addressing this hypothesis are presented in **Chapter 3**.

Review Article

The role of the tendon ECM in mechanotransduction: disruption and repair following overuse

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Overuse and Tendinopathy

Tendons play a critical role in locomotion and joint stability. Accordingly, tendon injuries lead to diminished quality of life, lost days in the workplace, and a significant healthcare burden[1]. There is particular occupational hazard in conditions of repetitive motion, such as military[2], athletes[3], and workplace-related labor[4]. Additionally, increased age, smoking, diabetes, and obesity are also associated with increased risk of tendinopathy[5,6]. Accordingly, tendon injuries are among the most common reasons for musculoskeletal consultations[7]. Despite the prevalence of tendinopathy, there are limited treatment options to halt disease progression. A major hurdle to the development of effective therapeutics is a poor understanding of disease mechanism. Later stage injuries often require surgical intervention with high rates of retear and limited recovery of function[8], highlighting the importance of treating injuries prior to irreversible disease progression. Poor surgical outcomes may be attributed to persistent damage at the surgical site, further highlighting the lasting role of overuse damage in tendinopathy at all stages of disease.

Mechanical signals drive the biological response of the tendon, enabling homoeostasis, adaptation, and repair in response to physical activity. As a result of the anisotropic and viscoelastic properties that give tendons their specialized function, the microscopic matrix environment undergoes tension, compression, shear, and fluid motion in response to macroscopic tensile loads. Cellular structures including cilia, focal adhesions, gap junctions, and cytoskeleton allow tendon cells to sense and convert these mechanical stimuli into cell signals. Sub-rupture damage that occurs with overuse compromises mechanotransduction at multiple scales, including the bulk mechanical function, local matrix environment, and cell structures that signal adaptation and repair[9]. Without sufficient repair, damage accumulates and eventually leads to rupture and disability[10].

The biological response to load is guided by a cell's deformation from a mechanical stimulus. These cellular deformations are mediated from the joint level by the extracellular matrix (ECM). This review will focus on the role of ECM on tendon homeostasis and repair: in particular, the upstream mechanical maintenance and downstream biochemical regulators of ECM composition and structure[11,12] (Fig. 1). As such, while the effects of load on mechanotransduction may only be evaluated at specific levels, understanding the upstream and downstream implications of multiscale transmission is key to describing the pathogenesis of tendinopathy and the effect of overuse damage (Fig. 2,3).

Whole Joint: Tendons transmit a wide range of mechanical forces dependent on their function and activity[13]. The rate of force development in human Achilles tendon increases 32% when increasing from slow to fast walking speed[14], with maximum forces as high as 12.5 times body weight during running[15]. Energy storing tendons, (ex. Achilles tendon), exhibit high levels of extensibility and recoil while positional tendons (ex. anterior tibial tendon) display stiffer, more time dependent behavior[16,17]. Human Achilles[18] and patellar[19] tendinopathy, common amongst athletes, is associated with increased tendon cross-sectional area, stiffness, and Young's modulus. Elbow, hand, and wrist tendinopathies are common work-related musculoskeletal disorders[20] and increase with amount of exposure to repetitive motion[21]. Swimmers undergo repetitive shoulder use during training, and have been found to have increased levels of rotator cuff tendinopathy[22]. Altered joint kinematics may cause subacromial impingement, and lead to abnormal compressive loads to the supraspinatus tendon, and further exacerbate tendinopathy with overuse[23,24]. Chronic downhill running of rats causes the supraspinatus tendon, which passes under the acromion, to exhibit signs of tendinopathy[25]. This protocol also induces degeneration to the whole joint[26], consistent with clinical studies that have found tendons adjacent to an osteoarthritic joint to have

scar tissue and degenerative appearance[27,28]. Further investigation is needed to evaluate whether this is caused by, or a contributing factor, to joint pathology. By altering function and mobility, joint-scale changes with tendon overuse likely affect downstream mechanical stimuli important for promoting repair.

Fascicle Matrix: Individual tendon fascicles consist of highly aligned and hierarchically organized type I collagen fibers, enabling transmission of tensile loads from the muscle to the bone. Helical structure of fascicles, especially in energy storing tendons[29], contribute to tendon recoil after unloading. Overuse injury disrupts the fascicle matrix at multiple scales, leading to structural damage that promotes loss in bulk mechanical properties. For instance, fatigue loading equine SDFT tendon fascicles increased hysteresis and decreased microstructural recovery, suggesting disruption of helical structure during overuse (Fig. 2a)[30]. Similarly, fatigue loading rat tail tendon fascicles decreased stiffness and maximum load and increased collagen damage area and fiber/fibril kinking as measured by SHG (Fig. 2b)[31] and TEM/SEM imaging[32,33]. Molecular collagen denaturation observed with collagen hybridizing peptide staining is also present in both fatigue loaded rat flexor carpi ulnaris tendon[34] and tail fascicles[35].

Interfascicle Matrix: The tendon interfascicle matrix (IFM) binds individual fascicles and has a distinct composition and mechanical properties that contribute to tendon extensibility and recoil[36]. In energy storing tendons, the IFM contains increased elastin[37] and displays more recoverable sliding[38] when compared with positional tendons. Fatigue differentially affects the elastin-rich IFM space[39], contributing to a greater number of fatigue cycles before failure in energy storing tendons[40].

Enthesis: At the enthesis, tendons can exhibit broader area of insertion to minimize stress

concentrations at the site of load transfer between stiff bone and compliant soft tissue. Accordingly, tendon enthesis has a fibrocartilaginous structure transitioning into mineralized tissue, varying collagen structure and viscoelastic properties along its length[41]. The highest strains occur within the microscale mineral gradient, creating an energy absorbing component that may prevent catastrophic failure[42]. However, non-uniform enthesis deformation may also result in stress shielding[43], where lack of tensile load results in atrophy and tensile weakening over time. Overuse results in greater damage at the enthesis of tendons[44], potentially due these structural and biomechanical variations and the presence of greater shear and compression.

Pericellular Matrix: Tendon cells are situated between collagen fibers and surrounded by a specialized pericellular matrix (PCM). The PCM's unique structure and composition, including collagen VI, fibrillin-2, and veriscan[45], create a local mechanical environment that modulates the fluid flow and matrix-induced deformation on tendons cells[46], analogous to that seen in meniscus[47] and cartilage[48]. At this scale, both quasistatic[49] and viscoelastic[50] variations in cell mechanical environment are detectable by atomic force microscopy, but surprisingly no differences between fatigue denatured and aligned areas was detected in fatigue loaded flexor carpi ulnaris rat tendons[51].

Interfiber spaces may become wider and less connected with overuse[32] resulting in altered interactions between cells and their surrounding matrix. A similar loss of contact between cells and PCM is associated with increased collagenase expression after stress deprivation[52], suggesting that under-stimulation as a result of overuse damage may signal catabolic PCM changes with overuse injuries[53]. Patellar tendinopathy in humans is associated with increased versican[54] and aggrecan and biglycan are upregulated with Achilles tendinopathy[55]. Chondrogenic PCM changes including aggrecan and collagen 2 upregulation is also observed in tendon explants cultured with

TGF-β2[56] and TGF-β1 injection model of tendinopathy in mice (Fig. 2c)[57]. Interestingly, pericellular aggrecan was reduced with 4 weeks of exercise after TGF-β1 injection in mice relative to cage controls[58]. Similarly, aggrecan decreased in damaged midsubstance regions of fatigue loaded rat patellar tendons with exercise initiated 2 weeks after injury, but had increased aggrecan in damaged insertion regions with exercise initiated immediately[59]. The pericellular variations in proteoglycans and associated glycosaminoglycans with injury, exercise, and repair suggest their important role in modulating PCM mediated mechanotransduction that may shift with time after injury.

Cell Phenotype and Location: Resident cells in tendon drive the biological response of the tendon to mechanical load. However, the relatively low cellularity of tendons contributes to their diminished intrinsic healing response. Tenocytes are the predominant cell type in tendon, and are elongated and highly aligned along the matrix[60]. In regions experiencing compression, such as the insertion site, cells appear chondrocyte-like and have a rounded shape[60]. Resident cells also include endothelial cells, pericytes, nerve cells, and immune cells, which may be modulated by injury[61]. For example, endothelial cells and pericytes associate with blood vessels, and their populations increase with hypervascularization observed in tendinopathy[62]. Similarly, immune cells aid in tendon repair but their dysregulation promotes fibrosis[63]. Further investigation of the temporal regulation of these cell populations following overuse will give insight into mechanisms of pathogenesis. Tendons also contain stem/progenitor cells that can differentiate in response to load, proliferate, and deposit new matrix[64]. For example, *in vitro* repetitive strain to tendon stem cells increased BMP-2 expression, which may explain ectopic calcification observed clinically in tendinopathy[65].

Interestingly, following overuse injury, cells that contribute to damage-induced matrix remodeling

are localized to the severely damaged IFM[66]. However, it is unknown whether this is caused by the higher cell population that is associated with this region in comparison to the fascicles, or by higher activity levels, thus their role in overuse injury warrants further investigation. The surrounding peritenon also has a rich supply of cells with high migratory and differential potential[67]. For example, myofibroblast lineage cells migrate from the peritenon during patellar tendon healing[68], and from the bursa in rotator cuff healing[69], warranting further investigation of the role of external contributions of cells to overuse injuries as well.

Aging ECM: Age-related degeneration is a major risk factor for tendinopathy, thus there is interest in how tendon mechanics, matrix structure, and cellular behavior are modulated with aging. Patellar tendons from patients older than 65 had a larger cross-sectional area and higher MRI signal intensity[70], indicative of structural and compositional changes with age. Collagen crosslinking associated with advanced glycation end-products (AGEs) increases in aging tendons[71], promoting a decrease in stress relaxation and collagen fiber sliding[72]. Aged tendons had greater IFM elastin quantity and organization in equine SDFT along with a corresponding increase in IFM area[39]. These aging related ECM changes correspond with compromised helix structure[73] and decreased fatigue life in equine SDFT fascicles and IFM with age[74].

Aging Cells: Surprisingly, one study found no correlation with aging and matrix synthesis in equine healthy SDFT[75]. However, this may instead point to the decreased ability of cells to respond to changes in their mechanical environment with age[76] as the contributing risk factor. Furthermore, the progenitor cells of aging tendons have decreased expression of tendon lineage markers, but an increase in adipogenic markers[77]. Additionally, the stem cell population becomes smaller, less proliferative, and more senescent[77]. Taken together, aging decreases the population of cells that are capable of contributing to matrix remodeling following injury.

Signalling Mechanisms

Interactions between the cells and the matrix (cell-matrix interactions) enable tenocytes to sense and respond to mechanical signals with a catabolic or anabolic response. The mechanical load at which cells' internal tension is balanced with the matrix forces maintains homeostasis and is known as the mechanostat set point. Understimulation of cells results in a degenerative cascade, while exercise induces an anabolic response to restore cell homeostasis with the matrix. With excessive loading leading to sub-rupture damage, fibrils lose the ability to transmit load[78], subsequently disrupting cell-matrix interactions[79,80] and ultimately also understimulating cells.

Cilia: Primary cilia project from the cell body and deflect in response to cyclic loading[81], suggesting they play a key role in mechanotransduction. They are observed in the majority of tendon cells and are highly oriented with respect to the ECM[82]. Cyclic loading also shortens cilia length[83] within the fascicle matrix but not IFM[84] , while stress deprivation increases cilia length and biomechanical degradation primarily in the IFM[85]. This highlights how altered composition and lower strains in the IFM may alter cilia-mediated mechanotransduction and cell response to load. Within mouse supraspinatus tendon enthesis, overloading induced cilia disassembly and mineralization while unloading induced cilia assembly, decreased mineralization, and reduced stiffness and ultimate stress[86]. Changes in tendon cilia in response to loading, stress deprivation, and matrix environment suggest a role in altering mechanostat set point during injury. Overloading with overuse may initially induce cilia disassembly and shortening and reduce mechanotransduction. However, overuse induced damage may cause local stress deprivation, inducing cilia assembly and lengthening and restoring a reparative response to load in damaged

areas.

Integrins: Integrins are transmembrane receptors that bind cells to the ECM and cluster to form the basis of focal adhesions, transferring mechanical force and signals between cells and surrounding matrix. Integrin-mediated adhesion may be preceded by hyaluronan-mediated adhesion by CD44, which facilitates maturation of focal adhesions[87] and can augment integrin-mediated cell spreading[88]. Gene and protein expression of collagen binding integrins α1, α2, and β1 and fibronectin binding integrins α5, αV, β3, and β5 is widespread in various tendons[89,90]. ECM induced tenogenesis in MSCs is mediated by integrins and TGF-β crosstalk, demonstrating how integrin-ECM interactions may induce tendon cell behavior and phenotype[91]. Integrin β1 stimulation in tendon cells activates AKT and mTOR pathways regulating collagen expression[92], suggesting a role for integrins in matrix remodeling. In response to cyclic strain, collagen binding integrins α1, α2, and α11 increased in cultured tendon stem progenitor cells[93] and integrin β1 increases[94] and aligns[95] in ligament fibroblasts. Correspondingly, stress deprivation decreased integrin α1 and increased α2 expression in engineered human tendon[96]. Rat PTs increased integrin β1 expression one day after 100 but not 7200 cycles of fatigue loading, demonstrating how integrin mediated responses to load may be inhibited with overuse[97]. Integrin increase with strain suggests they may reinforce cell-matrix interactions in response to injury specific changes in matrix composition and structure such as disruption of pericellular collagen VI and increase in proteoglycans and hyaluronan. Cells may respond to overuse associated changes with increased integrins, reconnecting cells with their surrounding matrix and restoring mechanotransduction.

Connexins: Cells can directly communicate with electrochemical signals through gap junctions. In tendon, Connexin32 (Cx32) and Connexin43 (Cx43) have been identified of interest. Cx32 connects cells longitudinally, whereas Cx43 connects cells both longitudinally and laterally[98].

Cx32 increases collagen synthesis under loading, whereas Cx43 has an inverse relationship[99]. This provides a potential mechanism by which cells balance their response to different levels of load. Although cells still respond to cyclic loading with gap junction inhibition by increasing collagen synthesis, there is a synergistic effect of load and cell-to-cell signaling[99]. Thus, connexins in tendon may integrate with the other mechanosignaling pathways to respond to applied stimuli. Cx43 has also been shown to be required for proper structural and mechanical development of murine postnatal insertion site and anabolic response to treadmill exercise[100]. However, the mechanistic role of connexins in tendon after injury or from aging are yet to be elucidated. On disorganized fiber substrates, as may be observed after injury, tendon cells exhibit decreased Cx43 expression localized to the cell nucleus rather than periphery[101], further highlighting impaired mechanosignaling as a result of injury condition.

Cytoskeleton: The actin cytoskeleton mechanically transmits load from cell-surface receptors to the cytoplasm and nucleus, and thereby regulates the cell shape by tensioning the cell to the matrix[102]. The cytoskeleton is largely responsible for mediating the cell's homeostatic set point. In response to the loss of tissue strain, cells' cytoskeleton become rapidly unorganized, and cells contract against the lax substrate until cytoskeletal tension is regained[103]. Similarly, under high levels of cyclic tissue strain (12%), cells undergo cytoskeletal depolymerization, cell rounding, and increased collagenase expression[104]. Additionally, the tendon crimp pattern is dependent on tensional homeostasis from the cells, reflecting a balance between cells' internal force from the cytoskeleton against the ECM forces that are externally acting on the cell[105]. Therefore, the crimp pattern may be a method for cells to modulate their local tissue mechanical environment.

Cell-mediated contraction of lax ECM is lower in aged rat cells compared to young cells, suggesting they may be less mechanoresponsive following overuse[106]. This is supported with *in*

vivo patient results, where cyclic loading lengthened tendons, and the extent of return was age-dependent[107]. Taken together, this unloading of the actin cytoskeleton may be implicated in the etiology of tendinopathy. Furthermore, studies have found correlation between applied tissue strain, collagen organization, F-actin, and cell nucleus strain, highlighting the multiscale role of mechanotransduction[12,108]. Modulation of cell nucleus shape is a method of physically regulating chromatin availability required for cell division, as well as other downstream transcriptional and protein activity[109].

Cell Fate in Overuse: The combined multiscale mechanical and structural changes with overuse ultimately affect numerous mechanobiological pathways[110]. While remodeling associated genes including Col-1, -XII, MMP2, and TIMP3 were increased in response to fatigue loading in rat patellar tendon, they shut down after a high level of induced damage and apoptosis[111]. Physiological levels of cyclic loading to tendon cells transiently activates the JNK signaling pathways in a magnitude-dependent manner[112]. This pathway is mitogenic in the short-term, allowing for healthy cell proliferation and matrix turnover, but can activate the apoptotic cascade if continually upregulated in the presence of other stresses in the matrix[113]. Apoptosis is also induced by loss of homeostatic tension through both tissue stress deprivation[79] and high mechanical loading[114] in organ culture. *In vivo* models have shown that a greater severity of induced sub-rupture fatigue damage is correlated with increased apoptotic activity, which may account for the correlated muted and ineffective biological response to injury[115]. Fatigue loading may initially overload cells, upregulating remodeling genes. However, higher induced damage to the PCM may disrupt cell-matrix interactions, under-loading the cells and inducing apoptosis and shut down of remodeling associate genes. Ultimately, a muted remodeling response results in persistent damage that does not resolve over a period of time[116]. Pharmacological inhibition of

apoptosis for five days following sub-rupture injury increased the number of cells producing ECM and PCM proteins, such as procollagen I and collagen VI but did not mitigate cellular stress markers[117]. Consequently, pharmaceutical inhibition of apoptosis ultimately led to deterioration of mechanical properties, suggesting that inhibition of apoptosis in the context of sustained matrix damage promotes a catabolic stressed state. A more effective therapeutic approach would require inhibition of apoptosis in conjunction with stabilization of the cell-matrix environment.

