THERAPEUTIC UTILITY OF THE MRL/MPJ REGENERATIVE MOUSE IN CLINICALLY-RELEVANT TENDON INJURIES

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Due to their critical role in joint stability and locomotion, injuries to the tendon cause pain, disability, and diminished quality of life. Tendon injuries are usually progressive, in which incurred damage does not repair, leading to chronic tendinopathy which can then progress to rupture. Existing therapies range from physical therapy, corticosteroids, and biologics to surgical repair in late-stages of disease, but all existing methods fail to effectively repair underlying damage, leading to further progression of injury. Thus, there is an unmet clinical need for effective therapeutics to halt the progression of tendinopathy. The MRL/MpJ regenerative mouse strain (MRL) has demonstrated enhanced recovery of structure and function in a variety of tissue injuries, including a tendon punch excision. However, this exaggerated injury fails to recapitulate the progressive nature of clinical tendinopathy. Accordingly, the enhanced healing capacity of the MRL in clinically relevant, progressive stages of tendinopathy is unknown.

To elucidate the utility of the MRL to recapitulate various tendon injuries, Chapter 2 investigates the MRL as a model of enhanced healing following surgical repair of the supraspinatus tendon in late-stage tendon disease. Our findings indicate that the MRL restore enhanced structure, composition, and function following rotator cuff repair through a temporal balance of collagens. This study establishes a platform to interrogate specific mechanisms that may underlie effective healing following

surgical repair.

Subsequently, because current surgical repair procedures experience high rates of re-tear, Chapter 3 investigates the efficacy of the MRL in earlier stages of tendinopathy following chronic overuse, which is associated with a markedly disparate biological cascade than previously investigated acute injuries. We demonstrate that the MRL is capable of recovering composition, structure, and function in these disparate injuries, highlighting its broad utility to harness as a therapeutic. Finally, our previously developed MRL cell- and matrix-derived components were evaluated as a therapeutic in early onset tendinopathy in Chapter 4.

Taken together, this work identifies a platform for future investigation of mechanisms associated with enhanced healing in various stages of tendinopathy. Furthermore, the ability of MRL-derived therapeutics to attenuate early onset tendinopathy motivates further investigation of these disease-modifying biologics to halt the progression of tendinopathy.

BIOGRAPHICAL SKETCH

Monideepa was born in India and grew up in Wilmington, DE. She graduated with an Honors Degree with Distinction in Biomedical Engineering from the University of Delaware in 2016. Her undergraduate research experiences in cartilage biology and cancer mechanobiology inspired her graduate school research interests in orthopedic mechanobiology and regeneration. In addition to her graduate research, Monideepa was involved in establishing new K-12 outreach initiatives with the local community. Additionally, she worked in the Diversity Programs in Engineering to mentor undergraduate students and establish pedagogical trainings for inclusive learning environments. After graduation, Moni is continuing her research career as a Senior Engineer at Medtronic.

Dedicated with all my love to my Mom and Dad for their sacrifices and support

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CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1 Background

Due to their critical role in joint stability and movement, injuries to tendons are a significant source of pain and disability. Existing therapeutics are largely palliative, and fail to effectively repair underlying damage. A major hurdle to the development of effective therapies is that intrinsic repair mechanisms in clinically relevant tendon injuries remain unknown. This first chapter details tendon physiology and function, the progression of tendon injuries, limitations of therapeutics, and introduces an adult mammalian model of regeneration, the MRL/MpJ mouse strain, and identifies gaps in the literature that would enable therapeutic development.

1.2.1 Tendon physiology and composition

Tendons are a connective tissue that play an essential role in movement and joint stability. Skeletal motion is enabled by tendons that transmit loads from muscles to bones. Tendons must meet very diverse functional and mechanical demands. These functions range from precise movements such as typing to repetitive motions such as daily walking activities and rapid sprinting[1–3]. Thus, tendons significantly improve quality of life due to their critical role in almost all activities.

Tendon function is enabled by its matrix composition and structure. The predominant constituent of tendon is highly aligned collagen I, which accounts for approximately 75% of the tissue's dry weight. Collagen I has large fiber diameter and packs in an organized manner, which largely contributes to tendon's high tensile

stiffness[4,5]. Trace amounts of other collagens are also present in tendon and serve differing roles. For example, collagen III, V, and XII serve to form rapid crosslinks between fibers, regulate collagen fibril diameter, and provide lubrication between collagen fibers, respectively.

Proteoglycans also are present in small quantities in tendon midsubstance. These proteins are composed of highly charged glycosaminoglycan side chains, which can affect fluid flow the tissue, thereby affecting the viscoelastic, or strain-dependent, mechanical behavior of the tendon[6]. Due to this ability to dissipate stress concentrations, proteoglycans are found in higher quantities at the tendon-to-bone attachment site[7]. GAGs in tendon can also promote fiber sliding during tensile loading[8]. In addition to mechanical regulation, proteoglycans also can affect biological processes, such as growth factor sequestration[9].

Finally, the remaining major component of tendon composition are glycoproteins. Elastin, which accounts for 2-5% of tendon dry weight, enables the tissue's recoil and resilience to repetitive motion[10,11]. Other glycoproteins, such as fibronectin, tenascin-C, and hyaluronan, also facilitate fiber assembly, tissue hydration, and mechanical integrity[12].

Tendon also has a resident cell population, composed predominantly of tenocytes, which are a population of fibroblasts that are elongated and highly aligned along the matrix[13]. Additionally, a small stem/progenitor cell population can differentiate in response to loading[14]. A recent study also identified small populations of resident endothelial cells, pericytes, nerve cells, and immune cells, which may be modulated in response to injury[15].

1.2.2 Tendon injury

Tendons have a poor innate healing capacity due to their acellularity and avascularity. Low cell density provides few cells to respond to damage in the matrix, and the lack of vascularity limits any potential systemic response to injury. Thus, tendon injury results in a persistent scar tissue with inferior mechanical properties to naïve tendon[16]. As a result, the damaged/poorly healed/scarred tendon is unable to meet the functional demands of the tissue and is susceptible to further injury[17]. Tendon injuries account for 30% of all musculoskeletal consultations[18]. However, certain populations have higher propensity of these injuries due to repetitive motions, such as the military, athletes, and work-place related labor[19–21]. Additionally, increased age, smoking, diabetes, and obesity are associated with increased risk of tendinopathy[22,23]. These injuries can result in disability and occupational challenges, lost days in the workplace, and pose a significant healthcare burden[24].

Tendons are mechanosensitive, and will respond with multiscale biological adaptations to mechanical loading[25] (Figure 1.1). In overuse, initial damage to the tendon is incurred by the matrix in loss of mechanical and structural integrity[26]. The resident cell population may attempt a biological repair response, but, when outpaced by further damage accumulation, will be ineffective, resulting in a chronic injury state[27].

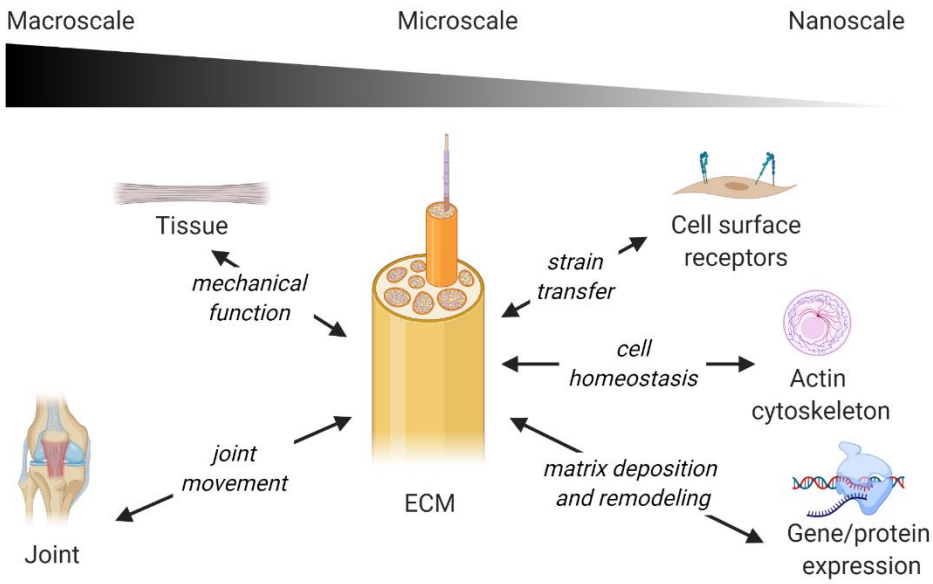

Figure 1.1 Tendons respond to mechanical loading through multiscale feedback mechanisms[25]

At the microscale, healthy tendons are characterized by sparse, elongated cells and highly aligned collagen fibers. When incurred damage does not repair, patients will experience chronic tendinopathy, marked by collagen fiber disruption, hypercellularity, and cell rounding. Further damage in this condition can lead to tendon rupture, which results in collagen disorganization and loss of mechanical integrity[28–30].

1.2.3 Clinical therapeutics for tendon injury

Treatment options for tendinopathy vary by severity of the injury. For tendon ruptures, surgical repair is the primary indication, for which the tendon is sutured back onto the bone and may be augmented with a tendon graft in large defects[31,32]. In earlier stages of disease, conservative treatment options are used, such as physical therapy

and exercise, anti-inflammatory medications, corticosteroid injection, or platelet-rich plasma (PRP) treatment[31,33]. The effects of these treatments remains poorly understood, and continue to have mixed outcomes that cannot consistently demonstrate improved recovery from placebo[34–36]. Ultimately, existing treatment options all fail to effectively repair underlying damage to the tendon, leading to further progression of injury. Even surgical repair experiences high re-tear rates (up to 94%), highlighting the lasting damage at the site of injury[37]. Thus, there is an unmet clinical need for effective therapeutics to halt the progression of tendinopathy.

1.2.4 The MRL/MpJ mouse strain to investigate adult mammalian regeneration

Several regenerative medicine approaches to prevent tissue scarring after injury have used the MRL/MpJ mouse strain (MRL). The MRL is the control strain for the MRL/MpJ-Fas^{lpr} mouse, which is used to study lupus[38]. In contrast, the MRL retains wild-type Fas expression, delaying the onset of autoimmune disorder until later in life. This inbred mouse strain was bred from C57Bl6 (0.3%), C3H (12.1%), AKR (12.6%) and Large (75%) mouse strains[39]. Following an ear punch injury the MRL originally was discovered to exhibit seamless wound closure, including naïve cartilage structure and recovery of sebaceous glands and hair follicles, unlike C57Bl6 (B6) mice[40]. Subsequently, the MRL mouse has been shown to have enhanced healing in injuries such as alkali-burned corneas, full-thickness articular cartilage defect, and myocardial tissues[41–43]. However, not all tissues or injuries show this regenerative capacity. For example, skin wounds, partial thickness cartilage defects, and

dopaminergic neuron lesions heal with scars in the MRL, thereby highlighting the tissue-specific enhanced capacity of the mouse[42,44,45].

The specific drivers of the enhanced healing response are unknown. Initial hypotheses implicated the systemic environment. For example, numerous quantitative trait loci have been associated with the enhanced healing outcomes in MRL, although no single gene has been identified as being responsible for the phenotype. Additional consideration was given to the immune system because MRL mice are predisposed to autoimmunity and exhibit an altered basal immune system. However, numerous studies have identified that, despite sharing the systemic environment, there is no correlation in enhanced healing outcomes between tissues within the same mouse. Thus, because scarless healing is tissue-specific in the MRL, despite a shared systemic environment, and because even different injuries within a tissue exhibit varying degrees of enhanced healing, the extent of enhanced tissue healing for a particular injury must be individually investigated and identified.

1.2.5 Enhanced healing of the MRL/MpJ mouse strain in tendon excision injuries

For tendon research, the MRL mouse offers several advantages for translational medicine as a model of regenerative healing compared to other model organisms. Organisms such as salamanders and starfish are capable of total limb regrowth following amputation, and zebrafish are capable of scarless healing[46–48]. However, these amphibians, invertebrates, and fish do not recapitulate mammalian physiology and biological processes, limiting the clinical translation of findings from these organisms. Neonatal models also exhibit regeneration, and have provided insight

into cascades associated with scarless healing. However, neonates exhibit a dampened systemic response due to their underdeveloped immune system, in contrast to a pronounced inflammatory cascade in adult injuries[49,50]. Additionally, neonatal tissues are rich in undifferentiated stem cells[51]. Despite a small progenitor cell population in adult tendons, most cells are terminally differentiated, motivating the need for study in an adult model[14]. Furthermore, the mechanical loading environment is critical to consider, due to the mechanosensitive behavior of tendon. Mechanical stimulation to the neonatal tendon is largely due to muscle contractions from limb lengthening during development [52]. In contrast, tendons in adults are responsible for much higher loading demands, up to 12.5 times body weight[53]. Therefore, preserving the mechanobiological environment is critical for investigating tendon injury and healing. In tendon, the MRL mouse displays enhanced recovery of structure and mechanical properties after a patellar tendon punch excision compared to scar-mediated healing in B6[54–56]. This exaggerated injury with a punch excision has been a valuable platform for mechanistic understanding of the enhanced healing cascades associated with the MRL. For example, using this injury, we have identified the local tendon environment to be a driver of this enhanced healing using an organ culture model, and elucidated sex-differences that highlight different genetic profiles associated with enhanced healing[57,58].

However, as previously described, clinical tendon injuries are caused by unrepaired damage accumulation. The chronic and progressive alterations to the tendon are not recapitulated in the punch excision model. Thus, the enhanced healing

capacity of the MRL in the clinically relevant, progressive stages of tendinopathy is unknown.

1.3 Chapter overviews

This thesis investigates the utility of the MRL mouse strain in different stages of clinically relevant tendon injuries to determine whether this mouse model is broadly applicable for investigation of tendinopathy. Subsequently, this work harnesses the structural and biological cues in the healing MRL tendon as a therapeutic to attenuate early onset tendinopathy in a non-reparative mouse model.

Chapter 2 investigates the MRL as a model of enhanced healing following surgical repair of the supraspinatus tendon in late stage tendon disease. Comparative assessments between the B6 and MRL are made in insertion site structure and composition, bone organization, and functional outcomes, and identify the MRL as a model of enhanced healing in rotator cuff surgical repair. However, because current surgical repair procedures experience high rates of re-tear, the following study investigated the efficacy of the MRL in earlier stages of tendinopathy. Accordingly, Chapter 3 examines the enhanced repair capacity of the MRL in a model of chronic rotator cuff overuse. Despite similar mechanobiological environments between the B6 and MRL mice, the MRL recover mechanical properties following overuse, unlike the B6 mice.

Thus, the MRL exhibits enhanced repair capacity in a wide variety of tendon injuries, despite differing injury mechanisms and cascades. To apply the broad applicability of the MRL as a therapeutic, previously developed MRL cell- and

matrix-derived components were evaluated as a therapeutic in early onset tendinopathy in Chapter 4. Results indicate that these MRL-derived therapeutics are capable of inducing a reparative phenotype associated with therapeutic exercise. Taken together, this work identifies a platform to investigate mechanisms of enhanced healing in various stages of tendinopathy. Furthermore, MRL-derived therapeutic testing opens methods for disease-modifying biologics to induce a reparative response and halt the progression of tendinopathy.

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CHAPTER 2: ENHANCED HEALING OUTCOMES IN MRL/MPJ TISSUES CONSERVED IN INSERTION SITE FOLLOWING SURGICAL REPAIR

The following chapter is in review for publication in *Journal of Shoulder and Elbow Surgery*. The anticipated reference to the work is:

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2.1 Abstract

Background: Surgical repair of supraspinatus tendons (SST) has a high failure rate at the insertion site. A significant hurdle to therapeutic development is that effective intrinsic healing mechanisms are unknown. The MRL/MpJ (MRL) mouse exhibits tissue-specific enhanced healing; however, these tissues exhibit disparate properties from the complex SST. The extent of SST healing in the complex environment of the rotator cuff is unknown. We hypothesize that the MRL exhibits enhanced restoration of the structurally complex insertion site, resulting in functional improvements.

Methods: B6 and MRL mice underwent SST detachment and immediate surgical repair. Gait was assessed after either 2 or 6 weeks, then mice were euthanized for immunohistological analysis.

Results: MRL SST demonstrated enhanced recovery of zonal architecture and bone structure than B6 SST. MRL SST exhibited decreased levels of collagen III at 2 weeks

and increased procollagen I at 6 weeks compared to B6 SST. At 2 weeks, MRL experienced initial gait deficits that were recovered by 6 weeks.

Discussion: The temporal balance of collagen in MRL suggests recovery toward naïve composition. Initial gait deficits in MRL may provide a protective loading environment that is ultimately beneficial. Mechanisms of enhanced healing observed previously in the MRL may be conserved in the complex SST, providing a platform to interrogate specific aspects of improved healing.

Level of Evidence: Level I

Keywords: enthesis; rotator cuff; gait; supraspinatus

2.2 Introduction

Rotator cuff surgical repairs are very common procedures, with over 250,000 repairs performed in the United States annually[1]. However, surgical repair of supraspinatus tendons (SSTs) has high failure rates ranging from 20% to 94%[2], [3]. Despite improvements in surgical techniques, persistently high failure rates highlight the need for additional biological supplementation to increase surgical success.

Functional assessments, such as the critical shoulder angle and shoulder range of motion, are commonly used clinically to track patient recovery[4]. These spatial parameters measure weakness and ability to use different muscles and associated tendons within the rotator cuff. Additionally, higher critical shoulder angle and lower post-operative American Shoulder and Elbow Surgeons (ASES) scores, indicative of

pain and disability, are correlated with increased rates of re-tear[5], [6]. Accordingly, measures in animal models of similar spatial parameters of injury and healing would guide biological development and serve as a pre-clinical benchmark of healing.

Tears primarily occur at the tendon-to-bone attachment site, mandating restoration of this structurally complex insertion site[7] for an effective healing outcome. A healthy insertion site is composed of a gradient of tissues that progress from tendon, unmineralized fibrocartilage, mineralized fibrocartilage, to bone. In contrast, healing of a torn insertion site is characterized by fibrovascular scar formation, failure to recreate the zonal microarchitecture, and diminished bone quality, thereby increasing the propensity for re-injury[8]–[12]. In particular, the attachment site remains weak largely due to poor integration between the bone and tendon. In addition to structural deficits, naïve composition of the insertion site is not restored following injury. For instance, a healthy insertion site is predominantly composed of collagen I, which is critical to the tensile load bearing capacity of the tendon, in addition to significant amounts of aggrecan and other proteoglycans to withstand compression[13], [14]. In contrast, poor healing of the insertion site is characterized by scar tissue that is predominantly composed of collagen III, and low amounts of proteoglycans[15]. A major hurdle to the development of therapeutics is that effective healing mechanisms that restore multi-zonal structure and function following surgical repair remain unknown.

We and others have previously shown that the Murphy Roths Large Mouse (MRL/MpJ (MRL)) is a model of enhanced healing in stark contrast to scar-mediated healing of normal-healer C57Bl6 mouse strain (B6). Furthermore, the MRL is an

advantageous model since it possesses fully developed immune and skeletal systems, thereby exhibiting normal, mammalian adult structure and function. The enhanced healing capacity of MRL mouse is tissue-specific, and has been observed to extend to midsubstance patellar tendon punch injury[16], [17], full-thickness cartilage defect[18], and bone fracture[19], the major tissue constituents of the insertion site. However, these tissues experience a complex, multidirectional mechanical loading environment in the context of the insertion site that differs from their characteristic loading demands [20], [21]. Furthermore, biological crosstalk between the tissues of the rotator cuff adds yet another complexity to the healing cascades and ultimate surgical outcome. For example, *in vitro* explant culture of the rotator cuff's bone-tendon-muscle unit shows altered protein synthesis, inflammatory behavior, and cell activity in comparison to tendon-only explant culture[22]. Furthermore, the cells responsible for SST healing are recruited from the surrounding tissues and not the tendon itself[12], [23], highlighting the direct effect that these surrounding cells have on the healing outcome. The extent of the MRL healing capacity in the structurally and mechanically complex environment of the rotator cuff insertion site is unknown.

Accordingly, the objective of this study was to evaluate the healing capacity of the MRL after SST surgical repair. The integrity of the healing SST was assessed histologically for structure and composition, and functionally using longitudinal gait analysis. We hypothesize that the MRL exhibits enhanced restoration of the

structurally complex insertion site, resulting in functional improvements after surgical repair.

2.3 Materials and Methods

SST Surgical Repair

Following IACUC approval, 16 week old male B6 and MRL mice (n=12-13/strain) underwent left SST detachment and surgical reattachment as previously described[24]. Briefly, mice were anesthetized and a skin incision was made from the elbow towards the ear. The deltoid was released to visualize the rotator cuff. A 7-0 PDS suture with BV-1 needle was placed in a figure-of-eight fashion through the SST, and the tendon was sharply released with a micro-scalpel and the insertion site was debrided. The needle was then used to create a tunnel within the bone of the humeral head from posterior to anterior. The suture was ligated, bringing the tendon back down to its original insertion site. The deltoid was reflected over the humerus and the skin incision was closed with running 6–0 prolene sutures. Buprenex was administered post-surgery and every 12 hours for the next 48 hours. Animals resumed normal cage activity. Mice were sacrificed at either 2 or 6 weeks post-operatively for histological assessment (n=4-6/timepoint). All of the remaining mice were evaluated for gait for this study and subsequently allocated for other studies post-sacrifice.

Gait Analysis

To assess functional deficits from injury, mouse gait was collected using Digi-Gait Imager 12.2 (Mouse Specifics, Inc.) before surgery (baseline), and 2-and 6-weeks post-operatively. To account for differences in size between B6 and MRL, gait was assessed at fixed treadmill speeds of 25cm/s and 30cm/s, respectively, as our preliminary data shows that these speeds result in a similar number of strides per second between the two strains. These speeds were chosen as they have been shown to result in consistent gait patterns on the treadmill[25], [26]. Left front and left hind paw areas, left and right stride lengths, and shared and front stance lengths were calculated and normalized to pre-injury levels. These parameters have been shown by others to be modulated by tendon injuries and were chosen for analysis in this study because they are indicators of pain or weakness of the injured and contralateral limbs[12], [27].

Immunohistochemistry and Histology

At sacrifice, the construct consisting of the humerus, scapula, the four attached rotator cuff musculo-tendinous units and coracoacromial arch was dissected, processed, paraffin-embedded, and cut into 6um sections.

Structural assessment: Sections were stained with toluidine blue and imaged (10x magnification) to analyze cellularity, vascularity, and restoration of zonal architecture of the insertion site. Metrics were assessed semi-quantitatively on a scale from absent (-) to increasing levels of presence (+, ++, +++, ++++) by two blinded graders and averaged[10]. The quality of newly formed bone and orientation of the bone fibers at

the site of repair was also evaluated[28], [29]. Gap size was manually measured by a blinded user and was defined as the distance from the suture hole to the bony attachment site in toluidine blue images. This metric was included since gapping has previously been shown to correlate with repair strength[30] and propensity for re-tear. Scaling the established threshold of gap formation in rat rotator cuff (> 1cm) to the smaller mouse anatomy is smaller than the degree of surgical precision (~350μm). Therefore, in this study, K-means clustering (MATLAB) was used instead to identify samples with gap formation. Analysis was conducted to as to stratify the samples in 2 clusters representative of either with or without gap formation.

Compositional assessment: To spatially assess matrix composition throughout the healing insertion site, sections were stained with collagen III (1:500, Abcam), procollagen I (1:100, SantaCruz), or aggrecan (1:500, Millipore-Sigma). Briefly, samples were de-paraffinized, rehydrated and incubated with either Chondroitinase ABC (Sigma-Aldrich) for 30 minutes (aggrecan) or Pro-K solution (Dako) for 5 minutes (collagen III and procollagen I) for antigen retrieval. During paraffin sectioning, naïve samples were more susceptible to shredding, and had to undergo brief SDS incubation to achieve analyzable sections. As SDS is also an antigen retrieval, these naïve sections underwent Pro-K antigen retrieval for only 2 minutes to prevent non-specific staining. Endogenous peroxidase activity was quenched with 10% hydrogen peroxide incubation for 10 minutes. Sections were blocked (Dako) for 30 minutes, then incubated with the primary antibody for 1 hour at room temperature (collagen III and procollagen I) or overnight at 4°C (aggrecan). Vector-Anti Rabbit secondary (Vector Laboratories) was applied for 30 minutes, then Diaminobenzidine

(DAB) (Vector Laboratories) was added for 3 minutes to visualize the brown positive stainning. Samples were counterstained with Toluidine Blue (Electron Microscopy Sciences) for 2 minutes, then rehydrated and coverslipped. Sections were imaged under 10x magnification (collagen III and aggrecan) or 40x magnification (procollagen I). Procollagen I is reported as percent of positively stained cells.

To quantitatively threshold and assess the percent of matrix area staining positive for collagen III and aggrecan, RGB images were imported into ImageJ by a blinded user. A Region of Interest over the new insertion site was selected, and the image was split into its respective channels [31]. The blue channel, representing the chromagen signal, was isolated for analysis, and a histogram of gray-scale intensity values was exported. To account for background signal and carryover signal from the counterstain, the gray-scale value corresponding to the cumulative highest 5% of intensity on the negative control section was used as an unbiased method to establish a threshold. Pixel intensities on experimental samples above this threshold were labelled as positive, and the percent of area staining positive is reported.

Statistical Analysis

Gait parameters were first compared to pre-injury baseline using paired non-parametric t-test, then normalized to baseline and compared between mouse strains at each timepoint with non-parametric t-test. P-values were corrected to account for the multiple comparisons. Similarly, histological composition values between mouse

strains at each timepoint were compared using non-parametric t-test. Significance was set at $p \leq 0.05$ (*) and trends were set at $p \leq 0.1$ (#).

2.4 Results

The surgical procedure was well tolerated, and all animals survived until the designated sacrifice time-point.

Gap Formation

Tendon-to-bone gapping was grossly visible at sacrifice. K-means cluster analysis identified only 3 samples with gap formation. Gap formation was not unique to any one experimental group or mouse strain (Fig. 2.1), indicating that gap formation alone was not indicative of strain-dependent differences in healing.

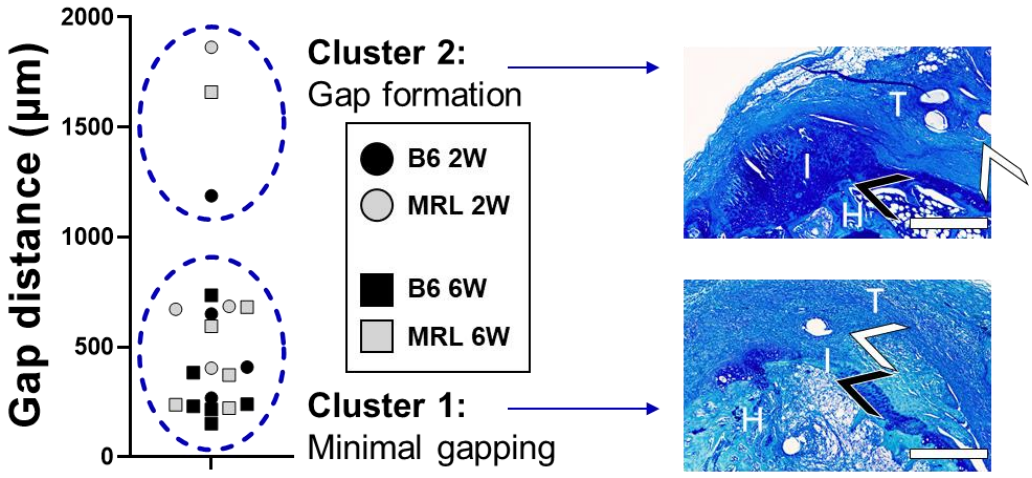

Figure 2.2 Gap distance between attachment site (black arrow) and suture hole (white arrow) was similar between groups, indicating gapping alone does not indicate strain-dependent differences in healing. T: tendon, I: insertion site, H: humerus. Scale bar = 400µm

Insertion Site Structure

The MRL SST exhibited greater recovery of the zonal microarchitecture than the B6 SST (Fig. 2.2a). Although grading indicated differences in cellularity, when adjusted for cross-sectional area, no differences were found between strains in cell density (data not shown). Little to no vascularity was observed in histological grading for either strain. MRL samples exhibited greater number of zones restored, including a distinct fibrocartilage transition zone (Fig. 2.2b). Additionally, the B6 bone remained fibrous at the site of repair, characteristic of woven bone, while the fibers in MRL bone were parallel to and interdigitated with the SST, indicative of mature, lamellar bone (Fig. 2.2c).