Interventions to enable and promote repair

Physical therapy and exercise are common, nonsurgical methods to treat tendinopathy, though type, frequency, and timing is contextual, and may account for disparate clinical outcomes[118]. This suggests that mechanical stimulation through exercise or physical therapy can induce repair but that an appropriate mechanical and biological environment must be present. For example, at early stages of tendinopathy with limited damage, mechanotransduction may be minimally disrupted and modulation of the ECM is unnecessary to enable repair. However, in the presence of more severe or widespread damage, cells have disrupted mechanotransduction, and have a minimal or aberrant response to mechanical load. Tear and rupture undoubtedly affect mechanotransduction at all scales, requiring surgical intervention and potentially multiscale ECM and biological modulation post-surgery to enable repair. Repair must be induced to a degree that outpaces further accumulation of damage to prevent progression of tendinopathy into later stages with inflammation, pain, rupture, and unrecoverable function.

Strategies to modulate ECM

Exercise to Restore ECM: The goal of exercise as a therapeutic intervention is to ultimately change tendon matrix composition to withstand a greater loading demand. Tendons respond acutely following exercise, which may promote matrix remodeling and increased mechanical properties and anabolic activity[119]. Exercise upregulates tenogenic genes, but supra-physiological loading additionally induces stem/progenitor cell differentiation towards chondrogenic, osteogenic, or adipogenic phenotypes[120]. Modulation of collagenous components is also a therapeutic area of interest. For example, the ratio of MMPs and tissue inhibitors of metalloproteinases (TIMPs) has been implicated in pathogenesis of tendinopathy[121] and their relative levels may serve as a therapeutic goal. The role of proteolytic matrix enzymes such as MMPs, which play a role in matrix turnover, warrants further investigation into their contextual and temporal role, as there are conflicting outcomes decrease[119] or to increase[122] following exercise.

Following a sub-rupture fatigue injury, initiation of exercise 1-day after onset of fatigue injury further exacerbates structural damage to the tendon. In contrast, 2 weeks of cage activity prior to initiation of exercise decreases structural damage[44]. Since there is no difference in macroscopic mechanical properties at the initiation of either exercise protocol, it is likely that this time frame is critical for development of biological mechanisms that aid in repair and restore the micromechanical environment. Furthermore, the increase in myofibroblast cell population that is observed with effectively therapeutic exercise[116] is hypothesized to tension the matrix at the site of collagen kinks and recover the stiffness loss associated with injury. Additionally, exercise also increased integrin α5, which can impart greater cell contractility from the cells to tension damage kinks and withstand greater loads through increased focal adhesions in exercise[123].

Although aging can diminish tendon's mechanical properties due to the presence of increased AGEs[124], exercise in old mice can decrease tendon stiffness through decreased AGE levels and increased collagen turnover[125]. Interestingly, this occurs independently of changes in cell density, suggesting that the local cell population can be reprogrammed through exercise to restore healthy tendon properties. Treadmill exercise prior to punch injury in aged rats lead to faster wound closure, improved collagen organization, increased stem cell markers, and decreased cell senescence[126].

Targeting cell signaling to restore ECM: Biochemical stimulation of cells provides a strategy for cells to restore their proper matrix environment in response to overuse. Clinically disparate outcomes with use of Platelet Rich Plasma (PRP) suggest varying protocols and potentially, formulation[127–129]. There are limited studies investigating efficacy of PRP in controlled animal models. Mechanistically, *in vitro* studies have shown that PRP increases differentiation of stem cells into tenocytes, increases collagen production[130], and enhances cellular proliferation[131], thereby increasing the population of cells that can respond to injury and remodel the matrix. PRP may allow for synergistic effects of combinations of growth factor therapy or may activate antagonistic pathways. Due to dysregulation of growth factors following overuse[132], therapeutic delivery of selective growth factors, such as FGF, TGF-β, and IGF-1 has been tested in animal repair models of acute injury[133–135]. This method can selectively target pathways to enhance cell proliferation, tendon vascularity, and collagen deposition, and likely plays a promising role in overuse injuries. Another strategy is to genetically transduce the commonly used mesenchymal stem cells with scleraxis, a tendon marker, which has been shown to improved mechanical properties of the healing tendon[136]. However, while these strategies are often improvements over untreated controls, current studies are still unable to restore naïve mechanical properties or

structure[137]. It is likely that attempts to modulate cell behavior are ineffective without concurrent modulation of the surrounding micromechanical environment.

One strategy to further enhance the efficacy of growth factors is to augment the therapeutic with a biomaterial scaffold with matrix cues to guide cell-mediated healing. For example, one study designed a hierarchical scaffold mimicking the gradient microarchitecture of the supraspinatus tendon insertion site, composed of hydroxyappetite and PLGA, and successfully induced cell differentiation along the gradient[138]. However, while commercially available scaffolds are widely used to augment tendon surgical repair[139], they have limited practicality in overuse injuries prior to rupture. Instead, therapeutic-loaded injectable nanospheres[140], which can be applied to tendons in the absence of a rupture, may provide a more promising avenue for early tendinopathy cases.

Non-collagenous matrix components consisting of glycoproteins, proteoglycans, and elastic fibers also affect both mechanical properties, mechanotransduction, and biological response and should therefore be considered for therapeutic modulation. For example, induced knockouts of decorin and biglycan altered fibril size and mechanical properties in mice[141]. Hyaluronan and proteoglycans like aggrecan and decorin in the PCM and interfascicular spaces are increased during overuse injury and clinical tendinopathy[142], potentially modulating tendon mechanics, cell environment, and cell-matrix mechanotransduction. Injections of hyaluronan[143] or lubricin[144] may help recovery of interfascicular sliding after overuse injury, especially in the presence of adhesions. On the other hand, enzymatic removal of hyaluronan[145] or other glycosaminoglycans after injury may allow for more dense or aligned collagen during repair, enhancing mechanical properties and mechanotransduction during recovery. ADAMTSs, enzymes associated with proteoglycan turnover, were increased with stress deprivation in rat tail tendon fascicles and downregulated with high strain in ovine tendinopathy, suggesting they may also be targeted to

modulate ECM with overuse injury[146,147].

Inflammation in ECM repair: Clinical tendinopathy often presents with granulation tissue and inflammatory cell infiltration[148], motivating the investigation of inflammation in the pathogenesis of tendinopathy and its consequences on mechanotransduction. When unresolved, chronic inflammation leads to excess matrix deposition and fibrosis that is characteristic of tendinopathy[149–151]. Immune cells, such as M2 macrophages and T cells are implicated in this inferior repair response[152,153]. A recent study suggests that NF-κβ signaling in overuse may activate these subsequent signaling pathways that sustain inflammation[154]. Certain aspects of inflammation that facilitate controlled matrix deposition should be considered for therapeutic use to promote matrix synthesis following overuse injury.

A limited number of studies have investigated the time course of inflammation in overuse models. Rotator cuff overuse in rats causes small increases in expression of the inflammatory genes COX-2 and FLAP, however, their decrease over time suggests an adaptive response to overuse[155]. Similarly, IL-6 and COX-2 protein expression is elevated within 24 hours after damage-inducing cyclic loading in an organ culture model[66]. Together, this suggests that the early inflammatory period may be critical for long-term repair. Supporting this notion, inhibiting inflammation in acute injuries during the early inflammatory phase is detrimental to healing[153,156]. Further study is needed into inflammation's mechanism of action in the pathogenesis of tendon overuse injuries to guide therapeutic use. Although nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used to treat patients to mitigate tendon pain, their mechanistic efficacy in overuse injuries is controversial. Some studies show that administration of NSAIDs following exercise is detrimental[157], while others show no beneficial effects over a placebo[158,159]. Future interventions should combine specific targeting of key pathways in the inflammatory cascade that enable controlled matrix

synthesis with temporal specificity to disease progression.

Remaining gaps and unknowns

Among other factors, overuse associated fatigue loading, local structural damage, and compositional changes at multiple scales disrupt tendon mechanotransduction. This persistent disruption may inhibit a reparative response and explain the chronic nature of tendon overuse injuries and high rates of reinjury. Significant progress has been made to understand the spatial, mechanical, and temporal changes that occur with overuse. As the foundation of tendon mechanics and mechanotransduction, ECM modulation should be a focus for therapeutic strategies and utilized for more mechanistic perturbation of tendon structure and function. Consequently, ECM modulation of mechanotransduction may enable repair in response to load and ultimately restore structural and mechanical integrity of the tendon. However, the mechanotransduction pathways covered in this review require direct study linking their effect on mechanobiology of tendon repair. Studies directly perturbing key ECM components following overuse and assessing effects on mechanotransduction and subsequent cell response are vital to enable clinical translation.

A major hurdle in the field is the lack of models that directly measure and relate tendon mechanotransduction across multiple scales. Existing studies emphasize the varied role of the ECM in modulating mechanotransduction in different tendon types and tissue compartments (IFM, enthesis, and PCM). The context-dependent role of the ECM composition and structure in modulating cell-matrix interactions should be investigated more thoroughly in healthy and damaged tendons. Subsequently, direct study of cell-matrix interactions and multiscale mechanics should be correlated with cell responses within overuse tendon models. In addition to collagen deposition as a metric of remodelling and recovery after injury, the clear mechanistic role of non-

collagenous matrix in modulating mechanotransduction following overuse indicates their inclusion in future studies.

In vitro tissue and organ culture models provide a powerful method to isolate mechanotransduction pathways and study their interactions with metabolic pathways implicated in repair. Incorporation of components of the overuse tendon ECM environment as reviewed would provide enhanced context of findings for translation. Animal models can recapitulate the overuse tendon environment in a controlled manner, removing confounding factors that result in varied clinical outcomes in humans. How *in vitro* and animal overuse models translate to the more complex human overuse injury conditions that make clinical management of tendinopathy difficult remains the subject of ongoing investigation, specifically with regards to lifestyle, pain, genetics, and age.

Studies show therapeutic intervention can induce either repair or further damage with tendon overuse injury and is dependent on dosage and timing of application. Exercise or physical therapy results in upregulation of ECM and cellular components associated with mechanotransduction pathways. However, the functional role of each component in these pathways and whether their upregulation is a prerequisite for reparative exercise, or a result of reparative exercise requires further study. In conclusion, to improve the timing and efficacy of current and future therapeutic strategies for tendinopathy, more comprehensive assessment of local ECM and cell environments with overuse and their impact on mechanotransduction are crucial. The connection between multiscale ECM changes with tendon overuse injury and their effects on mechanotransduction and subsequent repair remains an important and promising focus for current and future investigation.

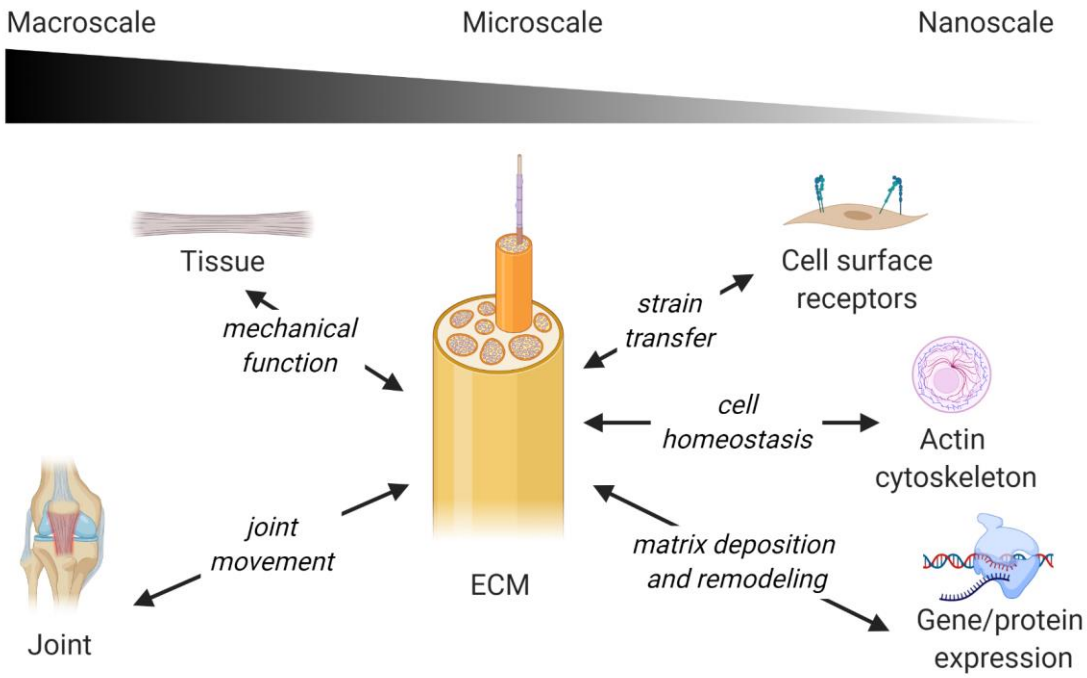

Figure 1. Tendon's hierarchical structure spans multiple length scales. Healthy tendons maintain ECM homeostasis through feedback mechanisms (arrows) between multiscale mechanotransduction components. (Created with biorender.com)

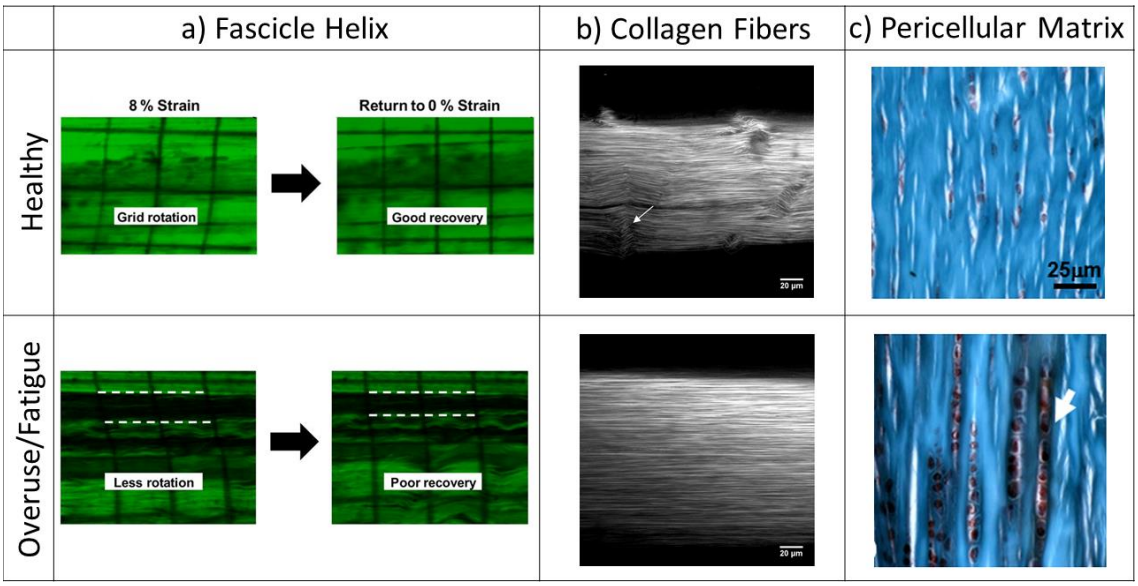

Figure 2. Damage and disruption of ECM structures in overuse injury models alters multiscale biomechanics, compromising tendon function and downstream mechanotransduction. a) Rotation and recovery with strain is reduced in fatigue loaded horse superficial digital flexor tendon[160]. (Reprinted from Acta Biomaterialia, 10, Thorpe, C. T., Riley, G. P., Birch, H. L., Clegg, P. D. & Screen, H. R. C., Effect of fatigue loading on structure and functional behaviour of fascicles from energy-storing tendons, 3217–3224, 2014, with permission from Elsevier). b) Collagen fibers and fibrils display damage areas and kinks (arrow) in fatigue loaded rat tail tendon fascicles[32]. (Reprinted from Journal of Biomechanics, 85, Ros, S. J., Muljadi, P. M., Flatow, E. L. & Andarawis-Puri, N., Multiscale mechanisms of tendon fatigue damage progression and severity are strain and cycle dependent, 148-156, 2019, with permission from Elsevier). c) Cell rounding and pericellular accumulation (arrow) of glycosaminoglycans 2 weeks after TGF-β1 injection in mouse Achilles tendon[58]. (Reprinted from Journal of Biomechanics, 46, Bell, R. *et al.*, Controlled treadmill exercise eliminates chondroid deposits and restores tensile properties in a new murine

tendinopathy model, 498-505, 2013, with permission from Elsevier).