A

	Cellularity insertion	Vascularity	Insertion site zones
B6 Naïve	++	-	++++
MRL Naïve	++	-	++++
B6 2W	++	++	++
MRL 2W	++	+	++
B6 6W	+	-	++
MRL 6W	++	+	+++

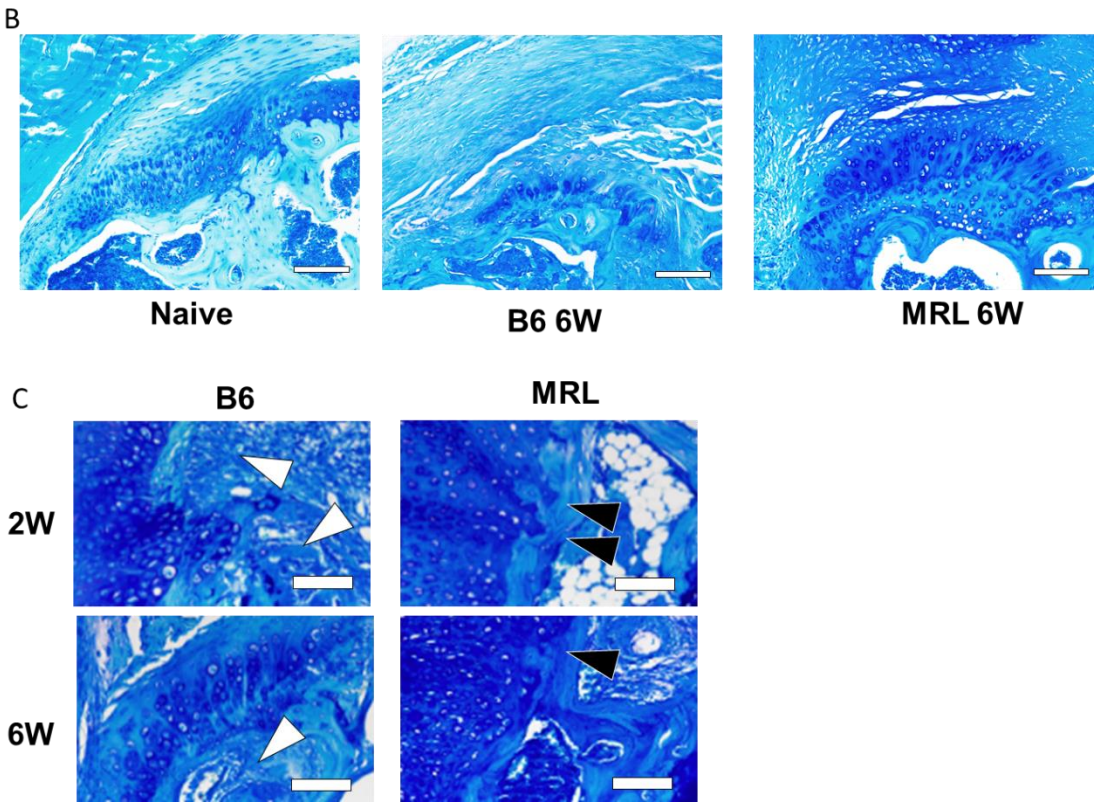

Figure 2.3 (A) Semi-quantitative grading indicates MRL samples recover zonal microstructure better than B6 samples. (B) Representative images showing B6 SST insertion site lacks a significant fibrocartilage transition zone. Conversely, MRL SST insertion site recovers zonal structure. Scale bar = 200µm (C) B6 bone at the attachment site is unorganized and fibrous (white arrows). In contrast, MRL bone is parallel and interdigitated with the insertion site (black arrows). Scale bar = 100µm

Matrix Composition

No differences in naïve levels of collagen III, procollagen I, nor aggrecan were found between B6 and MRL. Supporting our hypothesis, the percent of matrix positive for collagen III was higher in the healing insertion of B6 than MRL at 2W (trend: p=0.1) (Fig. 2.3) but did not differ between strains at 6W.

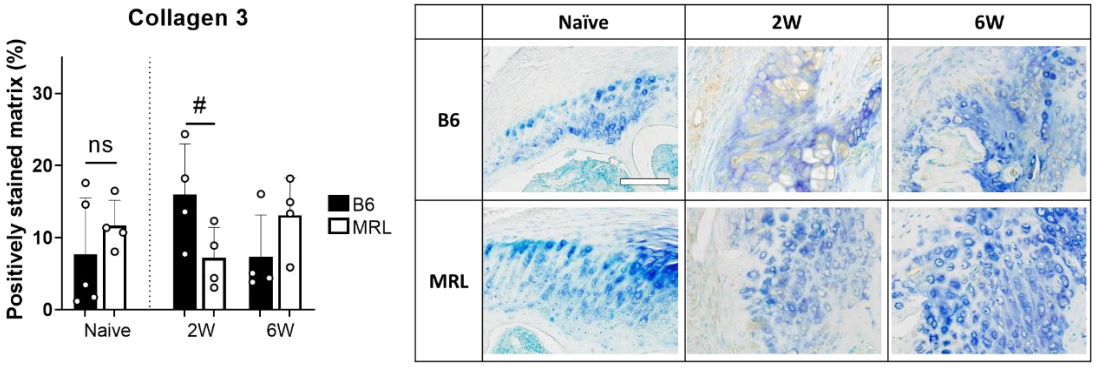

Figure 2.4 Collagen III levels are higher in healing B6 than MRL at 2W. Representative images of each group indicate positive matrix staining in brown. Scale bar = 100µm.

Further supporting our hypothesis, the procollagen I positive cell percentage was decreased in B6 at 6W relative to MRL (trend: p=0.06) (Fig. 2.4).

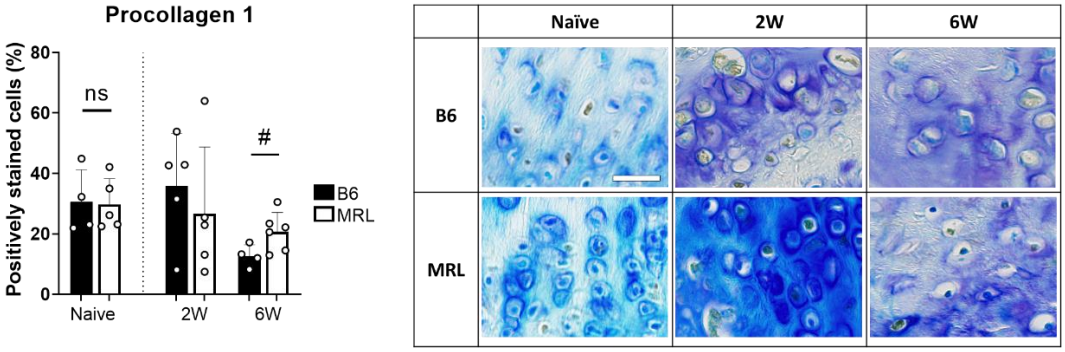

Figure 2.5 Procollagen I levels are higher in healing MRL than B6 at 6W. Representative images of each group indicate positive cell staining in brown. Scale bar = 25µm.

Contrary to our hypothesis, there was no difference in the percent of matrix positive for aggrecan between strains at either timepoint (Fig. 2.5).

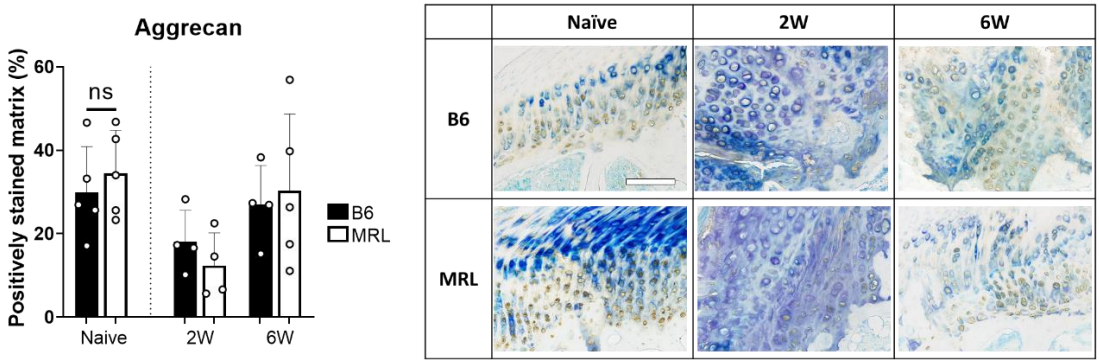

Figure 2.6 Aggrecan levels were not significantly different across strains at any time point. Representative images of each group indicate positive staining in brown. Scale bar = 100µm.

Functional Assessment

Contrary to our hypothesis, the injured arm in only MRL exhibited a decrease in paw area (p=0.01) (Fig. 2.6a), and an increase the corresponding hind paw area (p=0.01) (Fig. 2.6b) at 2W. However, supporting our hypothesis, these deficits were recovered by 6W, with the MRL preferring the injured paw relative to pre-injury (trend: p=0.1) and exhibiting increased stride length on the injured side (trend: p=0.1) (Fig. 2.6c). No differences were found relative to baseline nor between strains in right stride length, shared stance length, and front stance lengths.

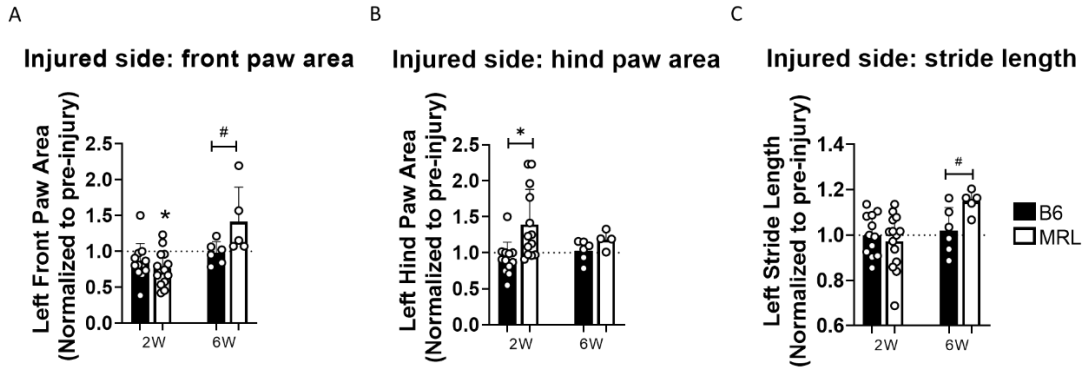

Figure 2.7 Gait parameters that were altered by injury. (A) Injured paw area decreases relative to pre-injury in MRL only at 2W, but is increased relative to B6 by 6W. (B) Hind paw area on the injured side increases in MRL only at 2W relative to B6. (C) Stride length on the injured side is increased relative to B6 in MRL only at 6W.

2.4 Discussion

Surgical management of rotator cuff repair remains a significant clinical challenge. Current strategies augment surgical repair with physical therapy[32], synthetic or biological grafts[33], or platelet-rich plasma (PRP)[34], but these continue to result in unacceptably high re-tear rates. Therefore, a model of enhanced healing in this persistent injury environment would expand insight into potential therapeutic targets. Consequently, we sought to evaluate whether the MRL/MpJ regenerative mouse strain exhibits enhanced restoration of the structurally complex insertion site, and whether this results in functional improvements after surgical repair.

Notably, the MRL recovered zonal structure at the insertion site compared to B6. Unlike the B6 SST, the MRL SST developed a distinct fibrocartilage transition region, which is critical to dissipate stress concentrations and prevent mechanical failure at the attachment site[27], [35]. The transition zone consists of a linear gradient

of mineralization from the compliant tendon to the stiff bone[36]. Others have shown that following bone fracture, the MRL has improved mineralization over B6[19]. The MRL bone contains more osteoblasts which also have higher proliferation capacity than B6 bone. Through this method of controlled bone remodeling, the MRL can effectively bridge the tendon-to-bone gap. Although not directly measured in our study, the formation of distinct fibrocartilage zones separated by a mineralized tidemark in the MRL suggests that its enhanced mineralization mechanisms are conserved in the context of the complex insertion site healing. Restoration of this mineral gradient is critical to protect the tendon from rupture caused by modulus mismatch between the tissues.

Similarly, the naïve SST has a uniform collagen fiber organization across the insertion site to minimize stress concentrations[37]. We have previously demonstrated the MRL's enhanced fiber alignment compared to the B6 as early as 1 week post-injury in the patellar tendon[16]. Furthermore, we show that MRL SST demonstrates enhanced tendon-to-bone integration that may be instrumental to protecting the attachment site at the site of potential re-tear. Taken together, mechanisms such as mineralization, fiber alignment, and bone healing that drive enhanced healing in other MRL tissues are likely also present in the MRL SST, leading to the observed structural improvements.

Subsequently, we investigated key compositional and structural proteins that could restore mechanical function. In the human SST, collagen III is correlated with low fiber alignment and modulus[13]. Following tendon injury, increased collagen III levels in B6 indicates scar formation, and suggests that its repair site is mechanically

inferior to MRL[38], resulting in diminished bulk mechanical properties. Elevated collagen III can affect the microscale mechanical environment of the cells by decreasing the local mechanical stiffness[39], and in turn, promote cells to become less mechanoresponsive than in a more collagen I rich environment[40]. Additionally, collagen III fibrils also have been shown to impede cell contraction and myofibroblast activation[40]. Collagen III deficiency causes a robust myofibroblast response in skin, thereby accelerating wound closure[41]. A similar recruitment of myofibroblasts may occur and resolve early in the MRL SST healing cascade to aid in wound closure without leading to prolonged fibrosis. At 6 weeks, increased procollagen 1 in MRL SST suggests that the MRL is producing more collagen I than the B6 SST. Collagen I is ubiquitous throughout the SST insertion site and imparts the tissue's ability to withstand high tensile loads[13]. Taken together, the MRL likely achieves structural improvements through its temporal balance of collagen I and III levels.

Aggrecan is commonly abundant in compressive load-bearing tissues, such as cartilage[42]. In contrast to the tendon midsubstance, elevated aggrecan levels in the tendon insertion site suggests it plays a critical role by dissipating the unique, compressive loads in this region[14]. Surprisingly, no difference was found in aggrecan levels between mouse strains at either timepoint. Others have shown that aggrecan-positive cells do not contribute to SST healing[43] and their modulation largely reflects the loading environment. Consistent with this notion, we found that the MRL SST exhibits improved structure compared to B6 despite the lack of difference in aggrecan levels. This suggests that aggrecan may likely be a result of the similar

post-operative loading environments, rather than a contributor to the enhanced healing cascade.

Finally, our gait analysis shows that, despite initial loss of function, MRL ultimately displays enhanced recovery of gait. Chronic pain in rats decreases morphological gait measurements, such as paw area[44]. Thus, the initial deficits in MRL paw areas are likely a mechanism to offload the injured limb by offloading body weight from the front, injured paw onto the corresponding hind paw. For example, others have shown that decreased loading following SST surgical repair through immobilization protects the healing tissue and improves tendon-to-bone integration[45]. Similarly, the initial decrease in the MRL injured paw area may be ultimately beneficial to shield the injured limb while it structurally recovers.

The initial gait deficits in the MRL may indicate poor healing at that timepoint in the surrounding tissues of the SST. For example, a similar surgical strategy showed a decrease in paw area with just a sham surgery, suggesting that the injury to the deltoid muscle during surgery can diminish function[12]. The MRL's enhanced healing capacity does not extend to skeletal muscle. A recent study found that tenecotomy in B6 and MRL mice decreased the muscle's isometric force by 40% in both strains of mice; however, since the MRL's baseline isometric force is more than double that of B6, the magnitude of the MRL's muscle recovery is impaired more significantly[46]. This is corroborated by decreased amounts of MHC IIB/IIX in the MRL, but not B6, during healing thereby affecting muscle fiber contractility[46]. Thus, early deficits in gait may be caused by impaired healing of the deltoid muscle in the MRL.

Stride length is a functional measurement of the animal's capacity to use the limb. At fixed speeds, stride length decreases with injury as the animal is less capable of using the limb[47]. In contrast, our finding that the MRL increased stride length 6 weeks following surgical repair highlights its ability to recover from injury. Although stride length increases linearly with the weight of the animal[48], which was increased in MRL from the time of collection of uninjured baseline data to post-injury 6 weeks data, the absence of differences in the corresponding right limb stride length confirms that our change in stride length can only be attributed to the MRL's healing response.