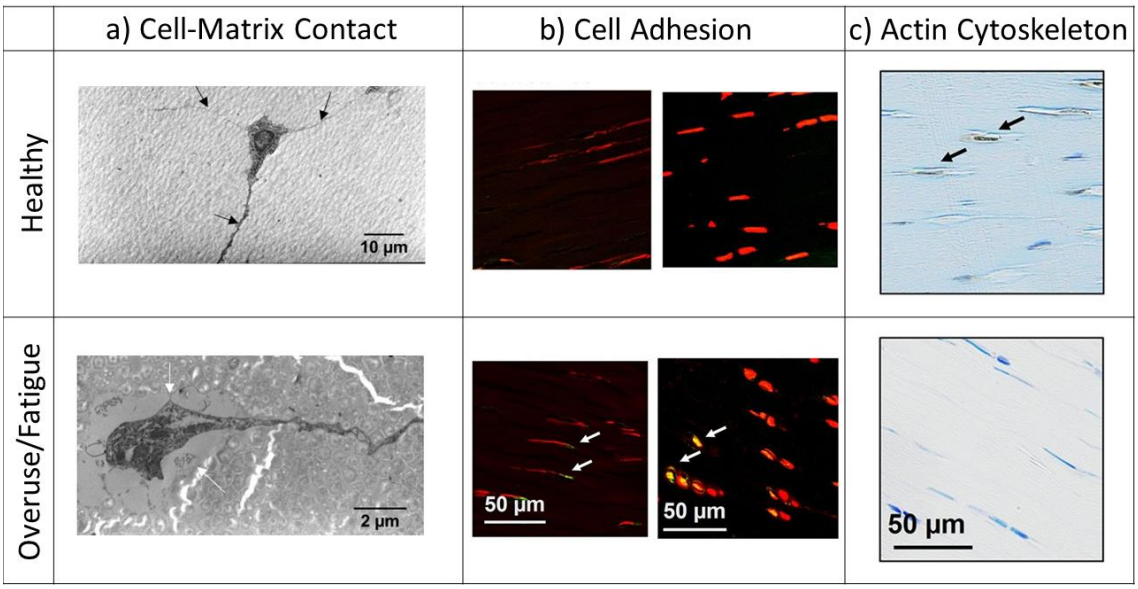

Figure 3. Cell-matrix interaction meditating structures are disrupted or alter expression in overuse injury models a) Cilia disruption (black arrows) and separation from matrix (white arrows) in fatigue loaded rat tail tendon fascicles[32]. (Reprinted from Journal of Biomechanics, 85, Ros, S. J., Muljadi, P. M., Flatow, E. L. & Andarawis-Puri, N., Multiscale mechanisms of tendon fatigue damage progression and severity are strain and cycle dependent, 148-156, 2019, with permission from Elsevier). b) Increased integrin α5 (left, arrows) and fibrillin (right, arrows) in fatigue loaded rat patellar tendons without reparative exercise[116]. (Reprinted from Scientific Reports, 8, Bell, R., Gendron, N. R., Anderson, M., Flatow, E. L. & Andarawis-Puri, N., A potential new role for myofibroblasts in remodeling of sub-rupture fatigue tendon injuries by exercise, 2018, CC BY). c) Decreased α-smooth muscle actin in fatigue loaded rat patellar tendons without reparative exercise[116]. (Reprinted from Scientific Reports, 8, Bell, R., Gendron, N. R., Anderson, M., Flatow, E. L. & Andarawis-Puri, N., A potential new role for myofibroblasts in remodeling of sub-rupture fatigue tendon injuries by exercise, 2018, CC BY).

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Original Article

Tendon Microscale Mechanics and Viscoelasticity are Modulated by Fatigue Injury Through an Increase in Glycosaminoglycans

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Introduction

Tendinopathies are common and debilitating conditions affecting a wide spectrum of the population. Overuse injuries occur from repetitive motion and result in fatigue damage and reduced mechanical properties. Without therapeutic intervention, fatigue injured tendons do not recover pre-injury mechanics or structure[1,2], suggesting that tendons do not innately repair fatigue damage. Fatigue injury is accompanied by reduced upregulation of remodeling associated genes and increased apoptosis[3]. This leads to damage accumulation outpacing repair, resulting in chronic disease and eventually rupture. Treatment strategies and outcomes vary greatly between patients as the progression from early-stage damage to late-stage disease is poorly understood.

Using our established *in vivo* fatigue loading model to induce sub-rupture damage in rat patellar tendons, we previously determined that physiological exercise could induce repair if initiated 2 weeks after injury, while earlier initiation may exacerbate damage[1]. At both timepoints, macroscale mechanical properties and collagen structure remain unaltered, suggesting that microscale, non-collagenous matrix changes may modulate mechanotransduction and enable a reparative response to load. Of note, glycosaminoglycans (GAGs) are increased in chronic overuse tendinopathy[4]. GAGs are polysaccharides often associated with protein cores to form proteoglycans (PGs) residing in between collagen fibers and in the peritendinous sheath. Dermatan sulfate (DS, associated with the PG decorin) is the most common GAG in the tendon midsubstance and facilitates fibril sliding[5,6], while chondroitin sulfate (CS, associated with the PG aggrecan) predominates in the tendon insertion to bone[7] and resists compression[8]. Hyaluronic acid (HA) is also present in the pericellular matrix of tendon cells[9], potentially mediating cell-matrix interactions. GAGs hold a negative charge that attracts water and regulates fluid motion, enabling lubrication, compression resistance, and viscoelastic behaviors including energy dissipation and

stiffening at high strain rates. They may also interact with other GAGs, ECM components, and directly with cells to mediate cell-matrix interactions and mechanotransduction. However, the mechanical role of GAGs in tendons and during post-injury repair is less understood. While clinical observations suggest an increase in GAGs is a prominent characteristic of late-stage, post-rupture tendinopathy, their presence and potential role in early-stage, sub-rupture injury and repair is unknown. Determining whether an increase in tendon GAGs is simply a hallmark of late-stage disease or part of an attempt to repair damage is crucial to defining therapeutic goals.

Assessing the multiscale mechanical role of GAGs after fatigue injury is integral to identifying the mechanical and biological environment necessary for tendon repair. The viscoelastic and multiscale contribution of GAGs in other tissues and the increase in GAGs after tendon injury and during reparative exercise suggests they may influence multiscale mechanics, mechanotransduction, and repair of tendon fatigue injuries. We hypothesize that an increase in GAGs during the period following fatigue injury modulates viscoelastic and microscale strain properties, ultimately influencing mechanotransduction and enabling repair in response to exercise and load. To test this hypothesis, we induced fatigue injury in the patellar tendons of rats and characterized the increase in GAGs two weeks after injury. We expected an aggrecan associated increase in CS in the insertion, a decorin associated increase in DS in the midsubstance, and an HA increase in the pericellular matrix to occur in the 2 weeks after fatigue injury, reflecting an attempt to restore location specific mechanics and cell-matrix interactions disrupted by injury. In addition, the mechanical role of GAGs was assessed by performing microscale strain and dynamic mechanical tests before and after enzymatically removing GAGs from fatigue injured tendons. We expected removing GAGs in fatigue injured tendons 2 weeks after injury would result in increased microscale strain, decreased strain-rate dependence, and a reduced viscous contribution to dynamic

modulus, reflecting multiscale mechanical and viscoelastic contributions for post-injury GAGs that may influence mechanotransduction and repair.

Methods

Fatigue Loading Injury Model

Experiments were approved and performed in accordance with the Institutional Animal Care and Use Committee at Cornell University. Sprague-Dawley rats aged 9-11 months were anesthetized and administered buprenorphine. As previously described[10], incisions on the left leg were made to expose the patella and tibia. The patella and tibia were gripped and connected to 50lbf load cell on a mechanical test instrument (Electroforce 5500) and the patellar tendon was fatigue loaded at 1 Hz from 1 to 40 N for 7200 cycles. Diagnostic testing was performed pre- and post-fatigue loading, consisting of loading at 1 Hz from 1 to 15 N for 420 (pre) or 120 (post) cycles. Diagnostic parameters previously described[10] were used to ensure induced damage was similar across groups. After fatigue loading, incisions were sutured, and rats resumed cage activity.

Microscale Shear Strain Testing

Two weeks after fatigue injury, animals were sacrificed and patellar tendons from fatigue injured and naïve, contralateral limbs were dissected, frozen in OCT compound, and cut in half longitudinally to create paired specimens (n=8). Samples were stained with 5-DTAF for matrix imaging, mounted in a tissue deformation imaging stage (TDIS), and positioned on a Zeiss LSM710 confocal microscope in a PBS bath to image across the length and thickness of the tendon sample. A grid consisting of 2x2, 100×100 μm squares was photobleached and imaged. Bulk shear strains of 0.08, 0.16 and 0.24 radians (rad) were applied sequentially and imaged after 8-min relaxation periods[11]. This strain tracking was repeated with both halves of each tendon, with each

half treated at room temperature in protease inhibitor (Halt, Thermo Scientific) and ChABC (from *proteus vulgaris*, 2 U/mL, SigmaAldrich) or PBS for 8-10 hours. The microscale matrix shear strain, or Lagrangian shear strain (*E*), was calculated at each applied strain (0.08, 0.16, and 0.24 rad) by comparing the location of grid intersections at each strain to those at 0 strain.

Bulk Viscoelastic Tensile Testing

Two weeks after fatigue injury, animals were sacrificed and patella-tendon-tibia complexes from fatigue injured and naïve, contralateral limbs were isolated and cross-sectional area was measured with calipers (n=5-6/treatment). The patella and tibia of each sample was gripped, attached to a 50lbf load cell on a mechanical test instrument (Electroforce 5500) in a PBS bath at room temperature, and preloaded to 0.1 N. Following preconditioning, stress relaxation was assessed by ramping to 4% strain and held for 300s. A frequency sweep consisting of 10 cycles of 0.125% amplitude sinusoidal strain (0.1, 1 and 5 Hz) spanning the physiological stride frequency of rats was then applied[12]. Stress relaxation and frequency sweep were then repeated at 6% strain. Tendons were incubated overnight in protease inhibitor (Halt, Thermo Scientific) and ChABC (from *proteus vulgaris*, 2 U/mL, SigmaAldrich), ChB (from *flavobacterium heparinum*, 2 U/mL, SigmaAldrich), HAase (from *streptomyces hyalurolyticus,* 2 U/mL), or PBS and retested using the same mechanical testing protocol. Dynamic modulus (|E*|) was calculated as stress amplitude divided by the strain amplitude at each frequency. Frequency or strain-rate dependence of dynamic modulus was calculated using linear regression and F-test for non-zero slope to assess viscoelastic, strain-rate dependent mechanical response. We expected dynamic modulus to increase monotonically with increasing frequency within the range tested[12]. Loss tangent (tanδ), defined as the ratio of viscous to elastic components of dynamic modulus (E”/E’, where E*=E’+*i*E”) was calculated as the tangent of the phase lag between stress and strain in radians (tanδ). Finally, a

pull-to-failure test was conducted at a rate of 0.3%/second to determine stiffness and maximum load.

Bulk GAG Quantification

After tensile testing, GAGs were quantified using fluorophore assisted carbohydrate electrophoresis (FACE). Tendons were separated from the tibia and patella and were lyophilized, pulverized, and digested in 10x proteinase K for 24hr at 60°C. After precipitation in ethanol, GAGs were depolymerized with chondroitinase ABC and fluorotagged by 2-aminocridone (AMAC). Acrylamide gels were loaded with samples and prepared disaccharide standards (ΔdiHA, Δdi0S, Δdi4S, and Δdi6S, AMSBIO) and electrophoresis was run at 150 V for 75 minutes to separate bands. Gels were imaged on a ChemiDoc system and GAGs were quantified by comparing sample band optical density with disaccharide standard bands. Final weights of hyaluronic acid (HA) and chondroitin and dermatan sulfate (CS+DS) were normalized to dry weight.

Immunohistochemistry for GAGs and PGs

At 2 weeks after fatigue, rats were sacrificed and patella-tendon-tibia complexes from fatigue and contralateral limbs were harvested and fixed under 2 N of tension in Z-fix (n=6). Samples were then decalcified, embedded in paraffin, and sectioned at 6 µm thickness. Antigen retrieval was achieved using chondroitinase ABC (ChABC) for PGs and HA, chondroitinase B (ChB) for DS, and chondroitinase AC for chondroitin sulfate (CS). Primary antibodies for CS/DS (clone 2B6, 1:20, AMSBIO), HA (HA binding protein, 2 µg/mL, VWR) decorin (1:100, abcam), biglycan (abcam), aggrecan (1:2000, abcam), versican (abcam), fibromodulin (abcam), and lumican (abcam) were applied, followed by a diaminobenzidine secondary antibody and toluidine blue as a counterstain. Sections were imaged using a 10x lens at the tibial insertion, midsubstance, and

patellar origin. GAG or PG amount was quantified by thresholding the image and counting the positively stained matrix area or number of positive cells per total tendon area in ImageJ. Each GAG positive ECM area measurement or cell density or was plotted with PG positive ECM area or cell density measurements from the same tendon and linear regression was performed to assess correlation between GAGs and PGs.

Statistical Analysis

Unpaired t-tests were used to determine increases in GAGs with fatigue injury in bulk tendons and by anatomical location and assess removal with ChABC/ChB/HAase treatment. Linear regression with F-test for non-zero slope was used to correlate CS, DS, and HA GAG types with decorin or aggrecan. Two-way ANOVA tests were performed to determine the effect of applied strain or fatigue injury on microscale strain or change in microscale strain with ChABC treatment, with Šídák's post-hoc analysis to determine effects of fatigue injury at specific applied strains. Two-way ANOVA tests with Šídák's post-hoc analyses were performed to determine effect fatigue injury or ChABC/ChB/HAase treatment on dynamic modulus or loss tangent at each frequency. Linear regression and ANCOVA comparison of slope was used compare frequency dependence of dynamic modulus with fatigue injury and with ChABC/ChB/HAase treatment.

Results

Bulk GAG Increase with Fatigue Injury

FACE quantification of chondroitin and dermatan sulfate (CS+DS) and hyaluronic acid (HA) were performed to quantify bulk increases GAGs after fatigue injury. As expected, GAGs were increased two weeks after fatigue injury when compared with naïve tendons. CS+DS were the

predominant GAG type present in naïve tendons and increased with fatigue injury (Fig. 1a). HA was also significantly increased with fatigue injury but present at lower concentrations (Fig. 1b).

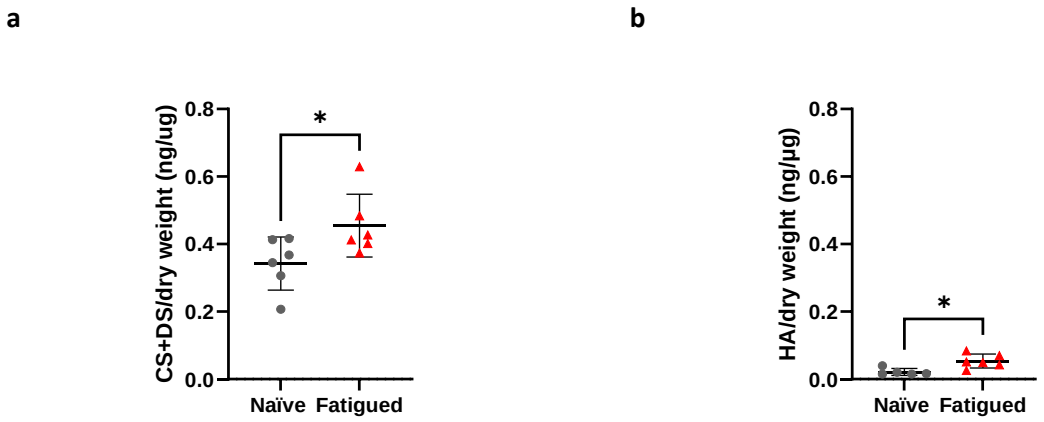


Fig. 1. Chondroitin and dermatan sulfate (CS+DS) (a) and hyaluronic acid (HA) (b) were both increased in fatigue injured tendons relative to naïve control tendons. *$p \leq 0.05$

Location Dependent GAG and PG Increase with Fatigue Injury

IHC staining of different GAG and PG types present in tendon ECM was performed to determine anatomical location of increased GAGs after fatigue injury and correlate these GAGs with PG increases. As expected, DS positive ECM area was increased in the midsubstance of fatigue injured tendons relative to naïve tendons (Fig. 2a) while CS (Fig. 2b) and HA positive ECM area (Fig. 2c) were not changed with fatigue injury. Interestingly, decorin ECM area in the insertion (Fig. 2d) was greater in fatigued tendons relative to naïve tendons whereas aggrecan ECM area (Fig. 2e) was not significantly different. Biglycan, versican, fibromodulin, and lumican ECM area were also not significantly changed with fatigue injury. As expected, increasing DS ECM area was positively correlated with decorin ECM area in fatigue injured tendons (Fig. 2f). In contrast, CS (Fig. 2g) and HA ECM area (Fig. 2h) were not correlated with PG increases. These findings identify a decorin-associated increase in DS in the midsubstance and an increase in decorin in the insertion as major components of the GAG increase after fatigue injury.