This study assesses differences in composition through immunohistochemistry analysis, which provides spatial context to the presence of our antigens of interest. Spatial information is critical in the SST insertion site, which has a heterogeneous structure and compositional gradient. However, immunohistochemistry is less sensitive than traditional quantitative approaches, such as ELISA. We expect that the limitation that only large differences can be detected using immunohistochemistry mean that our findings are biologically meaningful. Another limitation of this study is that we utilized a surgical repair model of an acute injury which may not recapitulate the clinically common chronic injury environment. Nevertheless, our findings provide insights into the utility of the MRL as a model of enhanced repair in the complex rotator cuff environment, and ongoing studies will examine the biological environment in chronic overuse. Finally, our study quantified the functional outcome of healing using gait, which assesses clinically relevant mechanical demands on the tissue, while also allowing for longitudinal tracking of the sample. Future studies will also incorporate mechanical assessment to further contextualize findings.

In summary, we have shown that the MRL/MpJ exhibits enhanced recovery of structure, composition, and function compared to C57Bl6 mice in the complex rotator cuff environment. Mechanisms of improved healing previously observed in surrounding tissues, such as fiber alignment and bone healing may lead to the ultimate structural and functional improvements. Additionally, the temporal balance of collagen levels in the MRL may play a critical biological role in restoration of structure and subsequent function. Our findings define a platform to interrogate and isolate specific aspects of improved healing following rotator cuff surgical repair.

2.5 Acknowledgements

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CHAPTER 3: EARLY MOLECULAR DAMAGE RESPONSE OF MRL/MPJ SUPRASPINATUS TENDON TO OVERUSE INJURY HIGHLIGHTS ENHANCED REPAIR CAPCITY

The following chapter is in preparation for publication in *Journal of Orthopaedic Research*. The anticipated reference to the work is:

Chatterjee M, Bell R, Andarawis-Puri N. (2021) Early Molecular Damage Response of MRL/MpJ Supraspinatus Tendon to Overuse Injury Highlights Enhanced Repair Capacity. *In preparation for publication in Journal of Orthopaedic Research*.

3.1 Introduction

Rotator cuff injuries affect up to 20% of the population, with higher prevalence in the elderly, athletes, and workers with repetitive overhead motion [1]. Current treatment options only manage pain symptoms and fail to repair the underlying accumulated damage. Upon chronic damage accumulation, surgical repair may be required, though high surgical failure rates (up to 94%) highlight the need for disease-modifying therapeutics to halt early progression of rotator cuff tendinopathy [2]. The supraspinatus tendon (SST) is a commonly injured tendon of the rotator cuff. Tendon's inherent poor intrinsic repair response is further impaired in the SST. In addition to the repetitive tensile loading that is characteristic of most tendons, etiology in SST tendinopathy is often multifactorial, arising from repetitive compressive impingement from the coracoacromial arch, intrasynovial injury environment, and

multidirectional loading environment [3]–[5]. Injury occurs when attempts at repair are outpaced by further damage accumulation.

Similar to humans, the SST of rats is also enclosed by a coracoacromial arch [6]. Consequently, rats have been used extensively in downhill treadmill running models, shown to reproduce key characteristics of human SST tendinopathy [7]–[9]. Interestingly, pathology in this model is limited only to the SST, and not to other tendons such as the patellar or Achilles tendons, highlighting the unique anatomical injury environment of the rotator cuff in SST injuries [10], [11]. However, rats, like humans, do not repair accumulated damage, limiting the model from investigation of effective intrinsic repair mechanisms after rotator cuff overuse. Furthermore, the use of pharmaceuticals to investigate effective repair mechanisms is difficult due to inherent drug-delivery challenges in tendon. In contrast, mouse models are a promising tool to study intrinsic repair, due to the wide availability of transgenic strains that can be perturbed to investigate these mechanisms.

The MRL/MpJ mouse strain (MRL) has emerged as a model of tendon repair due to its enhanced healing response to laceration injury in comparison to C57Bl6 (B6) mice in the patellar tendon [12]–[14]. Furthermore, we have demonstrated that mice also possess a coracoacromial arch, thereby recapitulating the anatomical constraints on investigative models of rotator cuff overuse [15]. We recently extended our investigation to the rotator cuff, and shown that the MRL also demonstrates enhanced healing of the SST after surgical repair. However, the healing mechanisms of acute injury or surgical repair are characterized by cell infiltration and a pronounced extrinsic inflammatory cascade; in contrast, overuse tendinopathy is marked by

intrinsic matrix remodeling, rather than an overt inflammatory response[9], [16]. The extent of healing in the MRL following chronic overuse in this unique injury environment remains unknown.

Accordingly, the objective of our study was to assess the MRL SST innate repair capacity to rotator cuff overuse. We hypothesize that the MRL repair response outpaces damage accumulation in overuse SST injury, demonstrated by recovery of functional, mechanical, and structural impairments.

3.2 Materials and Methods

Overuse damage induction

Following IACUC approval, 12-week old B6 and MRL male mice underwent a 2 week treadmill acclimation period of progressive downhill running, prior to being allocated to either Cage-Activity (CA) or Overuse (OV) groups (n=8). OV mice continued the running protocol at a 10° decline for 30min/day, 5day/week at 13m/min for either 4 or 8 weeks prior to sacrifice, with CA groups age-matched, for functional, structural or mechanical responses of overuse (Fig 3.1).

To assess the early tissue response prior to biological adaptation to running, an additional set of 10 OV mice per strain were given either 5 days of rest at the end of acclimation and then continued downhill protocol for 1 day, or 2 days of rest followed by 7 days of overuse. Mice were then euthanized 24 hours after the final running session for gene expression analysis.

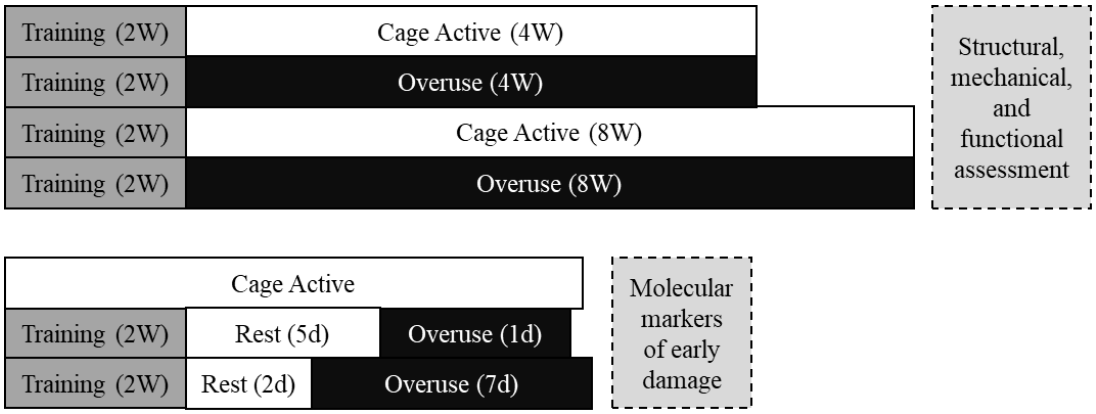

Figure 3.8 Experimental design and groups for rotator cuff overuse.

Gait

To assess the long-term functional outcome of rotator cuff overuse, a custom gait device recorded spatial gait parameters of CA and OV for the 8 week groups. Mice were recorded at a self-selected walking speed. 20 gait frames were randomly selected, and paws were manually segmented by a blinded user (ImageJ, NIH). Ratio of front paw to hind paw areas, width between front paws, width between hind paws, and angle between front paws were measured. To account for variances in walking speed and growth of the mouse, parameters were normalized to body length.

Mechanical Evaluation

The left humerus-SST units were dissected and frozen at the 4 and 8 week sacrifice timepoints and thawed at the time of tensile testing. Samples were preconditioned at 1% cyclic strain for 10 cycles at 0.1Hz. Following preconditioning, samples were tested for stress-relaxation at 5% strain with 300s recovery time, then returned to their

original position to recover for an additional 300s. Samples were preconditioned again, and then underwent a quasistatic pull to failure test at 0.1%/s to quantify maximum load and stiffness. Samples that slipped or failed at the grips were excluded (n=2).

To isolate the effects of SST overuse from potential exercise-induced adaptation in the running protocol, the patellar tendons after 8 weeks of running were additionally tensile tested using the same testing and analysis protocol.

Gene Expression

To identify the immediate mechanobiological effect of downhill running on the SST, gene expression of indicators of fibrocartilage and matrix turnover was measured. Left and right SSTs were dissected and flash frozen immediately upon sacrifice. RNA extraction was completed with Trizol and interphase separation (Phase Lock Gel, Qiagen). RNA cleanup and isolation using spin columns (RNeasy Mini Kit) with on-column DNA treatment (Qiagen). RNA quality and quantity were determined using a spectrophotometer (NanoDrop). Samples with a 260/280 $\geq$ 1.75 were used for gene expression analysis. cDNA was reverse-transcribed (SuperScript VILO, Thermo). Gene expression changes were measured for *Acan, Sox9, Col2* (fibrocartilage markers), *Col3*, and *Col1* (tensile remodeling) (Qiagen). Quantitative real-time PCR was performed using SYBR Green (Qiagen) and Viaa7 (Applied Biosystems). Gene expression data was analyzed using ddCt method, with results normalized to a housekeeping gene (*Gapdh*), and then to the corresponding strain's cage-active group.

Collagen Organization

The right humerus-SST unit was isolated and secured on a custom imaging device to keep the SST flat (~30 degree angle) and image the insertion site. A weight of 20g was applied to uncrimp collagen fibers. Second harmonic generation images of tendons were acquired on Zeiss LSM 880 upright multiphoton microscope using a 20x water immersion magnification lens. The multiphoton laser was tuned to 880nm and samples were imaged ~50um below the surface of the SST to avoid capturing potential surface damage from dissection. A custom MATLAB code was used to detect collagen disorganization in the form of collagen kinks.

Statistical Analysis

To test the effect of the overuse protocol on each strain, cage-active and overuse groups were compared using a Student T-test. Significance was set at $p \leq 0.05$ (*) and trends were set at $p \leq 0.1$ (#). All graphs represent mean ± standard deviation.

3.3 Results

Gait

Supporting our hypothesis, B6 OV exhibited aberrant gait, with a decrease in front to hind paw area ratio (p=0.02) and width between hind paws (p=0.04) compared to B6

CA (Fig 3.2). Supporting our hypothesis, no differences in MRL OV gait were found relative to MRL CA.

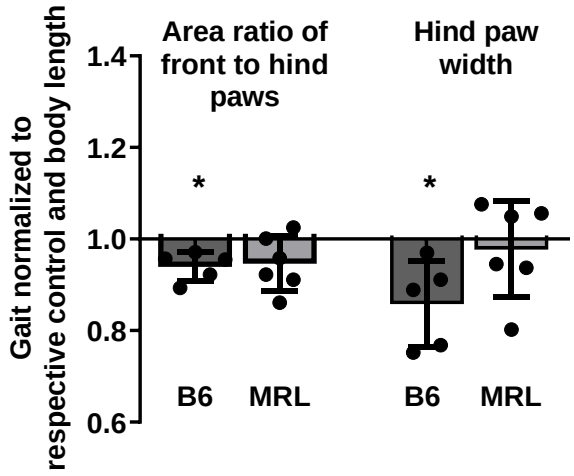

Figure 3.9 B6 mice display aberrant gait following 8 weeks of rotator cuff overuse by decreasing front paw areas and decreasing width between hind paws. No differences in MRL gait relative to cage-active mice were detected.

Mechanical Evaluation

Supporting our hypothesis, B6 OV SST demonstrated diminished mechanical properties, while MRL OV SST show some improvements (Fig 3.3). No differences were found in maximum load after either 4 or 8 weeks of overuse, however, stiffness decreased in in B6 OV ($p=0.003$), but increased in MRL OV (trend: $p=0.09$) after 8 weeks of overuse. Furthermore, relaxation increased in B6 OV ($p=0.001$), but decreased in MRL OV ($p=0.01$) after 8 weeks of downhill running.

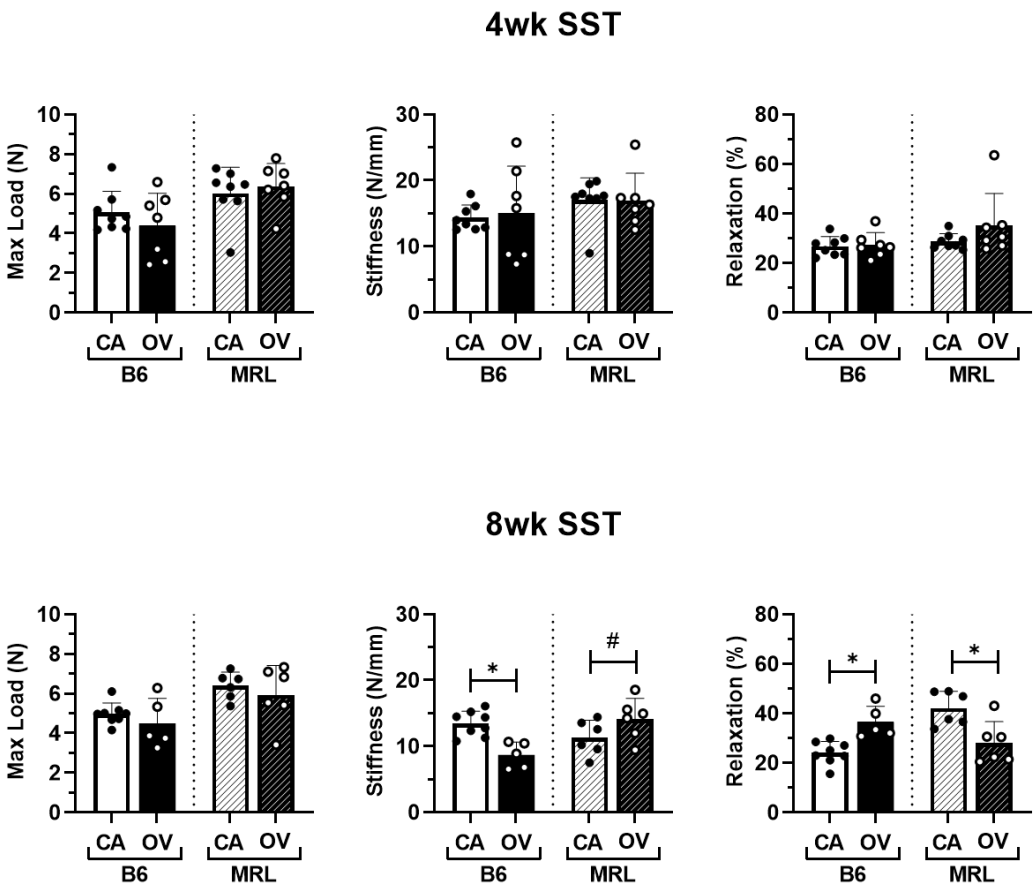

Figure 3.10 Overuse does not affect supraspinatus tendon (SST) mechanical properties in either strain after 4 weeks. At 8 weeks, B6 stiffness decreases and MRL stiffness increases with downhill running. B6 relaxation increases and MRL relaxation decreases at 8 weeks. (CA = cage-active, OV = overuse)

Supporting our hypothesis, there was no effect on maximum load, stiffness, or relaxation after 8 weeks of downhill running for either B6 or MRL PT (Fig 3.4).