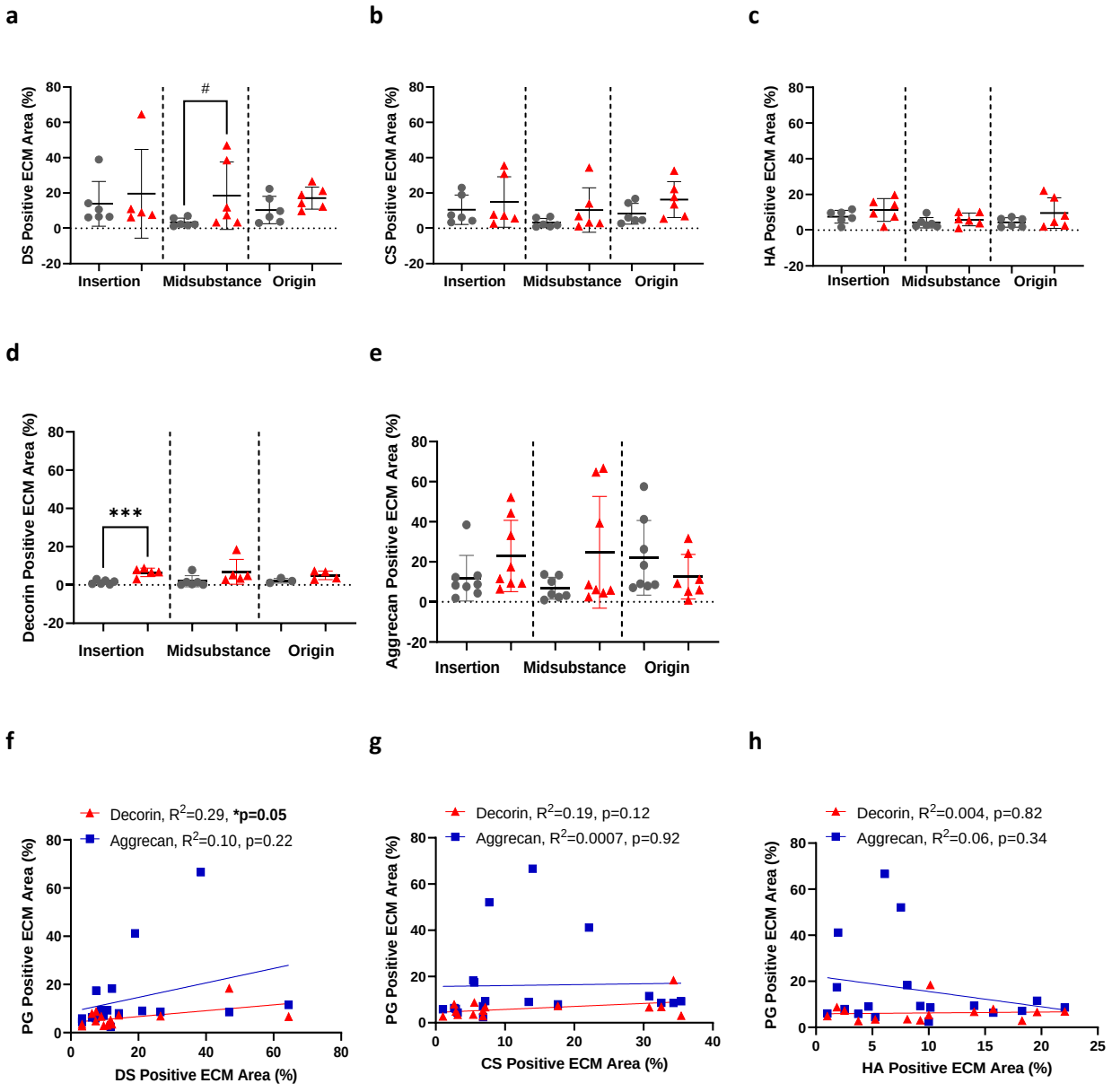


Fig. 2. DS positive ECM area (a) was increased in fatigue injured tendons relative to naïve tendons in the midsubstance. CS (b), and HA (c) positive ECM area were unaltered with fatigue injury. Decorin positive ECM area (d) increased in the insertion of fatigue injured tendons while aggrecan (e) was not significantly changed. Increased DS positive ECM area (f) was correlated with increased decorin positive ECM area. CS (g) and HA (h) positive ECM area were not correlated with increased decorin or aggrecan. #$p \leq 0.10$, *$p \leq 0.05$, ***$p \leq 0.001$

Pericellular GAG and PG Modulation with Fatigue Injury

IHC cell staining of different GAG and PG types present in or around tendon cells was performed to determine pericellular changes in GAGs after fatigue injury and correlate these GAGs with PG increases. DS positive cell density was decreased in the insertion and origin (Fig. 3a) while CS positive cell density was increased in the midsubstance (Fig. 3b) and HA positive cell density was increased in the origin of fatigue injured tendons (Fig. 3c). These pericellular GAG changes corresponded with an increase in aggrecan positive cell density in the midsubstance (Fig. 3e) and no changes in decorin positive cell density (Fig. 3d). Biglycan, versican, fibromodulin, and lumican cell density were also not significantly changed with fatigue injury. As expected, increased DS cell density was positively correlated with both decorin and aggrecan cell density (Fig. 3f). Despite both CS and aggrecan positive cell density increases in the midsubstance, CS was not significantly correlated with increased aggrecan or decorin cell density (Fig. 3g). HA cell density was not correlated with decorin or aggrecan cell density (Fig. 3h). These findings identify pericellular CS and aggrecan increases in the midsubstance and HA increases in the origin with fatigue injury. These findings also reveal a decorin correlated decrease in DS in the pericellular matrix of insertion and origin cells with fatigue injury.

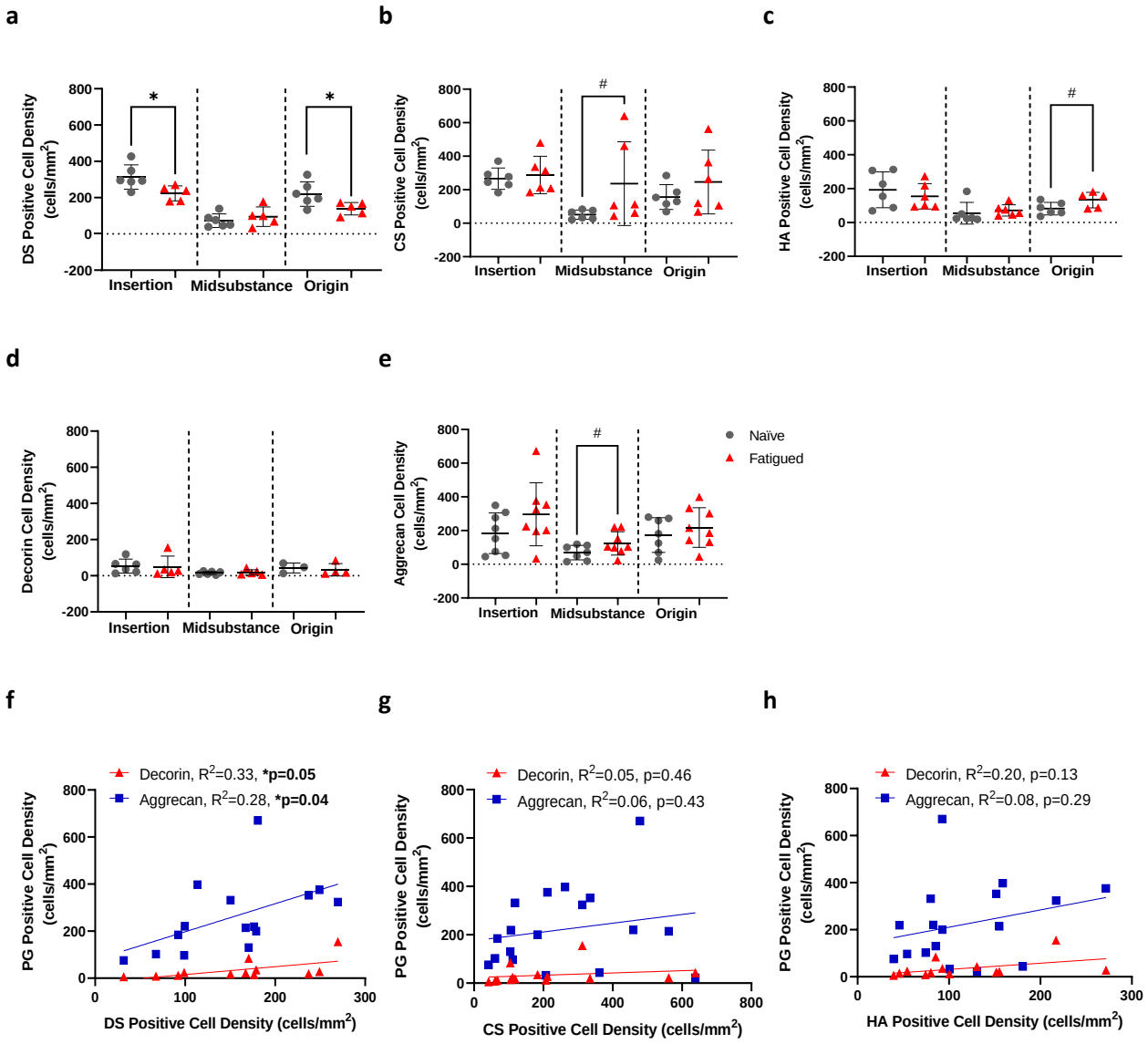
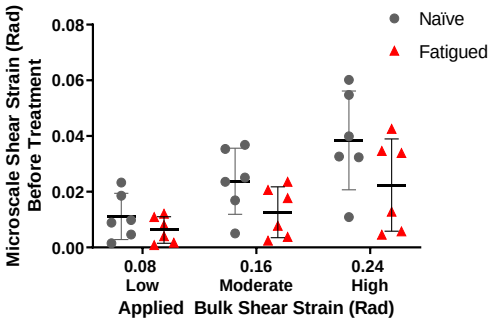


Fig. 3. DS positive cell density (a) in fatigue injured tendons was reduced in the insertion and origin while CS (b) and HA (c) positive cell density was increased in the midsubstance and origin respectively, relative to naïve tendons. Decorin positive cell density (d) was not changed with fatigue injury while aggrecan positive cell density (e) was increased in the midsubstance. DS cell density was correlated with both decorin and aggrecan cell density (f) while CS (g) and HA (h) cell density were not correlated with decorin or aggrecan. #$p \leq 0.10$, *$p \leq 0.05$

Microscale Shear Strain with Fatigue Injury

Microscale shear strain testing was performed to assess baseline changes in tendon microscale mechanical behaviors with fatigue injury before consideration of the effects of increased GAGs. As expected, applying increasing bulk shear strain to tendons increased microscale shear strain (Fig. 4a, b). Fatigue injured tendons had decreased microscale shear strain response to applied shear strain when compared with naïve tendons (Fig. 4a, b). No significant differences at specific applied shear strains were found.

a



b

Two-way ANOVA		
Source of Variation	% of total variation	
Strain x Fatigue	2.218	p=0.2
Strain	33.12	***p=0.0004
Fatigue	11.96	#p=0.1

Fig. 4. Microscale shear strain (a) varied with increasing applied bulk shear strains (b). Overall microscale shear strain was decreased fatigue injured tendons relative to naïve control tendons (b).

Bulk Viscoelasticity with Fatigue Injury

Dynamic tensile testing was performed to assess baseline changes in tendon viscoelastic properties with fatigue injury before consideration of the effects of increased GAGs. As expected, fatigue injury had a significant effect on dynamic modulus at every frequency at both 4% (Fig. 5a) and 6% strain (Fig. 5b). However, fatigue injury did not significantly alter the linear relationship between dynamic modulus and frequency as evidenced by similar slope in naïve and fatigue injured tendons, suggesting that fatigue injury did not alter strain-rate dependent viscoelasticity. At 4% strain, 0.1 Hz, loss tangent was significantly higher in fatigue injured tendons, suggesting

that the viscous component of dynamic modulus was greater at low strain-rate in fatigue injured tendons (Fig. 5c).

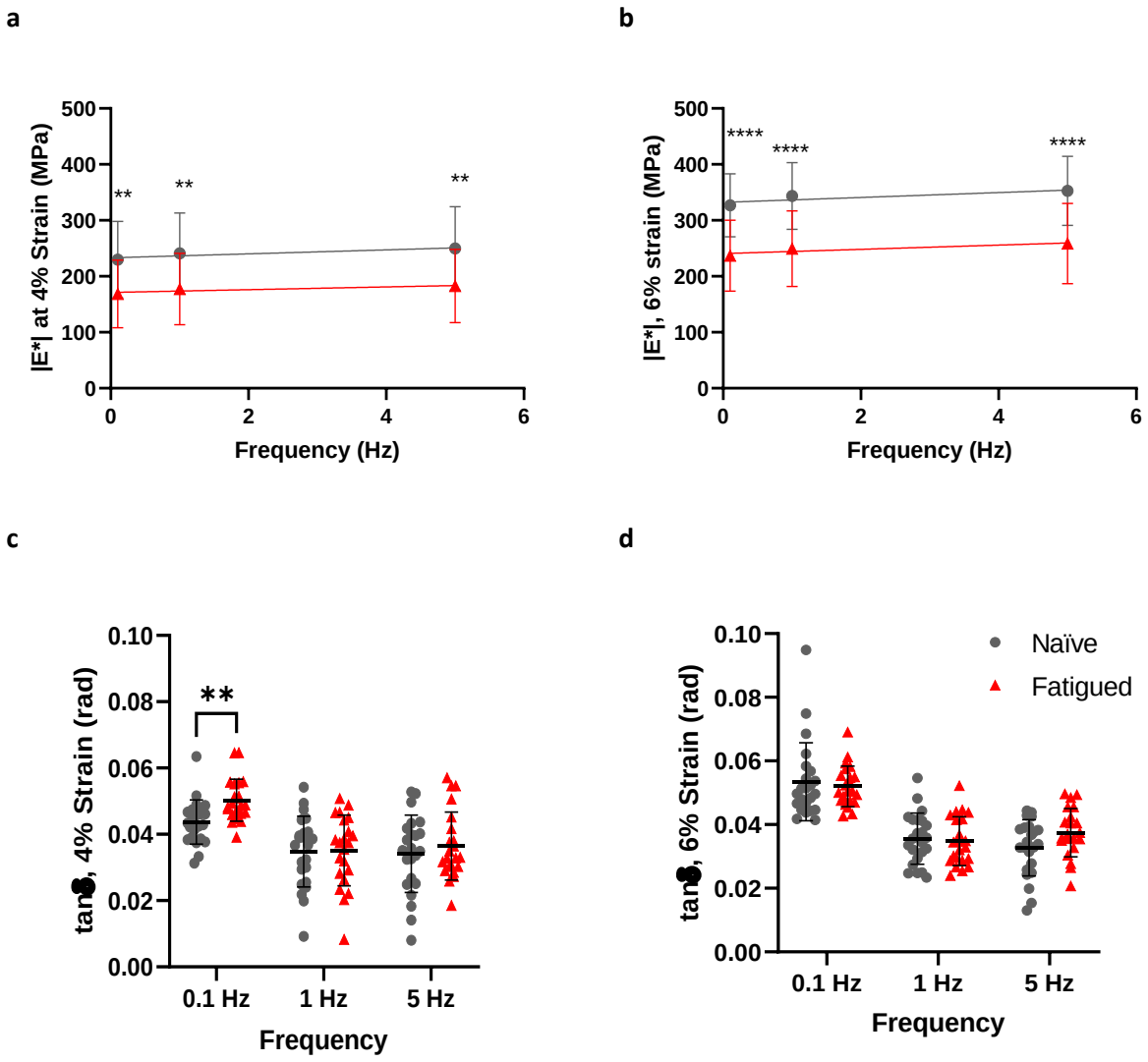
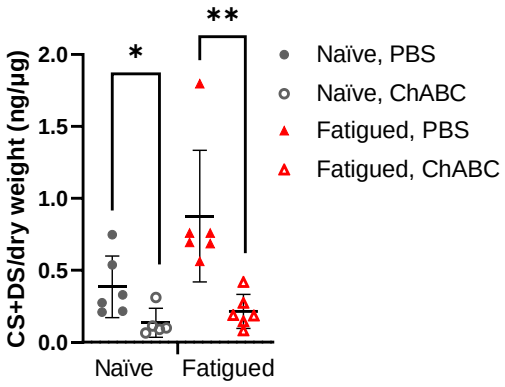


Fig. 5. Dynamic modulus (|E*|) at 4% strain (a) and 6% strain (b) were significantly decreased with fatigue injury at every frequency in a frequency independent manner. Loss tangent (tanδ) at 4% strain (c) was increased at 0.1 Hz with fatigue injury, suggesting higher viscous component of dynamic modulus at low strain-rate. Loss tangent at 6% strain was unaltered with fatigue injury (d). **$p \leq 0.01$, ****$p \leq 0.0001$

Bulk GAG Removal with Enzymatic Treatment

To confirm that the mechanical role of GAGs could be assessed by enzymatic removal, FACE was performed on naïve and fatigue injured tendons treated with PBS and ChABC, ChB, or HAase. As expected, FACE quantification of CS+DS confirmed that ChABC (Fig. 6a) and ChB treatment (Fig. 6b) reduced the concentration of CS+DS in both naïve and fatigue injured tendons. HAase similarly reduced HA in fatigue injured tendons while maintaining low amounts of HA in naïve controls (Fig. 6c).

a



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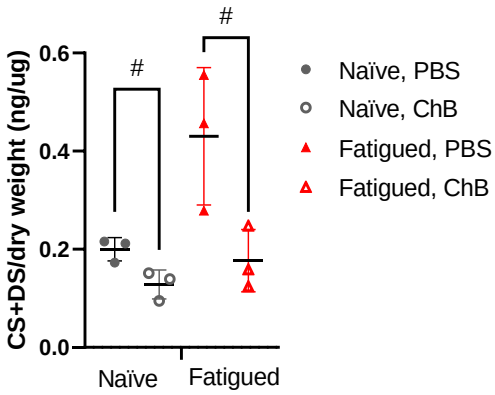

c

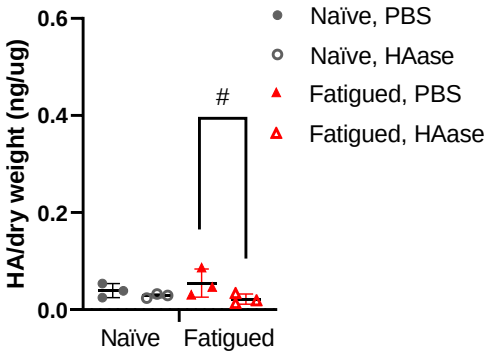

Fig. 6. CS+DS concentrations were reduced with ChABC (a) and ChB treatment (b) in both naïve and fatigue injured tendons. HA concentrations were reduced with HAase treatment (c) in fatigue injured tendons and remained low in naïve tendons. #p ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01

Microscale Shear Strain with GAG Removal

The change in microscale shear strain with GAG removal was compared between fatigue injured and naïve tendons to assess the microscale mechanical role of the post-injury GAG increase. As expected, the change in microscale shear strain in response to applied shear strain due to GAG removal with ChABC treatment was significantly positive at 0.24 strain (Fig. 7a), indicating that GAG removal generally increased microscale shear strain response at high strain. This GAG removal-dependent increase was greater in fatigue injured tendons at 0.24 rad applied strain when compared with naïve tendons. Change in microscale shear strain with PBS treatment (Fig. 7b) was no different between naïve and fatigue injured tendons. This finding suggests that there is a GAG-dependent decrease in microscale shear strain after fatigue injury that is most apparent at higher shear strain.