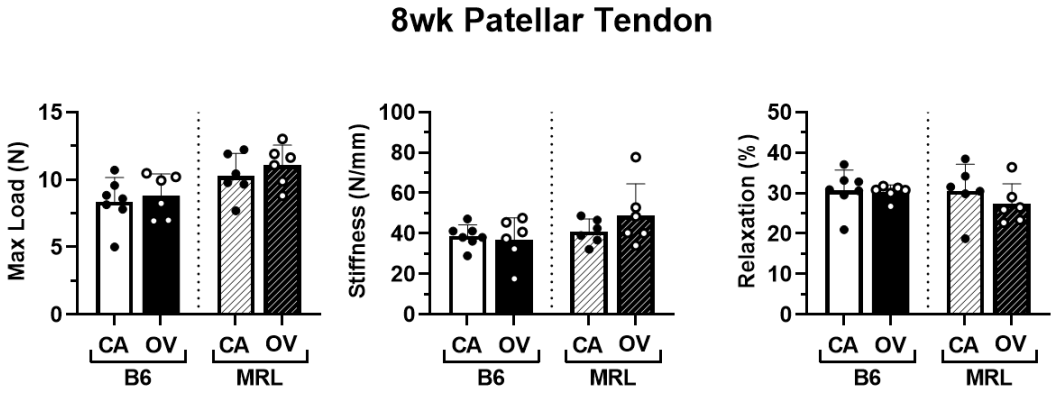

Figure 3.11 Downhill running does not affect patellar tendon mechanical properties in either strain after 8 weeks. (CA = cage-active, OV = overuse)

Collagen Organization

Contrary to our hypothesis, no structural damage in the form of collagen kinks were detected in either B6 or MRL samples after both 4 and 8 weeks of treadmill running (Fig 3.5). All samples showed a high degree of collagen alignment (>90%).

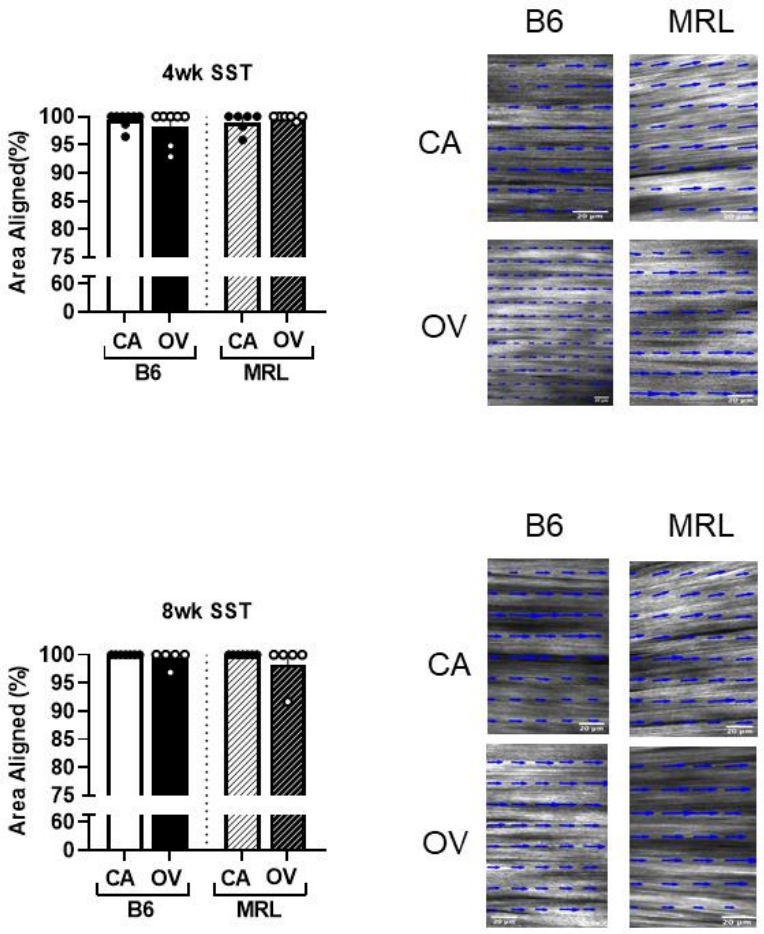

Figure 3.12 Overuse does not affect collagen organization in either strain after either 4 or 8 weeks. All samples show high degree of collagen organization in second harmonic generation imaging. Scale bar = 20um. (CA = cage-active, OV = overuse)

Early Molecular Response

Supporting our hypothesis, B6 SST increased gene expression at 1d of fibrocartilage, *Col2a1* ($p<0.0001$), *Acan* ($p<0.0001$), and *Sox9* ($p<0.0001$), and matrix turnover, *Col3a1* ($p<0.0001$) and *Col1a1* ($p<0.0001$) (Fig 3.6a). Further supporting our hypothesis, *Col2a1* ($p<0.0001$), *Acan* ($p<0.0046$), *Sox9* ($p=0.0005$), *Col3a1*

(p<0.0001), and *Col1a1* (p=0.0002) were all also increased in the MRL at 1d relative to cage-active mice (Fig 3.6b).

After 7d of overuse *Col2a1* (p=0.005), *Acan* (p=0.02), *Sox9* (p=0.0006), *Col3a1* (p=0.0002), and *Col1a1* (p=0.0005) were still upregulated in B6 SST (Fig 3.6c). Contrary to our hypothesis, MRL SST at 7d showed no upregulation in fibrocartilage genes, but did demonstrate increased col3a1 expression (p=0.008), indicating signs of adaptation to the running protocol (Fig 3.6d).

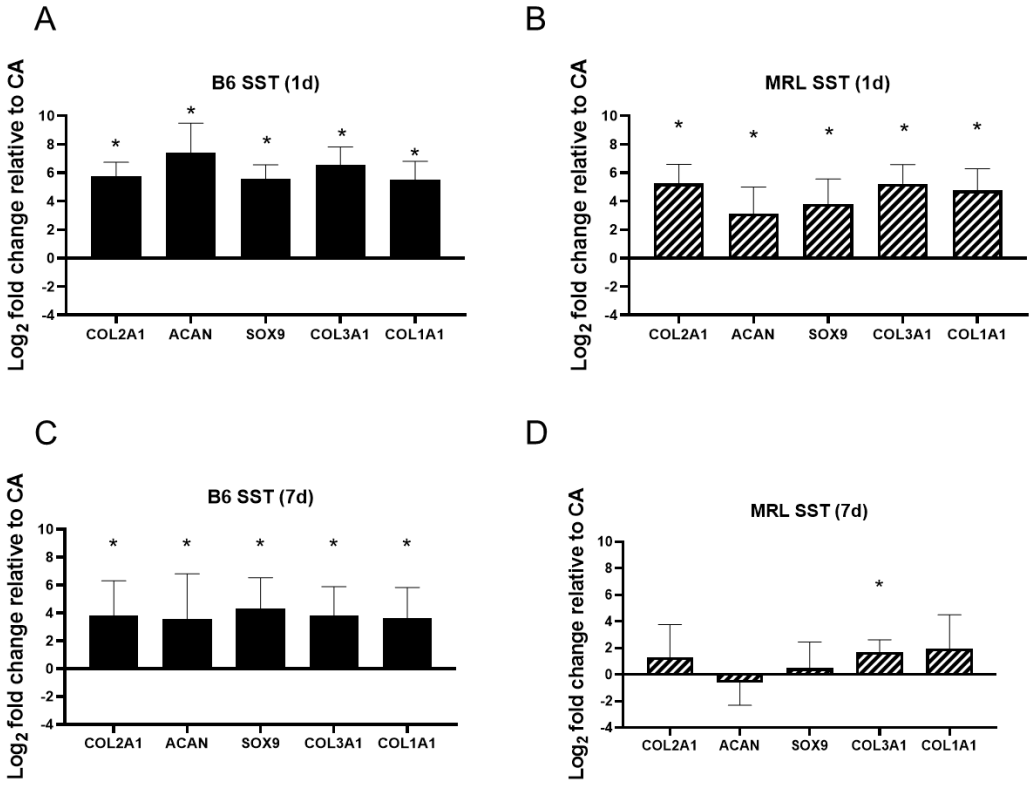

Figure 3.13 Gene expression of supraspinatus tendons (SSTs) relative to cage-active mice following downhill running indicate mechanobiological response to load. At 1day, both (A) B6 and (B) MRL have increased expression of compressive and tensile markers. By 7days, (C) B6 continues this expression but (D) MRL begins to show adaptation to compressive loading environment (CA = cage-active, OV = overuse, data represents –ddCT expression levels)

In contrast to the SST, B6 PT exhibited downregulation of *Col2a1* (trend: p=0.1), *Acan* (p=0.01), *Sox9* (p=0.001), *Col3a1* (p=0.0001), and *Col1a1* (p<0.0001) after 1d of downhill running (Fig 3.7a). Similarly, MRL PT exhibited downregulation of *Col3a1* (p=0.02) and *Col1a1* (p=0.02) after 1d of downhill running (Fig 3.7b). Confirming our hypothesis, both B6 (Fig 3.7c) and MRL PT (Fig 3.7d) showed no changes in molecular response from 7d of downhill running in any of the measured genes.

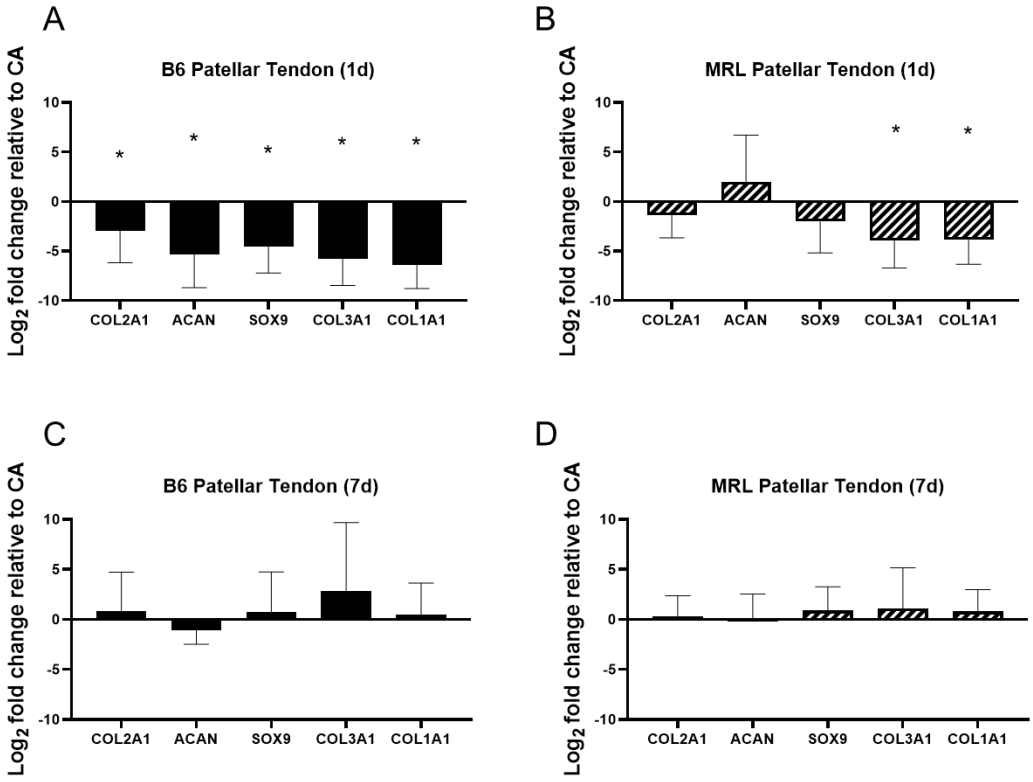

Figure 3.14 Gene expression of patellar tendons relative to cage-active mice following downhill running indicate mechanobiological response to load. At 1day, both (A) B6 and (B) MRL have decreased expression of matrix remodeling. By 7days, (C) B6 and (D) MRL show adaptation to running protocol with no differences in gene expression relative to cage active mice (CA = cage-active, OV = overuse, data represents –ddCT expression levels)

3.4 Discussion

The persistently high failure rate of rotator cuff surgical repairs, despite some improvements in surgical technique, motivates the investigation of enhanced repair responses necessary for early intervention following chronic overuse. Accordingly, the goal of this study was to evaluate the utility of the MRL/MpJ mouse as a model of enhanced healing in chronic rotator cuff overuse. Due to the multifactorial pathogenesis of rotator cuff tendinopathy, the overuse protocol in this study was intended to capture the effects of both repetitive tensile overuse and compressive loading from the coracoacromial arch[6]. For simplicity, the remaining discussion will refer to this protocol as 'overuse.'

As expected, the B6 SST showed diminished mechanical and functional characteristics following overuse, validating our downhill running protocol to induce mechanical degeneration and biological remodeling associated with similar rat studies[7]. Furthermore, the MRL exhibited increased SST stiffness and no loss in function. However, because increased stiffness may be the result of either adaptation to an exercise protocol, or the result of remodeling and repair after injury, we subsequently investigated the mechanical properties of the corresponding patellar tendons[17]. As the patellar tendon lacks the complex loading environment of the rotator cuff and does not incur damage from downhill running, potential exercise-induced adaptation would also manifest in increased mechanical stiffness in this tissue[10]. Since no mechanical tissue adaptations were found with overuse in either

strain, we can conclude that the observed changes in the MRL SST reflect a biological response to rotator cuff overuse.

However, due to anatomical differences in size between the B6 and MRL strains, we needed to further decipher whether the mode of damage induction during downhill running is comparable between the mouse strains. To this end, the acute mechanobiological response was measured to elucidate the tendon response to load. Genes associated with both compressive and tensile loading environments[10] were similarly upregulated in the SSTs of both strains, isolating the rotator cuff loading environment as the driver of the observed long-term biological outcomes and confirming that the mode of damage induction is the same for both strains. Additionally, while the B6 SST respond to pathogenic loading at both 1 day and 7 day of downhill running, the MRL pathogenic response is only seen at 1 day, with adaptation rapidly occurring by 7 day. Taken together, our findings indicate that despite similar mechanisms of damage induction to the B6 and MRL rotator cuff, the MRL are capable repairing the damage faster than it is accumulated.

To identify the driver of the observed mechanical response, we analyzed collagen organization. Other studies have identified that collagen disorganization is associated with and often precedes diminished mechanical properties[18]. Collagen kinks have been observed following tensile fatigue loading, where repetitive elongation of the fibers leads to permanent plastic deformations[19]. No collagen kinks were found following overuse in either mouse strain, supporting a different injury mechanism from downhill running than simply tensile overloading. Although some rat models of overuse show collagen disorganization, others report changes to

fiber crimping and packing[7], [20]. While not directly evaluated in our study, similar changes to fiber packing may result with overuse in B6 mice and be protected in MRL. Collagen spacing may be dysregulated through modulations in GAG-mediated crosslinks. In addition to structural regulation, GAGs can modulate the mechanical stress-relaxation response observed in the 8 week SST[21], and increase in animal models of overuse and clinical tendinopathy[22], [23]. Furthermore, different GAGs serve different biological roles. For example, chondroitin sulfate associates with aggrecan, and dissipates large, compressive loads[24]. Dermatan sulfate is found in decorin, which is responsible for fibrillogenesis in tendon[24]. Hyaluronan is a small proteoglycan critical in many cellular processes, such as wound healing, migration, and proliferation[24]. Ongoing experiments are parsing out the specific GAGs that arise in overuse and may be responsible for repair in the rotator cuff.

In addition to direct mechanical insult to the tendon, compositional alterations may arise from the unique biological environment of the SST due to its insertion into the synovial capsule. Unlike extrasynovial tendons, such as the Achilles and patellar tendons, which heal following injury, damage to intrasynovial tendons, such as the SST and flexor tendons, do not inherently repair[25]. Downhill running in rats detrimentally affects rotator cuff tissues in contact with the synovial fluid, including the humeral articular cartilage thinning and the fiber thinning in the tendon insertion site[26]. Clinically, rotator cuff damage severity is correlated to increased synovial inflammation and matrix proteases[27]. Taken together, the heightened pro-inflammatory and catabolic environment of the synovial fluid from downhill running may induce compositional, rather than structural, changes to the SST, thereby

resulting in mechanical deficiencies. Further supporting this notion, the patellar tendon lacks the pro-inflammatory synovial environment and therefore may not be susceptible to these compositional and mechanical alterations.

The MRL synovium may contribute to the biological repair of mechanical SST damage. In intraarticular fracture models, the MRL is protected from developing posttraumatic arthritis. This is associated with decreased inflammatory cytokines in the synovial fluid, as well as diminished early and late stage macrophage infiltration in the synovial tissue[28]. Similarly, by mitigating the adverse biological cascade in the synovium, the MRL SST may be protected from induced damage in downhill running.

Advances in early intervention for chronic tendinopathy are hindered by the lack of an animal model exhibiting enhanced repair following chronic injury. Studies are recently emerging using mice in downhill running due to the ability to perturb healing mechanisms with transgenic strains. One recent study showed downhill running did not induce adverse mechanical and biological outcomes in the murine rotator cuff[29], however, the running protocol applied a higher running speed at a steeper decline to put more weight on the joint. Thus, their running protocol may induce more tensile loading of the tendon, leading to beneficial mechanical adaptations. In contrast, our model appears to capture the compressive environment associated with rotator cuff overuse, highlighting the consequential role of the mechanical loading environment on inducing injury.

Here we also demonstrate enhanced repair of the MRL following rotator cuff overuse. Rotator cuff overuse is known to adversely affect the insertion site, with heightened proteolytic activity leading to structural loss[20]. Restoration of the

insertion site is critical to prevent further progression of tendinopathy and avoid failure at the tendon-to-bone attachment site between two tissues of vastly different material properties[30]. To this end, we have recently shown enhanced restoration of the insertion site, including the complex zonal fibrocartilaginous architecture, following surgical repair. In these acute surgical repairs, the cell response contributing to disrepair originates from multiple tissues[31], [32]. Accordingly, the MRL has shown enhanced healing in tissues surrounding the insertion site, including the bone and cartilage[33], [34]. However, in contrast, pathogenesis from overuse is characterized by an intrinsic response. Using an organ culture model, we have recently demonstrated that the MRL enhanced healing response is driven by the local environment[35]. This highlights that the innate cell population in the tendon may still be driving the enhanced repair response shown in this study. Furthermore, the ability of the MRL to repair damage in tendon injuries with differing underlying etiologies highlight the MRL as a broadly applicable model to investigate mechanisms associated with these enhanced healing outcomes.

Our findings mark an important first step in verifying the utility of the MRL to investigate intrinsic mechanisms governing enhanced repair following overuse. However, due to the destructive nature of the mechanical and biochemical tests in this study, further analysis of matrix composition and cell markers underpinning this phenotype could not be conducted. Future studies will supplement findings with histological analysis to identify spatial distribution of these markers. Additionally, the mouse is a quadruped, and does not fully recapitulate the human rotator cuff function and mechanical demands. However, our findings in the B6 mouse reflect pathology

associated with clinical tendinopathy, verifying the utility of mice in future preclinical studies.

In conclusion, these findings demonstrate that the MRL/MpJ mouse exhibit enhanced repair following rotator cuff overuse, despite the complex loading environment. This broadens available models to investigate intrinsic repair mechanisms that lead to improved outcomes in early tendinopathy.

3.5 Acknowledgements

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CHAPTER 4: MRL/MPJ-DERIVED THERAPEUTICS ELICIT REPAIR-ASSOCIATED PHENOTYPE IN SUBRUPTURE FATIGUE DAMAGED TENDONS

4.1 Introduction

Tendinopathy is a significant cause of pain and disability, accounting for 30% of musculoskeletal consultations[1]. The progressive condition arises from overuse, where incurred damage does not innately repair[2]. Furthermore, existing therapeutics remain ineffective due to failure to repair underlying structural damage and restore function. Consequently, unrepaired damage continues to progress and can lead to tendon rupture[3]. Thus, there is an unmet need for disease-modifying therapeutics to halt the progression of tendinopathy.

To investigate early onset tendinopathy, we have developed a rat patellar tendon fatigue injury model, which incurs an immediate and sustained 20% stiffness loss after a single bout of fatigue loading[4], [5]. Within the first two weeks following damage induction, tendons exhibit an increase in apoptosis that is correlated with a muted, and ultimately ineffective, matrix remodeling response[6]. Although this damage does not innately repair, therapeutic exercise can attenuate fatigue damage[7]. This repair is correlated with increased myofibroblasts that tension and remodel

damaged collagen, and decreased apoptosis, thereby increasing the number of cells to repair and remodel the induced damage[5].

Clinically, exercise or physical therapy is a common treatment regimen for conservative management of tendinopathy[8]. However, exercise is challenging to titrate to patient-specific needs. For example, sex, age, and treatment protocol may account for the disparate outcomes with exercise and physical therapy[9], [10]. The ability to instead mechanistically achieve these beneficial outcomes with a therapeutic would standardize and broaden treatment options for tendinopathy.

The MRL/MpJ (MRL) mouse strain exhibits innate broad enhanced tendon repair capacity – spanning midsubstance punch, rotator cuff surgical repair, or chronic rotator cuff overuse[11], [12]. We recently harnessed the MRL as a therapeutic by decellularizing and homogenizing the provisional extracellular matrix (MRL pECM) of the healing tendon 7 days after hole-punch injury and delivering it as a therapeutic to B6 mice following hole punch injury[13]. Treatment with MRL pECM substantially improved injured B6 tendon structure and mechanics, but did not restore naïve properties. This may be due to the inherent removal of cells from the MRL pECM during decellularization and their secreted factors (MRL secretome) that in turn modulate the matrix. *In vitro* culture of naïve B6 cells on MRL pECM elicits MRL-like behavior in the cells, but this phenotype is further enhanced with supplementation of the MRL secretome. The capability of MRL-derived therapeutics to shift cell phenotype, in conjunction with our findings that the MRL has broadly applicable mechanisms that may enable its enhanced healing response across the differing damage mechanisms associated with different injuries, suggests broad applicability of

MRL-derived components to induce beneficial outcomes following injury. However, the utility of these MRL-derived components, and particularly the role of the MRL secretome, to repair damage in the context of fatigue injury remains unknown.

Accordingly, the objective of this study was to evaluate the therapeutic potential of MRL pECM and MRL pECM + MRL secretome to attenuate post-fatigue damage. We hypothesize that treatment with MRL-derived therapeutics will promote an exercise-associated reparative phenotype through increased cell survival, myofibroblast phenotype, and matrix remodeling.

4.2 Materials and Methods

All murine studies underwent IACUC approval. Survival murine studies were administered analgesic at the time of surgery (Buprenorphine, 0.2 mg/kg). Isoflurane during surgical procedures was administered (2% by volume, 0.3 L/min).

Fatigue model validation

Our existing protocol to induce murine fatigue damage has not been validated with a particular age range of mice[14]. Since aging can have confounding effects on tendon function and repair[15], a protocol to standardize damage induction on 16wk old male B6 mice was developed. Left PT underwent pull-to-failure testing at 12% strain, akin to the strain rate during fatigue loading (n=7) to identify the maximum load. Subsequently, mice were anesthetized and subject to *in vivo* fatigue loading, in which the left patella and tibia were clamped and attached to an actuator and load cell. The

PT was sinusoidally loaded from 0.5N to 4.5N (10lb load cell, Transducer Techniques), corresponding to approximately 40% of the maximum load (10.8N ± 1.4N) at 2Hz for 7200 cycles to induce subrupture damage. Mice were immediately euthanized and the mechanical properties of the fatigue-loaded and contralateral tendons were evaluated using a pull-to-failure test at 0.1% strain to confirm mechanical damage to the tendon.

Therapeutic derivation

MRL pECM was obtained as previously described[13]. Briefly, 40 male 16wk old MRL mice underwent left PT hole-punch and allowed cage-activity for 7days prior to sacrifice. Tendons were harvested immediately, flash frozen, and stored at -80C until decellularization. Samples were lyophilized, weighed, then pulverized and decellularized through a series of washes using Latrunculin B (BioVision), KCl, and KI to remove cells. Samples were lyophilized for 72 hours, then solubilized at a concentration of 10mg dry weight/mL in a 1mg/Ml 0.1M HCl-pepsin (Sigma-Aldrich). The pECM diluted in 0.1M acetic acid to a final concentration of 7.5mg/mL, then aliquoted and frozen at -80C.

Secretome was collected from confluent MRL cells cultured in serum-free DMEM (Thermo Fisher Scientific) for 24 hours. Conditioned media was collected, concentrated in 3K MWCO centrifugal filters, aliquoted, and stored at -80C until use.

Therapeutic delivery

B6 mice underwent fatigue loading and were randomly assigned to a therapeutic testing group: 1) Vehicle control 2) MRL pECM (200ug) or 3) MRL pECM (200ug) + MRL secretome (10x). Therapeutics were delivered using the Alzet osmotic pump (0.25ul/hr) for 14 days to coincide with the peak of the biological activity after fatigue injury[6]. Pre- and post- fatigue loading diagnostics were evaluated to ensure all groups received similar amounts of damage at the time of fatigue injury. Mice were euthanized at the end of the 14-day therapeutic administration period.

Histology and Immunohistochemistry

At euthanasia, the injured patella-PT-tibia complex was fixed in formalin under 20g of load, then decalcified and paraffin-embedded. Sections were deparaffinized, then chromagen stained for either cleaved caspase-3 (apoptosis), aSMA (myofibroblast), collagen III (scar tissue and remodeling), or with collagen hybridizing peptide (CHP, collagen structure) or safranin-O (GAG content), as previously described[13], [16]–[18]. Caspase-3 and aSMA images were assessed for positive cell density by a blinded user (ImageJ, NIH). Collagen III and safranin-O positive signal was thresholded using the intensity histogram, and percent of positive matrix is reported. CHP signal is reported as average grey scale intensity in the tendon. All outcomes were measured in the tendon midsubstance, as defined by >100um from the tidemark[19].

Statistical Analysis

For protocol validation at Day 0, control and fatigue loaded limbs were compared using a paired T-test. The effect of the therapeutics was compared to Vehicle control using a non-parametric one-way ANOVA (Krusal-Wallis) with Dunnett's post-hoc

multiple comparison of the treatment to the control. Significance was set at $p \leq 0.05$ (*) and a trend at $p \leq 0.1$ (#).

4.3 Results

Fatigue loading protocol induces loss in mechanical function

As expected, the fatigue loading protocol led to an immediate 27% stiffness loss of the fatigue loaded tendon (Fig 4.1A) (p=0.002). Fatigue loading also affected relaxation (trend: p=0.09) (Fig 4.1B). There was no effect on maximum load from fatigue loading (Fig 4.1C).

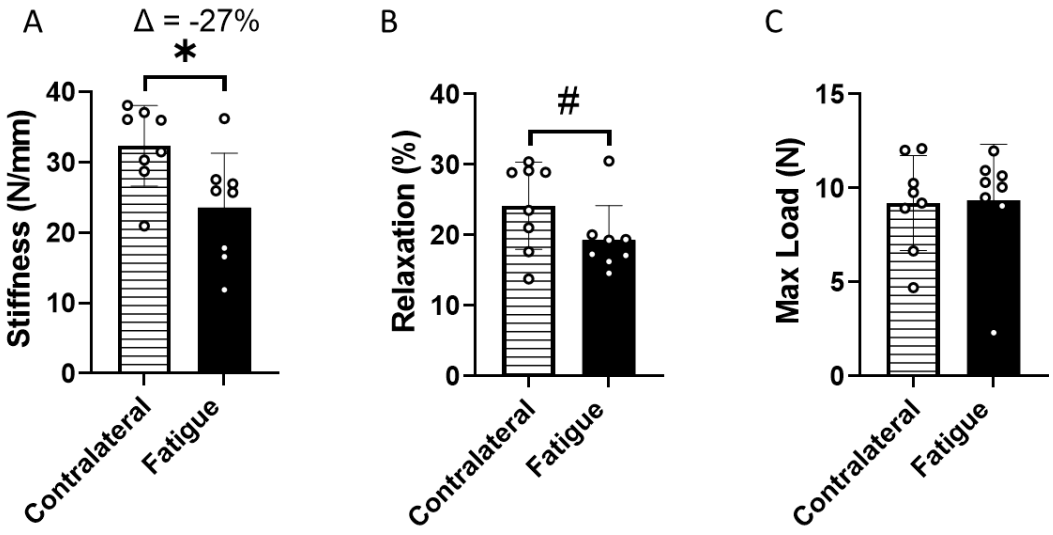

Figure 4.15 Fatigue loading led to an immediate 27% stiffness loss and affect relaxation. No effect was found on maximum load.

MRL-derived therapeutics alter cell behavior

Supporting our hypothesis, MRL pECM + MRL secretome increased myofibroblast levels relative to Vehicle control (p=0.04) (Fig 4.2).

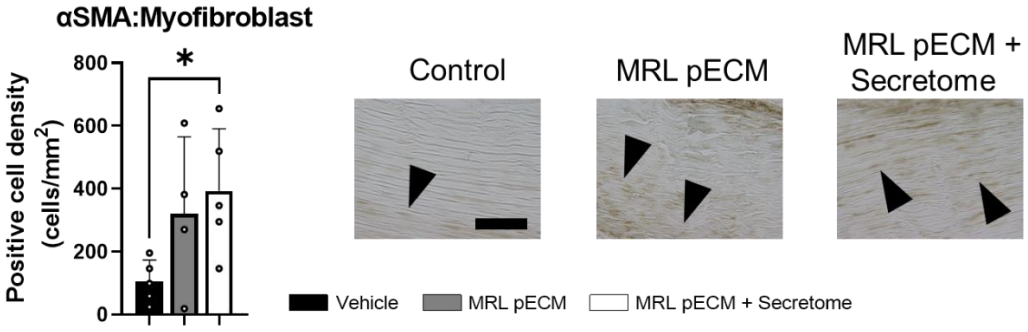

Figure 4.16 MRL pECM + Secretome increase myofibroblast levels relative to Vehicle. Positive cells are stained in brown (black arrowheads). Scale bar = 200µm

Further supporting our hypothesis, MRL pECM decreased apoptosis relative to Vehicle control (trend: p=0.1) (Fig 4.3). No other differences with the either therapeutic were found at this timepoint on these phenotypes.