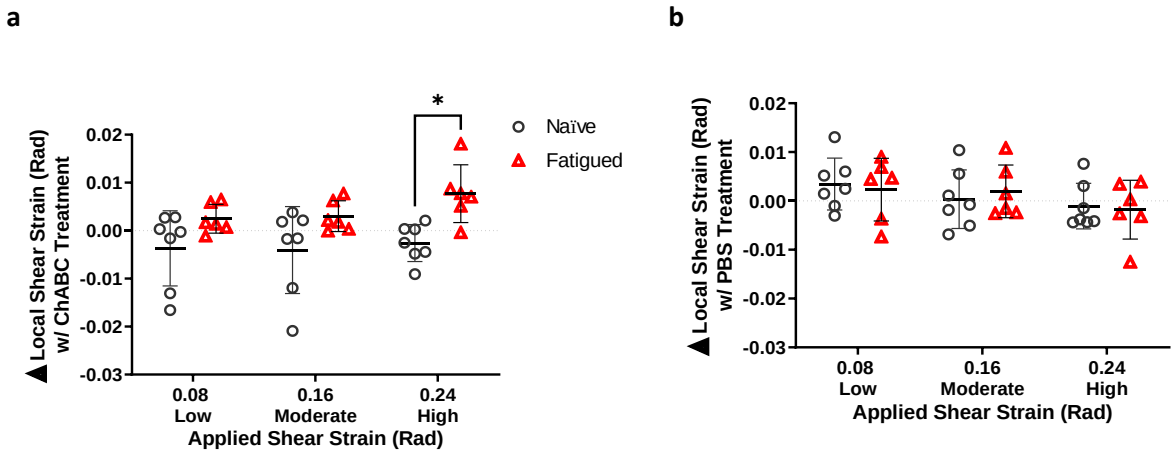

Fig. 7. Change in microscale shear strain with ChABC treatment (a) was greater in fatigue injured tendons at 0.24 rad strain relative to naïve tendons. Microscale shear strain change with PBS treatment (b) was no different between naïve and fatigue injured tendons. *$p \leq 0.05$

Bulk Viscoelasticity with GAG Removal

Dynamic tensile tests were performed before and after GAG removal with ChABC treatment of naïve and fatigue injured tendons to determine role of the post-injury GAG increase on viscoelastic properties including strain-rate dependence of dynamic modulus and loss tangent. Dynamic modulus was significantly reduced with GAG removal at every frequency in fatigue injured tendons only at 4% strain (Fig. 8a) but decreased in both naïve and fatigue injured tendons at 6% strain (Fig. 8b). Frequency or strain-rate dependence of dynamic modulus was not affected by GAG removal in naïve or fatigue injured tendons at either strain, as evidenced by unchanged slope before and after treatment. GAG removal increased loss tangent at 4% strain in both naïve and fatigue injured tendons at 5 and 1 Hz respectively (Fig. 8c). At 6% strain, GAG removal significantly increased loss tangent at 1 Hz in fatigue injured tendons only (Fig. 8d). These findings suggest that in naïve tendons, GAGs contribute to dynamic modulus at in the linear region (6% strain) and reduce the viscous component of dynamic modulus in the toe region (4% strain) at high strain-rate (5 Hz). With the increase in GAGs after fatigue injury, the role of GAGs is expanded, contributing to toe region dynamic modulus and reducing the viscous component of dynamic modulus at lower strain-rate and in the linear region.

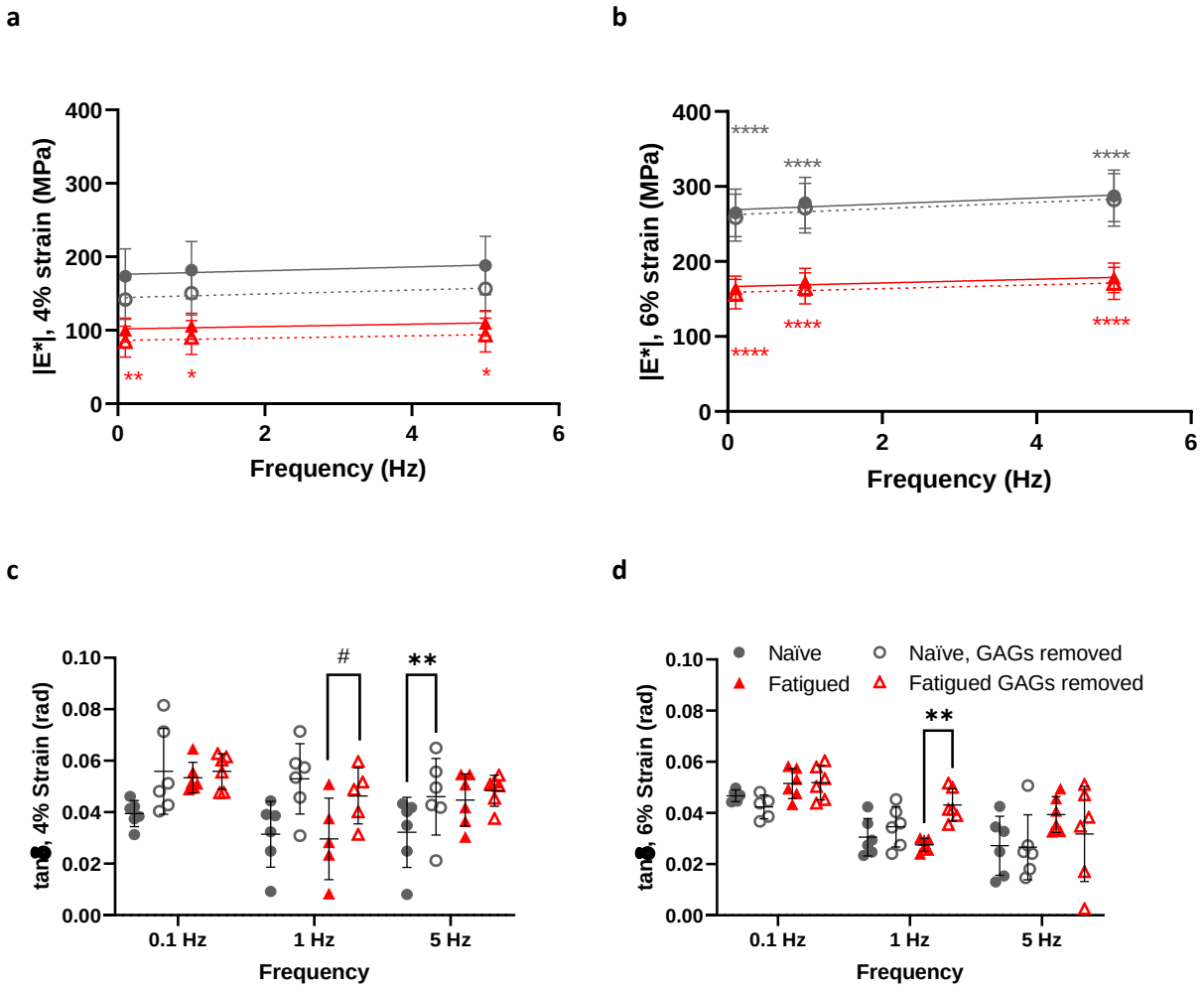

Fig. 8. Dynamic modulus (|E*|) with GAG removal using ChABC treatment was reduced at 4% (a) and 6% strain (b) in fatigue injured tendons at every frequency. Loss tangent (tanδ) at 4% strain (c) was increased at different frequencies in naïve and fatigue injured tendons and increased at 6% strain (d) at 1 Hz in fatigue injured tendons only. #$p \leq 0.10$, **$p \leq 0.01$, ****$p \leq 0.0001$

Bulk Viscoelasticity with DS Removal

Dynamic tensile tests were performed before and after DS removal using ChB treatment of naïve and fatigue injured tendons to determine the specific role of the post-injury DS increase on viscoelastic properties. DS removal reduced dynamic modulus at every frequency at both 4% (Fig. 9a) and 6% strain (Fig. 9b) in fatigue injured tendons, suggesting that the increase in DS after fatigue injury increases dynamic modulus. DS removal increased loss tangent at 6% strain (Fig. 9d) at 5 Hz in naïve tendons but did not affect fatigue injured tendons at any frequency or strain. This finding suggests that DS limits the viscous component of dynamic modulus in naïve tendons in the linear region (6% strain) at high strain-rate (5 Hz), but this effect is lost with fatigue injury despite greater amounts of DS after injury.

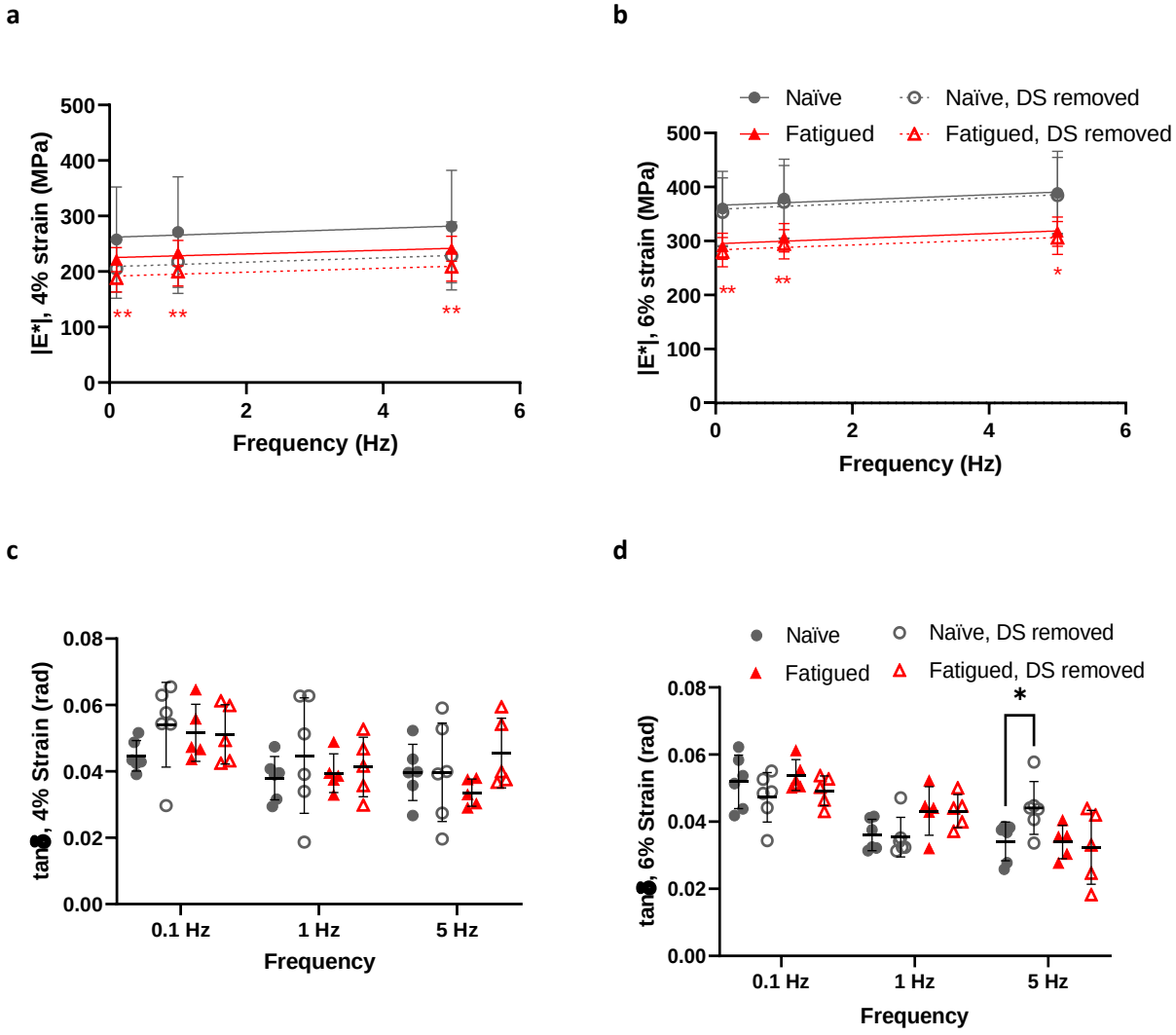

Fig. 9. Dynamic modulus (|E*|) with DS removal using ChB treatment at 4% strain (a) and 6% strain (b) was decreased in fatigue injured tendons. Loss tangent (tanδ) with DS removal was unchanged 4% strain (c) but increased at 6% strain (d) at 5 Hz frequency in naïve tendons only. *$p \leq 0.05$, **$p \leq 0.01$

Bulk Viscoelasticity with HA Removal

Dynamic tensile tests were performed before and after HA removal using HAase treatment of naïve and fatigue injured tendons to determine the specific role of the post-injury HA increase on viscoelastic properties. HA removal with HAase treatment decreased dynamic modulus at 4%

strain (Fig. 10a) at every frequency in fatigue injured tendons but not naïve tendons, suggesting that the increase in HA with fatigue injury contributes to dynamic modulus in the toe region. HA removal significantly increased loss tangent at 4% strain (Fig. 10c) at 0.1 and 1 Hz in naïve tendons and 5 Hz in fatigue injured tendons. This suggests that the increase in HA with fatigue injury shifted the contribution of HA to higher strain-rate (5 Hz) in the toe region (4% strain).