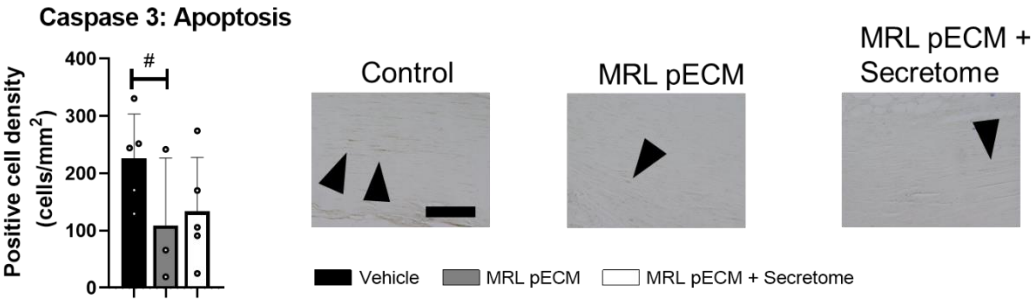

Figure 4.17 MRL pECM decreased apoptosis relative to Vehicle. Positive cells are stained in brown (black arrowheads). Scale bar = 200µm

<u>MRL-derived provisional matrix increases GAG content</u>

Supporting our hypothesis, MRL pECM delivery increases GAG content (p=0.05) (Fig 4.4). There was no effect from delivery of MRL pECM + MRL secretome on GAG levels.

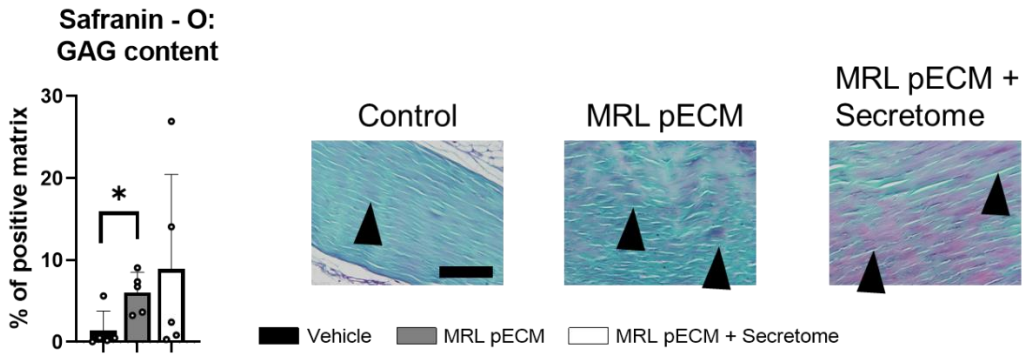

Figure 4.18 MRL pECM increased GAG levels relative to Vehicle. Positive matrix is stained in pink (black arrowheads) with green counterstain. Scale bar = 200µm

No effect from MRL-derived therapeutics on collagen remodeling

Contrary to our hypothesis, there was no effect of either therapeutic relative to Vehicle control on collagen III levels (Fig 4.5) or CHP levels (Fig 4.6).

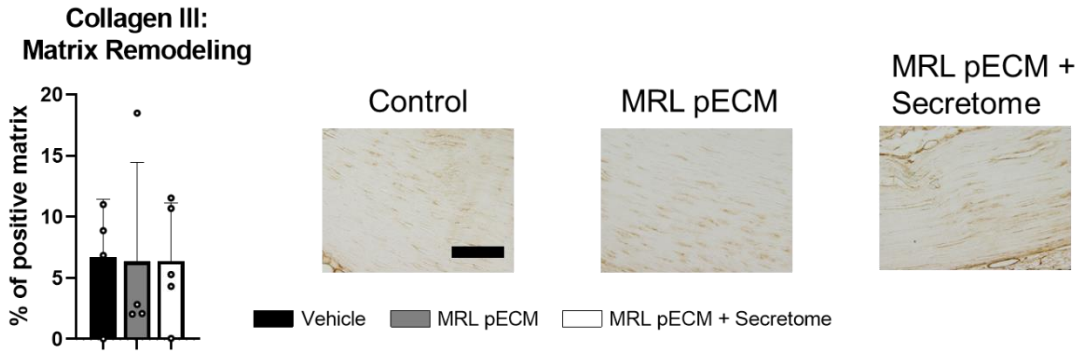

Figure 4.19 Neither MRL-derived therapeutic affect collagen III levels. Positive matrix staining is shown in dark brown. Scale bar = 200µm

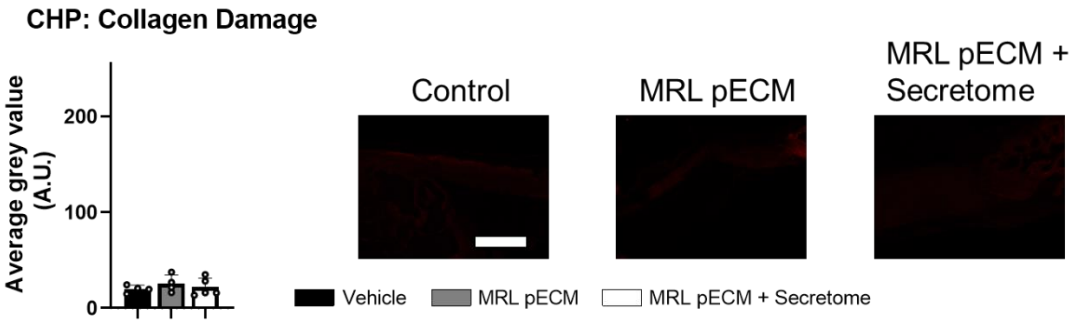

Figure 4.20 Neither MRL-derived therapeutic affected collagen structure and very little collagen denaturation was detected. Positive matrix staining shown in dark red. Scale bar = 1mm

4.4 Discussion

Overuse tendon injuries result in alterations to matrix organization and cell behavior. However, therapeutics such as cell therapies or growth factor delivery fail to effectively repair the underlying damage to restore function[20], [21]. We hypothesized that delivery of MRL-derived therapeutics following fatigue injury will achieve beneficial outcomes associated with therapeutic exercise. In contrast to other models of overuse that replicate tendinopathy, such as treadmill running[22], [23], fatigue loading allows for direct control and measurement of the loading regimen applied to the tendon. Furthermore, due to the chronic nature of treadmill running, it is difficult to discern at which point the tendon is injured to begin therapeutic administration[24]. A single bout of fatigue loading overcame these limitations by clearly delineating the fatigue injury from the repair response. We found that therapeutic treatment over 2 weeks following fatigue injury resulted in an early shift in phenotype through decreased apoptosis, increased GAGs, and increased myofibroblasts.

Clinically, apoptosis is markedly higher in degenerative patellar tendinopathy than healthy patients[25]. Apoptosis is associated with both high mechanical loading and with loss of homeostatic cell tension – highlighting the altered mechanical environment's effect on cell behavior[26], [27]. We expect that the observed decrease of apoptosis increases the cell population that can respond to the injury and remodel damaged matrix, but this effect is highly contextual. Direct pharmacological inhibition of apoptosis further exacerbates fatigue damage[16]. In contrast, the decrease in apoptosis associated with exercise attenuates fatigue damage[5] through altered pericellular composition and macroscale mechanical properties. Therefore, the reduction in apoptosis likely only is beneficial if the cells' mechanical environment is also remodeled.

One such modulator of the micromechanical environment is GAGs. In cartilage, GAGs alter the pericellular matrix mechanical properties and modulate cell response to mechanical loads load[28], [29]. In tendon, increased GAGs are with a hallmark of both early and late-stage pathology[30], [31], but whether this increase is a part of the degenerative cascade or simply a manifestation of the disease remains unknown. Harnessing the initial increase in GAGs can be ultimately beneficial for long-term outcome. Exercise is most therapeutic when initiated at the peak GAG level at 2 weeks post-fatigue, as opposed to 1 day after fatigue loading[7], suggesting GAGs modulate the cell micromechanical environment in tendon overuse. Similarly, the increase of GAGs with therapeutics may decrease mechanical stress on cells, and thereby prevent apoptosis.

MRL-derived therapeutics increased αSMA levels, suggesting the cells are mounting a collagen-remodeling response through myofibroblasts. Collagen III deposition was investigated due to its initial step in the wound healing cascade to support new collagen I formation[32]. However, fatigue injured tendons exhibit dampened gene expression of collagen III, contributing to the tendon's sustained loss of mechanical function[4]. Although not directly evaluated relative to uninjured tendons in this study, collagen III protein levels in all groups also remain low after fatigue loading (~5% of matrix). Similarly, CHP staining in all groups, including Vehicle control, shows very little staining of matrix damage. Thus, these therapeutics likely cannot rescue these markers of injury because they are unaltered by fatigue damage. Instead, myofibroblasts can contribute to recovering matrix damage through alternative mechanisms. For example, myofibroblasts also drive enzymatic collagen cross-linking and collagen I deposition[33]. Enhanced activity of these two cellular processes impart tendon tensile strength[34] and may lead to improved functional recovery.

Overall, both treatment groups induce similar effects on fatigue-loaded samples relative to Vehicle control. Thus, in this injury context, treatment with components of the MRL pECM alone may be sufficient to elicit behaviors associated with reparative outcomes. *In vitro* culture of naïve B6 cells on this MRL pECM shifts cell behavior to be MRL-like, including decreased cell rounding, increased cell area, and enhanced mitochondrial activity. Fatigue injuries are characterized by altered cell-matrix interactions, cell rounding, and subsequent aberrant mechanotransduction[2].

However, treatment with MRL pECM may stimulate cells to re-establish mechanotransduction pathways through these improved phenotypes.

To this end, previous proteomic evaluation of this MRL pECM relative to non-healing matrix-derived therapeutics identified elevated glycoproteins fibrinogen, fibronectin, and periostin, which may stimulate the rescue of the damaged cells and their microenvironment. Fibronectin and fibrinogen regulate cell adhesion and integrin interactions[35]–[37]. Periostin promotes stem-like cell behavior and tenogenic phenotype, which stimulates cells to restore their microenvironment[38]. HSP-90 is also enriched in the MRL pECM and is an anti-apoptotic agent[39]. Repetitive tensile loading causes cell stress, and can lead to apoptosis[40]. HSP-90 may modulate the cellular response to stabilize the cells, decrease apoptosis, and instead promote matrix repair.

Our findings show that MRL-derived therapeutics result in a clear shift in cell behavior and matrix composition during the administration period. Future studies will assess longer timepoints and mechanical manifestation of these early changes. Furthermore, due to the early GAG increase with therapeutic administration, future studies should evaluate whether taking advantage of this peak and initiating exercise results in long-term beneficial outcomes, or if this early increase is enough to itself result in enhanced repair. Additionally, while CHP staining paraffin sections is used as a proxy for collagen kinks, the two may not spatially co-localize[41], suggesting potentially independent modes of damage. Future studies will utilize second harmonic generation microscopy imaging of plastic sections to directly measure the effect of therapeutics on attenuating plastic deformations.

In conclusion, MRL-derived therapeutics may provide a disease-modifying method to achieve beneficial phenotypes associated with exercise. These findings provide a method to expand investigation of non-surgical and non-exercise based treatment options for tendinopathy.

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CHAPTER 5: SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

5.1 Summary

Mechanisms underlying effective tendon healing remain unknown, thereby hindering the development of disease-modifying therapeutics. The overall objectives of this thesis were to evaluate the utility of the MRL/MpJ regenerative mouse strain in the clinically-relevant, progressive stages of tendon injury (Chapters 2 and 3), then evaluate MRL-derived cell- and matrix- components as a therapeutic in a model of early onset tendinopathy (Chapter 4). The following studies were conducted to achieve these objectives:

Surgical repair following tendon rupture experiences unacceptably high re-tear rates at the tendon-to-bone attachment site. Chapter 2 identifies whether the MRL mouse, which displays enhanced healing in select orthopedic injuries, is capable of enhanced healing following rotator cuff surgical repair in comparison to scar-mediated healing in B6 mice. In this study, we demonstrate enhanced recovery of the zonal microstructure in the MRL insertion site and improved tendon-to-bone integration, both of which are critical to dissipate stress concentrations and prevent re-rupture at the site of repair. Furthermore, the MRL exhibits improved matrix composition through a temporal balance of collagens. Ultimately, these findings manifest in improved recovery of function in the injured limb in the MRL. Taken together, our findings conclude that the MRL exhibits mechanisms of enhanced repair in late-stage tendon disease. Thus, future advancements in surgical technique and pharmacological

interventions can utilize the MRL's healing outcomes as a benchmark for comparison of metrics associated with enhanced healing.

Subsequently, due to the high failure rate of surgical repairs, Chapter 3 investigates the use of the MRL to target earlier interventions in chronic tendinopathy. In particular, the pathology in the rotator cuff was investigated due to the joint's propensity to injury as a result of the complex (both tensile and compressive) loading environment. Our findings demonstrate superior recovery of the MRL mechanical and functional properties following overuse. Furthermore, despite similar modes of damage induction to both the B6 and MRL rotator cuff, the MRL begins to demonstrate mechanobiological adaptations to overuse within 7 days, whereas the B6 continue the degenerative cascade. Ongoing studies are parsing out compositional drivers of the observed mechanical changes. Thus, the MRL, which has previously demonstrated enhanced healing following a full-thickness midsubstance punch defect, also exhibits enhanced restoration following both late- and early-stages of tendinopathy. Accordingly, our findings suggest the MRL has broadly applicable mechanisms that may enable this enhanced healing response in tendon, despite the differing damage and healing mechanisms associated with the different injuries.

To this end, MRL-derived cell- and matrix- components that have previously shown therapeutic benefit in the punch excision injury were evaluated in a simplified model of early onset tendinopathy following fatigue injury. Although fatigue injury does not innately repair, damage can be attenuated with therapeutic exercise. Because exercise is clinically challenging to titrate based on patient-specific conditions, we sought to induce the phenotypes associated with exercise through use of a therapeutic

(Chapter 4). Supporting our hypothesis, these therapeutics result in a clear shift in cell behavior and matrix composition during the administration period that are associated with therapeutic exercise. These findings poise MRL-derived therapeutics as a method to expand investigation of treatments for tendinopathy.

5.2 Applications of MRL tendon biology

This thesis identifies the MRL as a suitable model for future investigation of enhanced healing in tendon biology. The specific contributions of the MRL's systemic and local environments to the enhanced healing outcomes remain under investigation. The MRL mice exhibit an altered basal immune system, which may contribute to the systemic response. Additionally, several quantitative trait loci have been identified in the MRL as correlated with the restoration of ear punch wounds. However, despite sharing a systemic environment, the MRL exhibit tissue-specific enhanced healing responses. Furthermore, MRL tendons isolated from the systemic environment in organ culture retain their enhanced healing outcome in comparison to organ-cultured B6 tendons, further supporting the hypothesis of the local environment.