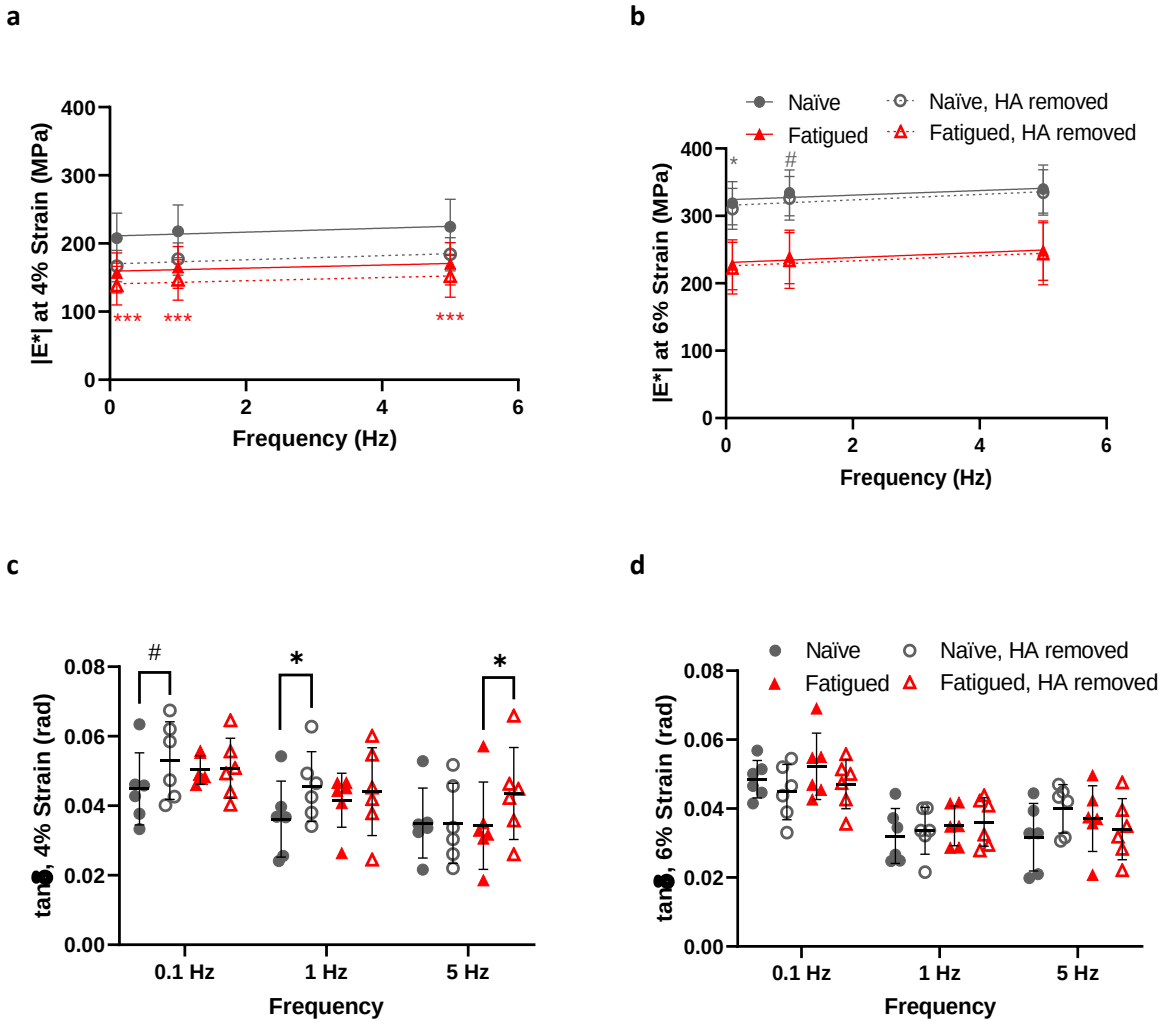

Fig. 10. Dynamic modulus (|E*|) with HA removal using HAase treatment decreased at 4% strain (a) but not 6% strain (b) in fatigue injured tendons only. Loss tangent (tanδ) with HA removal at 4% strain (c) increased at 0.1 and 1 Hz in naïve tendons and 5 Hz in fatigue injured tendons and unchanged at 6% strain (d). #$p \leq 0.10$, *$p \leq 0.05$, ***$p \leq 0.001$

Discussion

We have shown that the increase in GAGs after tendon fatigue injury may result in reduced microscale strains and altered viscoelastic behaviors. These GAG-dependent mechanical changes may influence mechanotransduction, influencing the reparative response to loading after fatigue injury. This study revealed the increase in GAGs with fatigue injury corresponds with increased decorin-associated DS in the midsubstance and insertion, along with increased pericellular CS, aggrecan, and HA in the midsubstance and origin. With the removal of these GAGs using ChABC, ChB, and HAase, microscale shear strain, dynamic modulus, and loss tangent were modulated in fatigue injured tendons in ways not seen in naïve tendons, suggesting the GAG increase with fatigue injury modulates multiscale mechanical properties and viscoelasticity.

Reduced microscale matrix strain and an increased viscous component of dynamic modulus with increased GAGs after fatigue injury suggests that the cell micromechanical environment and mechanotransduction are also altered during this period. This could explain cell behaviors known to change with fatigue injury, including expression of matrix remodeling associated genes[3], apoptosis[13], and inflammation[14]. These findings also suggest that the increase in GAGs limits shear strain transfer, potentially preventing cells from overloading during post-fatigue recovery exercise while still enabling the important cell-matrix interactions that promote remodeling[1,2]. These GAG-dependent changes in multiscale mechanical properties may also be linked to the observed changes in viscoelasticity through modulation of fluid motion and fiber sliding.

GAGs appeared to increase dynamic modulus in the toe region (4% strain) in fatigue injured tendons, suggesting a role for increased GAGs in fiber uncrimping behavior typical in the toe region. Increased GAGs in fatigue injured tendons decreased loss tangent in the linear region (6% strain) at moderate strain-rate (1 Hz), suggesting a role for GAGs in modulating viscoelasticity

during fibril sliding and strain transfer that occurs with linear region strain. HA reduced loss tangent in the toe region (4% strain) at high strain-rate (5 Hz), making high strain-rate uncrimping more efficient. Interestingly, a reduction in loss tangent with increased GAGs did not occur at low strain rate (0.1 Hz), where loss tangent was increased with fatigue injury. Contrary to our hypothesis, this suggests that GAGs do not necessarily increase to recover naïve viscoelastic properties but do appear to alter moderate and high strain rate behavior. While increased dynamic modulus and reduced loss tangent with increased GAGs are physiologically relevant behaviors affecting tendon function[15], altered viscoelastic properties may also modulate the mechanotransduction and mechanical signaling that influences cell response to loading.

Increases in DS and decorin with fatigue injury reflect previous findings that decorin expression is stimulated by tensile loading [16] and reparative exercise of fatigue injured tendons[17]. Decorin, a small leucine rich proteoglycan with a single GAG chain composed primarily of DS[18], is the most common PG in healthy tendons, accounting for 80% of total PG content. The mechanical role of decorin and DS on mechanics of tendon has been studied at multiple scales, with suggestions that their close association with collagen fibrils may enable strain transfer[5] or sliding between fibrils[19]. These findings suggest a similar role for DS in reducing the viscous component of dynamic modulus and microscale shear strain. Surprisingly, despite showing an effect at high strain rate (5 Hz) in the linear region in naïve tendons, increased DS with fatigue injury did not appear to alter viscoelasticity in fatigue injured tendons. This suggests a disruption of DS's mechanical role with fatigue injury or suggests that CS and HA may play a greater viscoelastic role after injury. Interestingly, the increase decorin positive ECM area in the insertion was also associated with a decrease in pericellular DS in the insertion and origin. In these areas, increased decorin may play a non-mechanical role in regulating calcification[20], collagen fibrillogenesis[21] fiber size and

organization[22], and binding of growth factors[23], which are relevant to repair and should be investigated in future work.

Besides decreased DS, other pericellular changes in GAGs with fatigue injury included increases in CS and aggrecan in the midsubstance and HA in the origin. Given the association between HA and aggrecan and the pericellular matrix in tendons, increased HA and CS post-injury could enhance mechanotransduction in the cell-scale viscoelastic environment[24], explaining differences in the cellular repair response with delayed reparative exercise[1]. HA may also serve a biological role in fatigue injured tendons, as it is closely associated with inflammation through interaction with CD44 and regulation of cytokines[25]. Pericellular HA-cell interactions also precede the formation of integrins and focal adhesions[26], which play an important role in mechanotransduction and tendon repair in response to exercise[2].

In conclusion, this study demonstrates that the location specific increases in GAGs after fatigue injury modulate the multiscale mechanical and viscoelastic properties of tendons. Decorin and DS in the insertion and midsubstance and CS and HA in the pericellular matrix may be used as diagnostic or therapeutic targets to modulate mechanotransduction and enable reparative exercise and loading after fatigue injury. Future work should consider the role of post-injury, pericellular and location specific GAGs in modulating mechanisms for mechanotransduction, including integrins and connexins, cytoskeletal and nuclear strains, and other downstream mechanobiological pathways.

Supplemental

Bulk Viscoelasticity with PBS Treatment

With PBS treatment, there was no significant variation in dynamic modulus at 4% strain (Fig. S1a) in fatigue injured tendons. In contrast, PBS treatment reduced dynamic modulus at 6% strain (Fig. S1b) at every frequency. PBS treatment did not significantly alter the frequency dependence of dynamic modulus at 4% or 6% strain in fatigue injured tendons. Loss tangent was unaltered by PBS treatment at both 4% (Fig. S1c) and 6% strain (Fig. S1d).

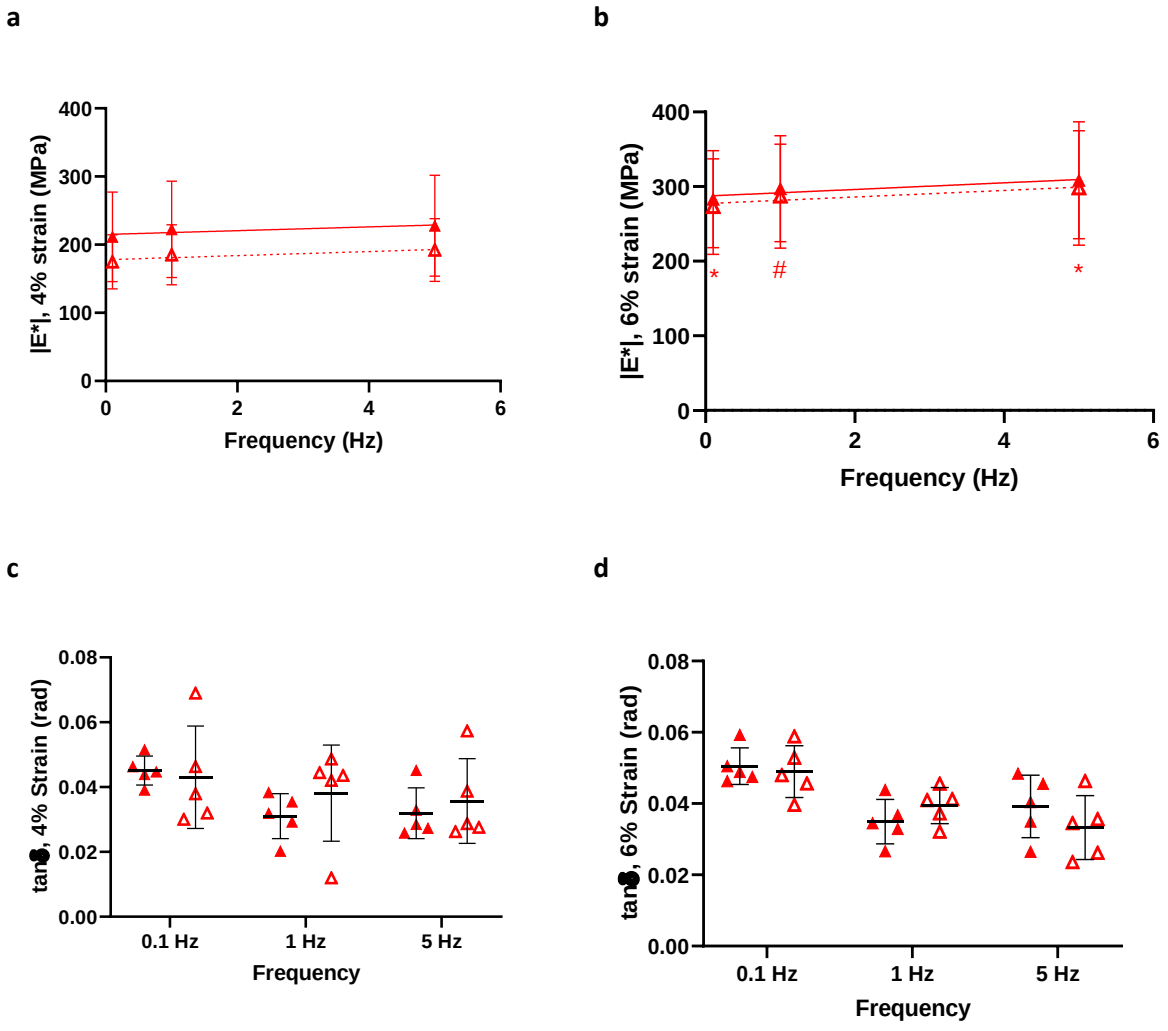

Fig. S1. Dynamic modulus at 4% strain (a) with PBS treatment was unaltered in fatigue injured tendons. Dynamic modulus at 6% strain (b) with PBS treatment was reduced at every frequency. Loss tangent at 4% (c) and 6% (d) strain were unaltered with PBS treatment. #$p \leq 0.10$, *$p \leq 0.05$

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Original Article

Post-Fatigue Injury Glycosaminoglycans Limit Tenogenic Phenotype and Modify Repair

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Introduction

Tendinopathies are debilitating conditions with limited therapeutic options. These conditions occur when damage accumulation from overuse outpaces repair, eventually leading to pain and loss of function. The progression from early-stage overuse to late-stage chronic disease is poorly understood, limiting early diagnostic and preventative treatment strategies. Previous studies have shown that a single bout of tendon fatigue loading results in reduced upregulation of remodeling associated genes[1] and an increase in apoptosis[2], which hinders recovery of microscale collagen organization and bulk mechanical properties[3,4]. Overuse injury is also associated with an increase in glycosaminoglycans (GAGs)[5], highly charged polysaccharide chains often attached to protein cores to form proteoglycans (PGs). However, whether this increase in GAGs is simply a disease outcome or a mediator of tendon repair remains unknown.

Given their interfibrillar and pericellular localization, GAGs can affect a wide range of biological activities in tendons. Dermatan sulfate (DS), a GAG associated with the PG decorin, modulates multiscale tendon mechanics by facilitating fibril sliding[6,7] and viscoelasticity[8], ultimately influencing mechanotransduction and response to load. Decorin also inhibits collagen fibrillogenesis[9], regulates fiber size and organization[10], and suppresses calcification[11]. Correspondingly, decorin expression is stimulated by tensile load[12] and decorin and DS are associated with tissues under tension such as skin and the tendon midsubstance[13]. Hyaluronic acid (HA) GAGs interact with cell surface proteins CD44 and RHAMM, resulting in downstream effects on expression of matrix components and inflammatory cytokines[14,15]. HA interactions with CD44 also mediate apoptosis[15], and high molecular weight HA can protect cells from damage due to reactive oxidative species (ROS). Given its mediating role between cells and their surrounding

matrix, HA is often associated with the pericellular matrix of cells[16]. GAGs can also bind growth factors and cytokines, serving as a co-factor or regulating availability[17].

The diverse role of GAGs and their associated structures and interactions in healthy and injured tendons suggest that they may play a role in tendon repair. We have previously shown that exercise can induce repair in fatigue injured tendons, but only when initiated after a post-injury increase in GAGs[3,4]. We hypothesize that an increase in GAGs after fatigue injury promotes a tenogenic phenotype and reduces apoptosis, enabling a reparative response to load. To test this hypothesis, we induced fatigue injury in the patellar tendons of rats as a model for early-onset, sub-rupture tendinopathy. In the period after injury, we disrupted the increase in GAGs with continuous *in vivo* treatment with chondroitinase b or hyaluronidase to enzymatically reduce DS and HA respectively. We expected that both DS and HA enzymatic disruption would alter bulk tensile mechanical properties in the weeks after injury due to reduced tenogenic phenotype and increased apoptosis.

Methods

Fatigue Loading Injury Model

Experiments were approved and performed in accordance with the Institutional Animal Care and Use Committee at Cornell University. Sprague-Dawley rats aged 9-11 months were anesthetized and administered buprenorphine. As previously described[18], incisions on the left leg were made to expose the patella and tibia. The patella and tibia were gripped and connected to 50lbf load cell on a mechanical test instrument (Electroforce 5500) in a PBS bath at room temperature, and the patellar tendon was fatigue loaded at 1 Hz from 1 to 40 N for 7200 cycles. Diagnostic testing was performed pre- and post-fatigue loading, consisting of loading at 1 Hz from 1 to 15 N for 420 (pre)

or 120 (post) cycles. Diagnostic parameters previously described[18] were used to ensure induced damage was similar across groups.

Disruption of GAG Increase with Continuous In Vivo

After fatigue loading, osmotic pumps (200µL 0.5µL/hr, Alzet) filled with chondroitinase B (ChB) (from *flavobacterium heparinum*, 40 U/mL, SigmaAldrich), hyaluronidase (HAase) (from *streptomyces hyalurolyticus*, 100 U/mL), or PBS were inserted subcutaneously via the initial incision. Catheters attached to the pump were sutured to the fascia lateral to the tendon to ensure the catheter opening stayed near the center of the tendon. Incisions were sutured, and rats resumed normal cage activity. Animals were sacrificed either after 14 days (for GAG quantification or ELISA) or 56 days (for tensile testing). Animals sacrificed 56 days after injury had osmotic pumps and catheters removed after 21 days via a small incision after anesthetization and buprenorphine administration.

GAG Quantification

After sacrifice 14 days post-injury, GAGs were quantified using fluorophore assisted carbohydrate electrophoresis (FACE) (n=6/treatment). Tendons were separated from tibia and patella and were lyophilized, pulverized, and digested in 10x proteinase K for 24hr at 60°C. After precipitation in ethanol, GAGs were depolymerized with chondroitinase ABC and fluorotagged by 2-aminocridone (AMAC). Acrylamide gels were loaded with samples and prepared disaccharide standards (ΔdiHA, Δdi0S, Δdi4S, and Δdi6S, AMSBIO) and electrophoresis was run at 150 V for 75 minutes to separate bands. Gels were imaged on a ChemiDoc system and GAGs were quantified by comparing sample band optical density with disaccharide standard bands. Final amounts of hyaluronic acid (HA) and chondroitin and dermatan sulfate (CS+DS) were normalized to dry weight.

Bulk Viscoelastic Tensile Testing

At 56 days after fatigue injury (n=6/treatment), animals were sacrificed and patella-tendon-tibia complexes from fatigued and contralateral limbs were isolated and cross-sectional area was measured with calipers. The patella and tibia of each sample was gripped, attached to a 50lbf load cell on a mechanical test instrument (Electroforce 5500) in a PBS bath at room temperature, and preloaded to 0.1 N. Following preconditioning, stress relaxation was assessed by ramping to 4% strain, corresponding with the toe region of the stress-strain curve, and held for 300s. A frequency sweep consisting of 10 cycles of 0.125% amplitude sinusoidal strain (0.1, 0.5, 1, 2, and 5 Hz) spanning the physiological stride frequency of rats was then applied[8]. Stress relaxation and frequency sweep were then repeated at 6% and 8% strain corresponding with the low and high linear regions of the stress-strain curve. Dynamic modulus (|E*|) and loss tangent (tanδ) were determined at each strain and frequency. The frequency dependence of dynamic modulus was calculated using linear regression for slope to determine, viscoelastic, strain-rate dependent response. We expected dynamic modulus to monotonically increase with increasing frequency within the range of tested[8]. Finally, a pull-to-failure test was conducted at a rate of 0.3%/second to determine stiffness and maximum load.

Tenogenic phenotype and apoptosis measurement

At 14 days after fatigue injury (n=8/treatment), tenogenic phenotype and apoptosis were measured using ELISA assays to determine tenomodulin and caspase-3 amounts in each tendon. Tendons were separated from the patella and tibia, snap frozen, and minced. Samples were placed in tendon protein extraction buffer (Invent Biotech), pulverized, and centrifuged, with supernatant taken for ELISA assay. Rat tenomodulin and caspase-3 ELISA kits (LSBio) were performed to determine protein amounts in each sample.

Statistical analysis

Unpaired t-tests were performed to confirm increases in HA and CS+DS and determine changes in tenomodulin and caspase-3 in fatigue injured, PBS treated tendons relative to naïve controls Unpaired t-tests were also used to assess decreases in HA and CS+DS with ChB and HAase treatment relative to PBS treated controls. Ordinary one-way ANOVA tests with Dunnett's multiple comparison to PBS treated controls were performed to determine changes in tenomodulin and caspse-3 with ChB or HAase treatment. Two-way ANOVA tests with Dunnett's multiple comparison to PBS treated tendons were performed to assess changes in dynamic modulus and loss tangent with fatigue injury or ChB and HAase treatment at each frequency. Linear regression and ANCOVA comparison slope were used to compare differences in frequency or strain-rate dependence of dynamic modulus between PBS and ChB or HAase treated tendons.

Results

GAG Reduction with ChB and HAase Treatment

As previously observed, fatigue injured tendons had higher concentrations of HA and CS+DS two weeks after fatigue injury when compared with naïve tendons (Fig. 1a). With 2 weeks continuous *in vivo* treatment of fatigue injured tendons with both ChB and HAase, CS+DS was reduced when compared to tendons treated with PBS (Fig. 1b, c). Surprisingly, HA was not significantly reduced with HAase treatment when compared with PBS treatment (Fig 1c).

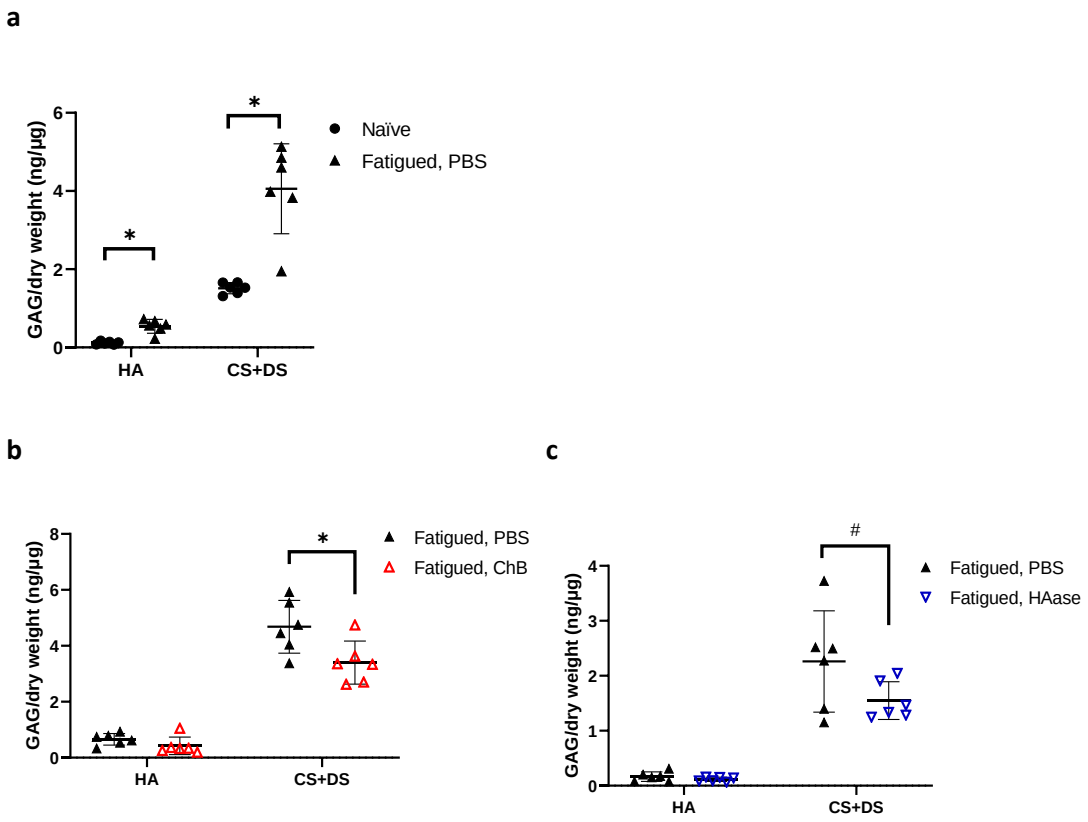

Fig. 1. Hyaluronic acid (HA) and chondroitin and dermatan sulfate (CS+DS) concentrations (a) were both increased in fatigue injured tendons treated with PBS relative to naïve tendons. CS+DS concentrations were decreased with continous *in vivo* ChB (b) and HAase (c) treatments after fatigue injury relative to PBS treated tendons. #p ≤ 0.10, *p ≤ 0.05

Tenogenic Phenotype and Apoptosis with GAG Removal

Fatigue injured tendons treated with PBS for two weeks had increased amounts of tenomodulin relative to naïve controls (Fig. 2a). CS+DS reduction with HAase treatment increased tenomodulin to a greater extent than fatigue injury and PBS treatment alone (Fig. 2a), suggesting that a further increase in tenomodulin with fatigue injury is limited by a concurrent increase in GAGs. Surprisingly, ChB treatment did not change tenomodulin amounts despite causing similar reductions in CS+DS with HAase treatment (Fig. 2a), suggesting an alternative mechanism for

tenomodulin upregulation besides CS+DS reduction. As previously observed, fatigue injury increased caspase-3 relative to naïve controls (Fig. 2b). However, neither ChB nor HAase treatment significantly changed caspase-3 amounts relative to PBS treated tendons (Fig. 2b). These findings suggest that CS+DS reduction with HAase treatment increases tenogenic phenotype but does not alter apoptosis in fatigue injured tendons.

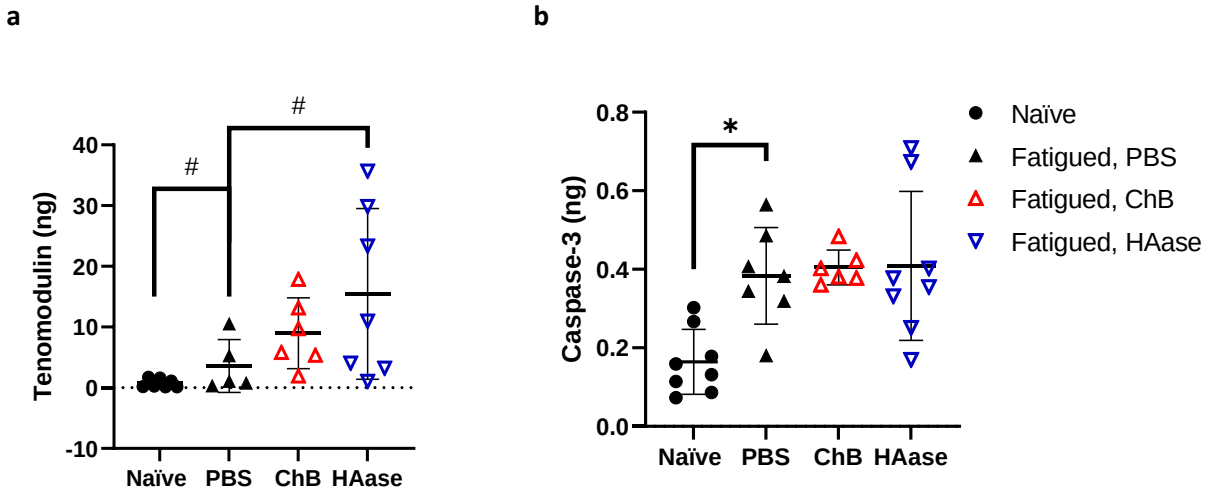

Fig. 2. Tenomodulin (a) and caspase-3 (b) amounts were increased in fatigue injured tendons treated with PBS relative to naïve tendons. Tenomodulin increased with continous *in vivo* HAase treatment after fatigue injury (a) relative to PBS treated tendons. #$p \leq 0.10$, *$p \leq 0.05$

Dynamic Modulus with Fatigue Injury and In Vivo GAG Reduction

While dynamic modulus 8 weeks after fatigue injury was reduced at every strain and frequency relative to naïve tendons, at no strain or frequency was dynamic modulus affected by CS+DS removal with ChB or HAase treatment relative to PBS treatment (Fig. 3a, b, c). Dynamic modulus frequency or strain-rate dependence was not altered with fatigue injury at 4 or 6% strain, as evidenced by similar slopes between naïve and fatigue injured tendons treated with PBS (Fig. 3a, b). Dynamic modulus frequency dependence was also unaltered with CS+DS removal using ChB or HAase treatment (Fig. 3a, b, c). These findings suggest that reduction of CS+DS in the weeks

after fatigue injury do not alter dynamic modulus at any frequency or affect strain-rate dependence of dynamic modulus.

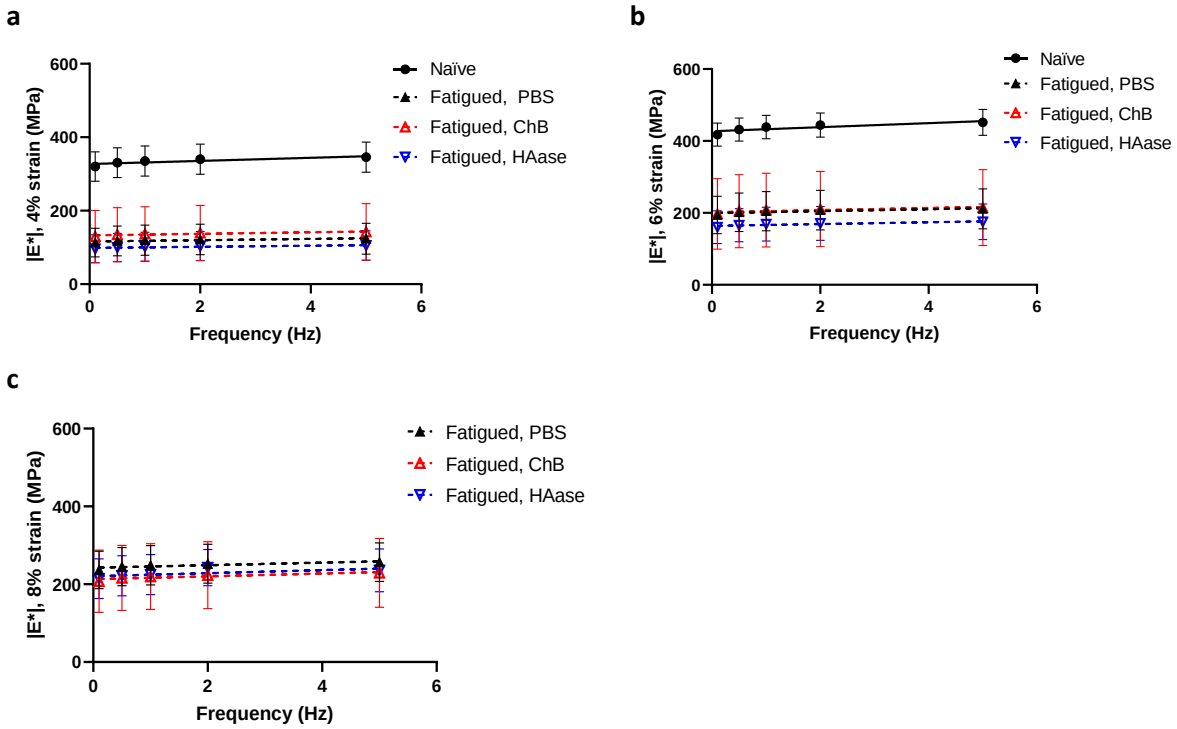

Fig. 3. Dynamic modulus (|E*|) was reduced at 4% (a) and 6% strain (b) in fatigue injured tendons relative to naïve tendons. Dynamic modulus at 4% (a), 6% (b), and 8% (c) strain was unaltered in fatigue injured tendons with CS+DS reduction after ChB and HAase treatment.

Loss Tangent with Fatigue Injury and In Vivo GAG Reduction

Loss tangent 8 weeks after fatigue injury was increased with fatigue injury at 4% strain and 2 and 5 Hz frequency relative to naïve tendons, suggesting fatigue injury increased the viscous component of dynamic modulus (Fig. 4a). CS+DS reduction for 2 weeks with continuous *in vivo* ChB treatment reduced loss tangent at 4% strain and 5 Hz frequency, returning the viscous component of dynamic modulus to naïve levels (Fig. 4a). In contrast, at 8% strain and 1 Hz frequency, CS+DS reduction with ChB and HAase treatment increased loss tangent and the viscous component of dynamic modulus (Fig. 4c). These findings suggest that CS+DS disruption

in the weeks after fatigue injury reduced the viscous component of dynamic modulus to naïve levels in the toe region (4% strain) at high strain-rate (5 Hz) while increasing the viscous component of dynamic modulus in the linear region at moderate strain-rate (1 Hz).

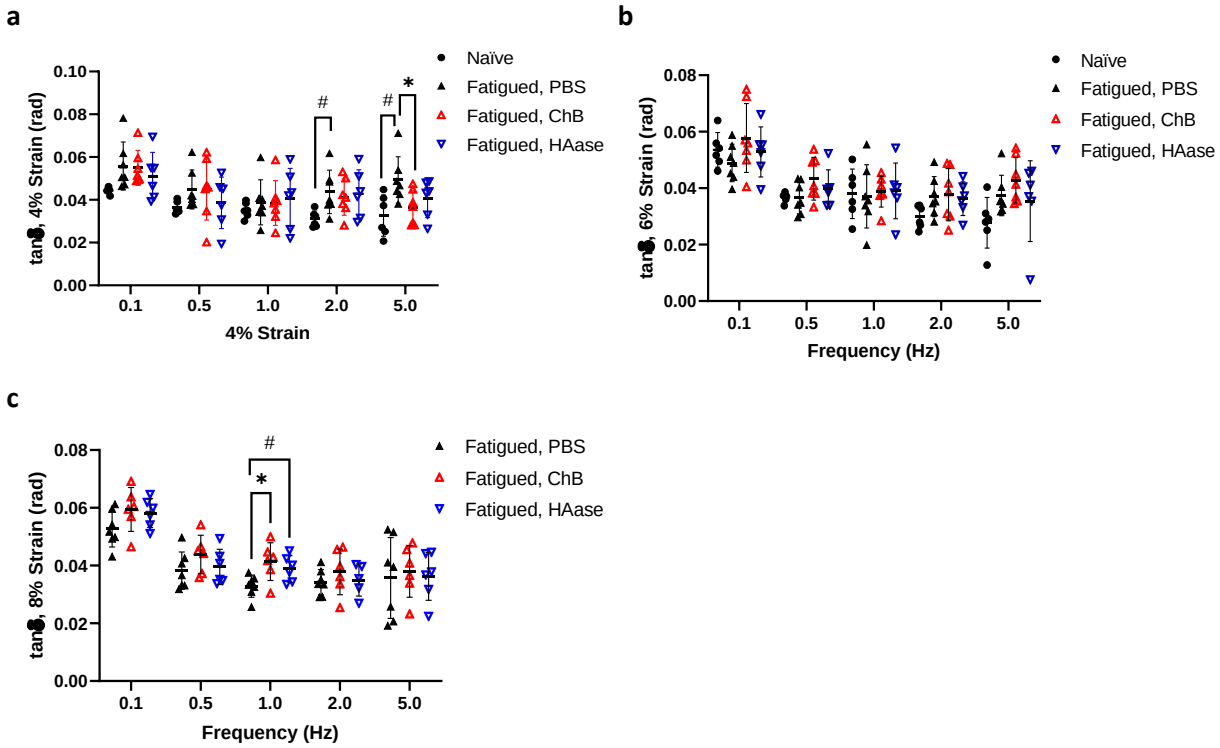

Fig. 4. Loss tangent (tanδ) at 4% strain (a) was increased at 2 and 5 Hz frequency in fatigue injured tendons treated with PBS relative to naïve tendons but returned to naïve levels with ChB treatment. Loss tangent at 6% strain (b) was unaltered with fatigue injury and ChB and HAase treatment. Loss tangent at 8% strain (c) at 1 Hz was increased with both ChB and HAase treatment. #$p \leq 0.10$, *$p \leq 0.05$

Pull to Failure Properties with Fatigue Injury and GAG Reduction

As expected, modulus (Fig. 5a), stiffness (Fig. 5b), and max stress (Fig. 5c) were reduced 8 weeks after fatigue injury with PBS treatment. However, ChB and HAase treated tendons had no changes in these parameters relative to the PBS treated group.