The specific differences in the local tendon environment of the MRL that drive enhanced healing are still under investigation. In MRL cardiac tissue, the resident cells express high levels of embryonic stem cell markers and retain features of embryonic metabolism. Recent *in vitro* work from our group found several unique basal MRL tenocyte behaviors. Specifically, these cells self-aggregate to become more aligned, express more of the mechanosensitive gap junction marker Cx43, and increased aligned fibronectin deposition compared to B6 tenocytes. This unique cellular

behavior may enable the enhanced outcomes in the MRL *in vivo* in both rotator cuff studies (Chapters 2 and 3). For example, establishing proper Cx43 signaling is essential in restoring the zonal structure and fibrocartilage of the insertion site. Similarly, aligned fiber deposition during rotator cuff overuse is critical to withstand loads. Thus, achieving these MRL-like cell behaviors can in turn alter matrix synthesis, composition, and response to injury in the provisional matrix.

The MRL provisional matrix is rich in glycoproteins and signaling factors that can also modulate cell signaling. Harnessing these MRL-derived components in an *in vitro* platform further enhanced cell alignment and fibronectin deposition. Similarly, delivery of these components can shift cell phenotypes to become more MRL-like and ultimately enable beneficial matrix turnover and repair.

5.3 Impact of current findings

Tendon injuries occur in complex mechanical environments and are often chronic in nature[1]. However, surgical interventions and exogenous biologics therapies remain ineffective. This is largely due to existing animal models of tendinopathy fail to recapitulate the complexity of the injury environment, and effective intrinsic repair mechanisms repair unknown.

Our findings indicate that MRL exhibits enhanced healing in both late and early stages of clinically relevant tendon injuries. Moreover, these outcomes are conserved across injuries. For example, in rotator cuff surgical repair, temporal regulation of collagen remodeling through a balance of procollagen I and collagen III levels is essential in restoring insertion site structure and composition (Chapter 2).

Similarly, when MRL-derived components were delivered following a patellar tendon fatigue injury, increased αSMA indicates a similar regulation of collagen-associated remodeling (Chapter 4). At the macroscale functional level, MRL mice following rotator cuff surgical repair no longer exhibited gait deficiencies after 6 weeks of recovery (Chapter 2). Likewise, MRL gait is protected from overuse injuries after 8 weeks of downhill running, unlike the B6 mice (Chapter 3).

This introduces a platform to allow for mechanistic perturbation the specific mechanisms that lead to an enhanced healing outcome. Additionally, the enhanced healing response of the MRL in tendon injuries sets therapeutic goals and standards for development of new therapies, including physical therapy, small molecules, biologics, or surgical repair. Our finding of the MRL-derived therapeutics to shift cell behavior and matrix remodeling following fatigue injury to that associated with therapeutic exercise suggests broad applicability to a variety of tendon injuries.

5.3.1 Future directions: Additional evaluation

Findings from these studies open investigation for further development in varying stages of tendon injuries. Ongoing work in the lab is evaluating the effects of the specific proteins in the cocktail of MRL-derived components on shifting cell phenotypes towards MRL-like behaviors. Additionally, the specific proteins that drive this response are being evaluated as a therapy utilizing a cocktail of these recombinant proteins. These proteins of interest are being evaluated in all stages of tendon injury, spanning early onset tendinopathy, chronic rotator cuff overuse, rotator cuff surgical repair, and patellar tendon punch excision. Despite differing drivers and mechanisms

of injury across the models and tendons, the goal remains the same for therapeutics to re-establish the basic structure and function of the tendon to transmit high tensile loads. Accordingly, identification of the proteins of interest that are broadly applicable across mechanisms will lead to the development of more effective, disease-modifying therapeutics.

Effective delivery of these therapeutics remains a non-trivial challenge. Previous work in our lab has utilized a PEG-MAL hydrogel delivery system following a patellar punch excision[2]. However, treatment with this carrier alone led to more severe degeneration than an untreated tendon injury. Subsequently, this study used an osmotic pump and catheter system to deliver solubilized components to the patellar tendon. However, catheter positioning over the course of the administration period may shift and also result in delivery to off-target tissues. Furthermore, a rigid foreign body, such as the catheter, adjacent to the tendon may apply abnormal or pathological loading onto the tendon, thereby confounding the mechanism of injury in the study. Nevertheless, the positive results in our findings despite the limitations of the delivery system motivate further investigation of MRL-derived components using alternative delivery methods, such as grafts, injections, or adhesive biomaterials.

5.3.2 Future directions: Mechanistic investigation

These studies used various methods to assess matrix composition and structure due to their role in tendon mechanical function and modulation of cell behavior. However, further investigation of the ultrastructure would give more insight into the specific micromechanical regulators of the cell environment. For example,

Transmission Electron Microscopy (TEM) can parse out differing mechanisms of collagen damage following fatigue loading, such as fibrillar bends, twisting, or rupture[3]. Additionally, changes to the cell morphology and its attachment to the matrix can be visualized with this method. Ongoing work in our lab is establishing a method to measure strain transfer from the matrix to cells under load[4]. Together, these methods can provide more insight into the mechanobiological environment associated with the enhanced healing outcomes observed in the MRL or with MRL-derived therapeutics. Establishing the specific mode of structural damage incurred will serve as therapeutic targets and evaluation following intervention. Furthermore, identifying the different ultrastructural changes following various tendon injury protocols in the MRL and their subsequent effects on cell behavior will establish goals for therapeutic intervention.

Evaluation of the MRL-derived therapeutics following fatigue injury, along with previous work of therapeutic exercise[5], highlights the potentially critical role of myofibroblasts tendon repair. The role of myofibroblasts in the healing cascade is highly contextual. While their presence following subrupture injury can likely re-tension damage kinks, the persistence of myofibroblasts in laceration injuries are associated with excessive matrix deposition leading to scar formation[6]–[8]. Similarly, in other soft tissues, early myofibroblast activity is necessary for matrix turnover and ultimate healing, but their late-stage activity activates fibrotic pathways[9]. The temporal role of myofibroblasts in clinically relevant tendon injuries and how they are modulated with MRL-derived therapeutics is unknown. Use of an inducible αSMA knockout mouse to turn off myofibroblast activity at varying points

during the healing cascade would elucidate the role of the myofibroblasts throughout disease progression or therapeutic administration. Furthermore, correlating this activity with known markers of matrix repair and turnover can pinpoint the biological contexts that cause the shift in myofibroblast activity from beneficial to detrimental in tendon healing. Furthermore, combining this approach with *in vivo* imaging with an optical window at the site of injury[10] would allow for longitudinal assessment of the same cell populations. Mechanistic understanding of the regulation of αSMA activity opens new avenues for cell and gene therapies. For example, these therapies can harness and prolong the early, beneficial response of myofibroblasts, and dampen their later biological cascades to avoid fibrosis.

5.3.3 Future directions: Clinical Translation

A major hurdle to translation of many animal studies is that preclinical studies fail to provide clinical contextualization. For example, tensile mechanical testing or functional gait evaluation was used as the primary outcome metric to assess the utility of the MRL in different injury models. Mechanics and gait measurements are gold standards in mouse studies, due to the quantitative ability to measure the tendon's primary functions of load transfer and joint stability. However, clinically, the primary assessment of efficacy is joint function following a return to activity[11]. Thus, further clinical context could be evaluated of the MRL response to surgical repair or effect of therapeutics by subjecting the animal to moderate exercise, rather than cage activity, and evaluating the therapeutic capacity under these loading conditions. These metrics

can guide clinical practice for a patient to return to activity or work following injury or therapeutic administration.

Additionally, inbred mice were used in these studies due to their low variability, cost-effectiveness, and anatomical similarities to humans[12]. However, mouse models fail to incorporate challenges associated with drug delivery to the larger, human tendons. No single animal captures the complexity of human tendinopathy, but different animal models recapitulate varying aspects. For example, sheep models may be optimal to match the human sustained inflammatory conditions in acute injuries, while horse tendons capture human matrix remodeling behavior characteristic of chronic injuries[13]. Thus, future preclinical studies should consider scaling up to larger animal models.

5.4 Future Directions

5.4.1 The role of synovial inflammation in rotator cuff overuse and repair

In this thesis, the structural and functional consequences of mechanical insult were evaluated in B6 and MRL rotator cuffs to evaluate the MRL as a model of enhanced healing in overuse. However, the biological contributions from surrounding tissues of the supraspinatus tendon were not evaluated. The rotator cuff is a very complex environment and understanding the role of the surrounding tissues can pinpoint tissue targets for therapeutic intervention. In particular, the role of the synovium and the inflammatory environment are under-researched, despite known correlations to increased disease severity. I hypothesize that decreased inflammation in

the synovium during rotator cuff overuse will mitigate the associated adverse mechanical and functional consequences.

I propose applying the downhill running protocol to B6 mice, with one group injected with anti-inflammatory therapeutics directly to the synovium. Following 8 weeks of running, which has been shown to cause mechanical and functional deficits (Chapter 3), the mice will be similarly assessed for mechanical and functional recovery. I expect that inhibiting inflammation will attenuate the induced damage. Subsequently, the synovial fluid can be isolated and assessed for inflammatory cytokines between the treated and untreated groups to identify particular proteins that may contribute to this discrepancy. Furthermore, the presence of macrophages in both the synovium and supraspinatus tendon can be assessed using F4/80 staining to identify potential inflammatory cell mediators of this outcome.

To date, no study has evaluated the protein level inflammatory mediators and cells associated in a controlled model of tendinopathy. Thus, findings from this study can have far-reaching implications of our understanding of rotator cuff tendinopathy pathogenesis and inform targets for intervention.

5.4.2 The role of cell cycle checkpoint regulation in enhanced tendon healing

This thesis identifies the MRL/MpJ regenerative mouse strain as a model of enhanced tendon repair in both early and late stage tendinopathy. The MRL also exhibits regenerative healing of other, non-orthopedic injuries, such as full thickness ear punch excision. In that model, the aberrations in the MRL cell cycle have been identified, such as accumulation of cells in the G2/M phase due to dysregulation of the

G1 checkpoint[14]. Through this manner, MRL cells are poised to proliferate soon after injury, bypassing DNA damage repair mechanisms that lead to cell cycle arrest, thereby responding to injury prior to additional damage accumulation. Interestingly, this aberration of the G1 checkpoint is a hallmark also associated with classic models of regeneration, such as in hydra, stem cells, and the liver[15]. In particular, the MRL ears show decreased levels of p21, which canonically causes cell cycle arrest or senescence, and instead causes damaged cells to undergo apoptosis. A recent study using RNA-seq identified that MRL p21 expression is negatively enriched following partial tendon laceration[16]. However, the role of these aberrations in more clinically-relevant, subrupture tendon injuries remains unknown. Accordingly, I propose a study to target G1 activation to improve tendon repair after injury. I hypothesize that inhibiting cell cycle checkpoint activation after injury reduces cell cycle arrest, promoting beneficial matrix remodeling and improvements in mechanics and function.

Using the subrupture fatigue injury model to delineate the injury and repair phases, I propose inhibition of p21 during the early apoptotic cascade through delivery of a small molecule inhibitor, which allows for a transient and reversible approach[17], [18]. Following validation of mitigation of cell cycle arrest, matrix turnover at longer timepoints post-injury will be assessed. I expect that p21 inhibition will decrease cell cycle arrest and senescence, thereby enhancing matrix turnover and deposition of collagen I, ultimately leading to improved structure and function following injury. Findings from this study will elucidate the mechanistic role of cell cycle control in tendon repair, thereby expanding mechanisms to target in therapeutic interventions. For example, development of a controlled, transient delivery method of

a p21 inhibitor or alternative methods to decrease cell senescence in the tendon may clinically be a potent therapeutic to rescue tendon injury.

5.4.3 The multiscale mechanobiological role of elastin in tendon overuse and repair

Tendon's high tensile properties are imparted by its hierarchical structure. Thus, macroscopic degeneration in turn leads to an altered cell environment. Since tendons are a mechanosensitive tissue, any damage to the matrix is transduced to the cells, which attempt to mount a biological repair response. However, the compositional components that increase the propensity of certain populations to incur tendon damage following repetitive overuse remains unknown. Elastin is abundantly found in repetitively loaded tissues, such as arteries or skin, and provides extensibility and resilience to the matrix. Pure elastin is highly extensible and resilient, and can recover large strains without permanent deformations[23]. Clinical tendinopathy is associated with decreased levels of elastin and increased elastase[24]. In tendon and ligament mechanics, enzymatic elastin depletion has been shown to affect cyclic tensile properties, toe region mechanics, and shear mechanical properties[13], [25], [26]. Recently, a paper by Godinho *et al.* found elastin depletion to decrease the fatigue life in only the intrafasicular matrix[27]. However, the effects of elastin on the conjoined fasicular and interfasicular matrices remains unknown.

Furthermore, elastic fibers are directly adjacent to the embedded tenocytes, and therefore significant contributors to the mechanical environment of the cells and subsequent cellular functions[13]. However, the mechanical role of this incurred fatigue damage on the resulting innate cell repair cascade has yet to be addressed.

Accordingly, I propose a study to determine the multiscale mechanical role of elastin in fatigue damage accumulation and subsequent cell repair cascade. I hypothesize that elastin plays protective role in preventing fatigue damage accumulation by mitigating multiscale strain and damage.

Since this proposed study investigates *in vivo* effects of elastin on fatigue injury and subsequent biological cascades, the *ex vivo* methods of enzymatic elastin depletion are limited by effective delivery methods to the tendon and perfusion out of the tissue. Thus, I propose using an elastin haploinsufficient mouse for this study, which retains 50% of naive elastin levels and can survive into adulthood, unlike elastin knockout mice[28]. To first delineate the multiscale effect of elastin on the propensity for the whole patellar tendon to incur damage, pre- and post-fatigue diagnostics, collagen structure, and bulk tensile mechanics will be assessed immediately following fatigue loading. Subsequently, the effect of elastin on the cells following injury will be assessed by quantifying the cell-matrix strain transfer[4] at various timepoints post-injury to elucidate the mechanobiological environment of the cells. Specifically, due to elastin's role in enabling tendon recoil[13], tensile-loaded and unloaded conditions will be compared. Additionally, I propose immunohistological quantification of cell behaviors and matrix composition that may drive the repair response.

I expect that elastin deficiency will increase the amount of fatigue damage incurred by the tendon, through increased structural and mechanical damage, thereby decreasing tendon recoil. In turn this will affect the degree of strain transfer to the cells during loading following fatigue injury, decreasing cell strain due to higher levels

of incurred matrix damage, as well as diminished sensitivity to loaded and unloaded conditions. Ultimately, this will lead to a highly degenerative cascade in which the matrix remains unremodeled following damage. This proposed study will mechanistically perturb the role of elastin at various stages of fatigue injury to parse out its contribution in tendinopathy. Results will open investigation into whether elastin is implicated in overuse injuries of at-risk populations in studies of sex-differences and aging.

5.5 Conclusions

Together, the work in this thesis and proposed future directions aim to elucidate mechanisms that contribute to the damage and repair responses in tendon. Mechanistic studies should be given further consideration to distill the biological processes that underlie enhanced tendon healing for clinical translation.

5.6 References

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