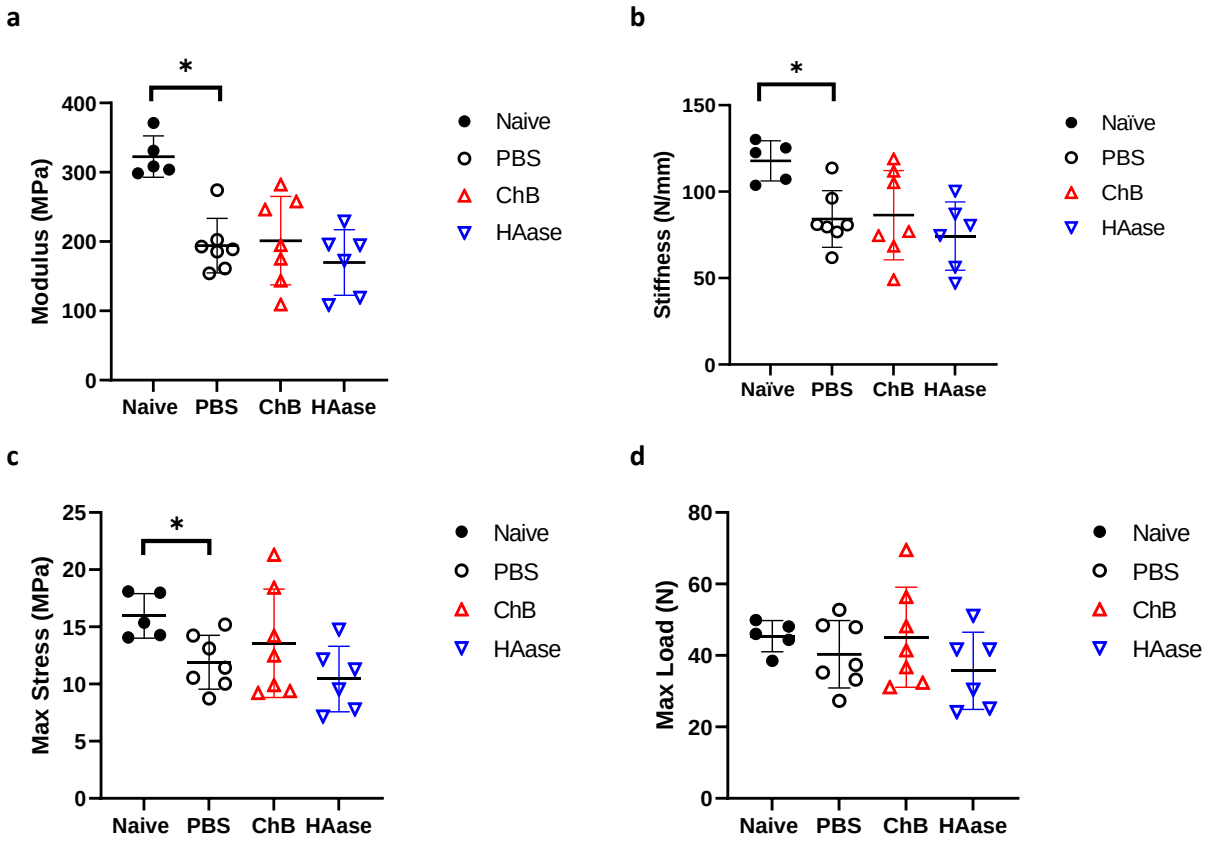

Fig. 5. Modulus (a), stiffness (b), and max stress (c) were reduced by fatigue injury and PBS treatment relative to naïve tendons. Max load (d) was unaltered with fatigue injury. CS+DS reduction with ChB and HAase treatment did not alter these properties relative to PBS treatment. *$p \leq 0.05$

Discussion

We have shown that the increase in glycosaminoglycans in the first two weeks after tendon fatigue injury may be involved in modulating tenogenic phenotype and behavior. This GAG driven influence on phenotype may affect viscoelastic mechanical properties in the weeks following fatigue injury that influence repair in response to exercise. When the increase in CS+DS associated with fatigue injury was limited in the first two weeks after injury using HAase treatment, tenomodulin was increased, suggesting that increased GAGs and their associated interactions limit

tenogenic phenotype after injury. While this contrasts with the previous finding that inhibition of CD44, a receptor for HA, reduced tenomodulin expression in tendinopathy[15], HAase treatment did not significantly reduce HA amounts in this study, suggesting that CD44-HA interactions were not necessarily disrupted. The turnover of HA is relatively quick (<24 hour half-life in cartilage[19]) and tendons have markedly higher turnover of GAGs relative to cartilage[20], suggesting HA may have been replaced faster than it could be depleted. In addition, HAase treatment in this study may have only disrupted the structure of HA or its interactions with cells and other matrix components without affecting the total disaccharide amount measured by FACE. The fact that ChB treatment did not have the same effect on tenomodulin as HAase treatment despite both treatments reducing CS+DS suggests that either HA disruption or off-target HAase effects, not DS reduction, resulted in the increase in tenogenic phenotype.

HAase treatment may have resulted in increased microscale matrix strains that occur with the removal of GAGs (Muljadi and Andarawis-Puri, 2022, in preparation), as greater strains in tenocytes have been correlated with increased expression of tenomodulin [21]. Interestingly, fatigue injury with PBS treatment alone increased tenomodulin relative to naïve control tendons. We have previously shown that fatigue injury reduces microscale strain (Muljadi and Andarawis-Puri, 2022, in preparation), suggesting that increased strains due injury do not drive this initial post-injury increase in tenomodulin. Alternatively, this tenomodulin increase may be an outcome from increased strains during fatigue loading itself, which is subsequently limited by reduced microscale strains due to the post-injury GAG increase.

Contrary to our hypothesis, any disruption of the pericellular matrix with both ChB and HAase GAG removal did not affect apoptosis as measured by caspase-3 levels. It is possible that interactions with other pericellular components, such as collagen VI[22], may be sufficient to prevent

further apoptosis even after reduction of GAGs. In addition, as previously noted, CD44-HA interactions important to inhibiting apoptosis may have not necessarily been disrupted by ChB or HAase treatment.

Loss tangent 8 weeks after fatigue injury with PBS treatment was higher in the toe region (4% strain) at high strain-rate (2-5 Hz) relative to naïve tendons, suggesting compositional and structural changes after fatigue injury increased the viscous component of dynamic modulus during fiber uncrimping that occurs in the toe region. With ChB treatment, loss tangent was reduced to naïve levels at this strain and frequency. This contrasts with the increase in loss tangent that occurred in the toe region with *ex vivo* treatment of fatigue injured tendons with ChABC and HAase (Muljadi and Andarawis-Puri, 2022, in preparation). These viscoelastic changes may be due to increased fiber size, reduced collagen organization[10], or increased calcification[11] occurring in the weeks after decorin and DS disruption by ChB treatment. Ultimately, these findings suggest that the increase in DS after fatigue injury results in matrix changes that alter fiber uncrimping behavior and increase the viscous component of dynamic modulus beyond naïve levels during high strain-rate activity.

Supporting our hypothesis, loss tangent 8 weeks after fatigue injury at high linear strain (8%) and moderate strain-rate (1 Hz) was increased with CS+DS reduction with both ChB and HAase treatment, suggesting that the disruption of GAGs resulted in an increased viscous component of dynamic modulus during fibril sliding and strain transfer occurring in the linear region. This corresponds with our previous finding that *ex vivo* GAG removal at 2 weeks after fatigue injury increases loss tangent in the linear region at 1 Hz. In addition to their own inherent mechanical contributions, the post-fatigue GAG increase may also result in other matrix changes that alter

viscoelasticity, including initial signaling for the formation of integrins and focal adhesions[23] which may have improved mechanotransduction and influenced repair[4].

In conclusion, the increase in GAGs with fatigue injury appears to limit tenogenic phenotype after fatigue injury. This GAG increase subsequently alters viscoelasticity, increasing loss tangent in the toe region while decreasing loss tangent in the linear region, either through inherent structural contributions or influencing matrix composition and structure in the weeks after fatigue injury. From a clinical standpoint, while disruption of post-injury GAGs may increase tenogenic phenotype, this may also modulate viscoelastic mechanical properties at multiple strains that contribute to tendon function and mechanobiology. Future work should consider the role that tenomodulin, viscoelastic mechanical properties, and other GAG influenced factors such as growth factors and inflammation play in mechanotransduction as well as downstream signaling of repair of early-onset subrupture tendon injuries.

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CONCLUSION

This dissertation has demonstrated how tendon overuse injuries and early-onset tendinopathy are mechanically and biologically modulated by an increase in glycosaminoglycans. This GAG increase is location and type dependent, with decorin-associated dermatan sulfate increasing in the tendon midsubstance ECM and chondroitin sulfate and hyaluronic acid increasing in the pericellular matrix after fatigue injury. We found that this increase in GAGs may alter the cell micromechanical environment, as the GAG increase reduces microscale strains and modulates bulk viscoelastic behaviors by increasing dynamic modulus and reducing loss tangent. This supports our hypothesis that the post-injury GAG increase influences mechanotransduction and repair. We also found that the increase in GAGs after fatigue injury limits tenomodulin, either due to reduced microscale strains, altered viscoelasticity, or direct interactions with cells. In the long term, these GAG-dependent mechanical and biological effects increase loss tangent in the toe region and decrease loss tangent in the linear region, further suggesting a role for the post-injury GAG increase in modulating tendon repair and influencing functional outcomes. Together, these findings suggest that GAGs could serve as a diagnostic or therapeutic target to enhance repair of tendon overuse injuries.

Contributions to the field

The work reviewed in the first chapter clearly identify changes in the tendon ECM as a driver of impaired mechanics and repair response after tendon overuse. These previous studies help explain the multiscale mechanical changes and impaired repair response observed in clinical tendinopathy and persistent damage leading to high retear rates after surgical repair. They also emphasize the need for diagnostic and therapeutic strategies that assess and restore damaged ECM in ways that

not only restore joint function, but also enable the multiscale mechanotransduction necessary to promote repair. Consequently, additional investigation into how unique tendon types, ECM compartments, and compositions are altered with overuse injury, as well as their contributions to upstream mechanical function and downstream biological repair response, is required. The original work in this dissertation aims to address these gaps through investigation of the multiscale mechanical and biological role of GAGs in fatigue injured tendons.

The role of GAGs in tendons is less established than that of collagen, and their increased presence in tendons is mainly associated with pathology, as demonstrated in animal models of acute injury or in patients with late-stage, chronic tendinopathy. However, any modulation of GAGs in early-onset tendinopathy was not previously well characterized. In the second chapter of this dissertation, we not only measured an increase in GAGs during early-onset tendinopathy, but also differentiated GAG and proteoglycan types in different tendon locations including the insertion to bone and pericellular matrix. These findings suggest diverse multiscale mechanical and biological roles for GAGs at earlier timepoints in the progression of tendinopathy than previously considered.

The role of GAGs in the transfer of macroscale loads to microscale deformations and cell responses during tendon mechanotransduction is an ongoing area of study. While the role of GAGs in mediating fibril sliding, fluid motion, and swelling had been considered in healthy and acutely injured tendons, the role of increased GAGs in combination with early-stage, subrupture damage was not well understood. In the second chapter of this dissertation, we measured scale and frequency dependent effects of increased GAGs, suggesting unique impacts of increased GAGs on multiscale phenomena after fatigue injury, including fiber crimping and sliding, microscale strain transfer, and viscoelasticity. This demonstrates an expanded role for GAGs during early-

onset tendinopathy that had not previously been observed or well characterized in healthy or diseased tendons.

In addition, the timing and conditions under which exercise or physical therapy is beneficial as opposed to harmful is a major hurdle to developing safer, earlier, and patient-specific therapeutic strategies for overuse tendon injures. In contrast the fatigue loading model used in this dissertation, previous models of overuse tendinopathy involving exercise could not control for systemic or joint level effects of exercise. Our finding that GAGs increase after fatigue injury suggests they may influence mechanotransduction during early-onset tendinopathy, as the GAG increase affects microscale strains and bulk viscoelastic properties, and reparative exercise is enabled only after these GAG-dependent mechanical changes occur.

Finally, GAGs have previously been shown to have important roles in cell phenotype and behavior, influencing fibrillogenesis, calcification, inflammation, and apoptosis. However, these biological roles for GAGs had mainly been assessed in acute injury and late-stage chronic tendinopathy models, which present with inflammation, proliferation, and/or hypertrophy in contrast to the fatigue injury model used in this dissertation. Contrary to previous late-stage models, in the third chapter of this dissertation we showed that the increase in GAGs during early-onset tendinopathy limits tenomodulin, a key marker of tenogenic phenotype that had not previously been assessed in our early-stage overuse injury model. This GAG-associated limitation on tenomodulin was correlated with long-term viscoelastic properties, either via alterations to multiscale mechanics and mechanotransduction or due to direct interactions with cells. These findings demonstrated that the biological effects of the early-onset GAG increase can affect long-term repair and mechanical properties in contrasting ways to their previously observed effects during late-stage, chronic tendinopathy and on short-term mechanics.

Future work

Future work should further investigate how other unique ECM and cellular changes that occur with tendon overuse injuries are affected by GAGs and ultimately influence mechanotransduction. Our work suggests a potential role of GAGs in modulating fibrillogenesis in the tendon midsubstance, calcification in the tendon to bone insertion, and integrin and focal adhesion formation in the pericellular matrix. The effect of these GAG-dependent changes on mechanotransduction should be measured directly, through multiscale mechanical assessment of cell and matrix strains. How GAG-altered mechanotransduction influences the activation of mechanobiological pathways and determines cell fate and behavior should also be further investigated.

The ways in which GAG-dependent mechanical changes affect post-injury tendon function, such as long-term joint stability and locomotion, should be also further considered. Decreased microscale strains and altered viscoelasticity with increased GAGs post-injury may not only affect tendon performance but may also be a mechanism for preventing further injury with continued activity. Energy storing tendons, like the patellar tendon used in our fatigue injury model, have improved resistance to fatigue injury compared with positional tendons. However, a direct correlation between viscoelasticity and fatigue damage has not previously been made. Future work should consider how GAGs present in both naïve and fatigue injured tendons impact the accumulation of fatigue damage that ultimately influences repair. The multiscale mechanical and viscoelastic techniques and assessments outlined in this dissertation provide the basis and initial data for such investigation.

The fatigue injury and GAG increase response in other tendon types is also warranted given the unique mechanical and biological environment of each tendon and joint. For example, tendons

with a high incidence of impingement, such as the supraspinatus tendon, experience greater amounts of shear and compression, likely resulting in altered mechanotransduction and mechanical contributions of GAGs. Tendons with greater exposure to synovium may also experience an altered inflammatory environment that is likely influenced by GAGs. While the fatigue injury model in rat patellar tendon used in this dissertation enabled consistent induction of the damage associated with early-onset tendinopathy, the use of larger animal models with anatomy more closely resembling human patients may provide more clinically translatable data.

Finally, the findings in this dissertation promote numerous therapeutic applications for the measurement or modulation of GAGs in the treatment of tendinopathy. GAGs could be used as a diagnostic marker to determine timing and modality of physical therapy and improve tendon repair in response to exercise. With sufficient technological advancement and clinical data, non-invasive measurement of GAGs may be achieved using imaging modalities such as MRI or ultrasound elastography, potentially by correlation of GAG amounts with swelling, fluid motion, or viscoelasticity. Direct application of GAGs, such as HA injections, is currently used with the goal of improving tendon healing and performance after injury. Our work suggests that inducing a location and type specific increase in GAGs, particularly in tendon insertion, interfibrillar spaces, and the pericellular matrix may further improve tendon repair, especially in overuse and early-onset cases of tendinopathy. General modulation of GAGs is possible through physical therapy or application of growth factors such as platelet derived growth factor (PDGF). However, more precise modulation of GAGs may be achievable through targeted drug delivery or gene therapy.

Despite the widespread impact of tendinopathy, additional research in the progression and repair of early-onset injury is required to develop more effective diagnostic and therapeutic approaches to prevent and treat the underlying causes of disease. The reviewed and original work in this

dissertation have only begun elucidating the progression of early-stage tendinopathy, tendon mechanotransduction, and the role of GAG and other post-injury ECM changes in tendon repair. However, this dissertation offers numerous opportunities for further research and clinical translation that may help us better understand and address common and debilitating tendinopathies.