I, Jessica R Arble, hereby submit this original work as part of the requirements for the degree of Master of Science in Biomedical Engineering.

It is entitled:
An Examination of the LG/J Murine Strain as a Model of Tendon Regeneration

Student's name: Jessica R Arble

This work and its defense approved by:

Committee chair: Jason Shearn, Ph.D.
Committee member: Chia-Ying Lin, Ph.D.
Committee member: Kumar Vemaganti, Ph.D.
An Examination of the LG/J Murine Strain as a Model of Tendon Regeneration

A thesis submitted to the

Graduate School

of the University of Cincinnati

in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE

in the Department of Biomedical, Chemical, and Environmental Engineering

of the College of Engineering and Applied Science

2016

By

Jessica Renée Arble

B.S. Virginia Commonwealth University 2014

Committee Chair:

Jason T. Shearn, Ph.D.
ABSTRACT

Musculoskeletal injuries are common in the United States, with injuries to tendons accounting for 30% of reported injuries\(^2\). Injuries to tendons are difficult to repair and usually do not heal to normal properties, resulting in a high risk of rerupture and often a need for further medical intervention. Understanding how tendons heal after injury is a vital part of creating successful strategies to improve healing outcomes. We have previously evaluated the tendon healing of the MRL/MpJ murine strain, which is known for regenerative healing. In this study, we evaluate the healing of the LG/J murine strain, which comprises 75% of the MRL/MpJ strain, to determine if the LG/J strain exhibits improved healing.

A full-length central patellar tendon defect was introduced at 16 weeks of age. Mechanical properties and histology were assessed at 2, 5, and 8 weeks post surgery. Tissue stain markers were placed on the tendon and photographed throughout the tensile test to allow for calculations of regional strain. Tendons were loaded into grips and preloaded to a value of 0.02 N, at which point the tendons were photographed for cross-sectional area optical measurements. Tendons were tested in 37°C PBS, preconditioned from 0 to 1% strain for 25 cycles, and then ramped to failure at 0.1% of length/second.

Average LG/J structural properties improved to near-native values at 8 weeks, with normal tendons displayed an average ultimate load of 4.29 ± 1.5 N and stiffness of 10.88 ± 2.34 N/mm. Tendons after healing displayed an ultimate load of 4.17 ± 1.2 N and stiffness of 10.52 ± 3.40 N/mm. At 8 weeks, stiffness was at 96.7% of normal and ultimate load was at 97.2% of normal. Elastic modulus reached 92% of normal and maximum stress reached 86% of normal by 8 weeks.
The LG/J strain returns to normal mechanical properties by 8 weeks, with a steady increase in properties at each time point, in contrast to the MRL/MpJ strain which does not return to normal properties by 8 weeks and exhibits a drop in properties from 2 to 5 weeks post surgery. Future studies will focus on analyzing the transcriptome and the proteome to understand the healing process employed by the LG/J strain, with a long term goal of utilizing the genes and pathways responsible for increased LG/J healing in regenerative medicine and tissue engineering applications.
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Dr. Jason Shearn. Thank you for giving me the opportunity to research in your lab, and thank you for understanding when my goals changed. I would also like to thank Dr. James Lin and Dr. Kumar Vemaganti for serving on my committee and providing their unique insight into my research. Thanks to Cindi Gooch for being an outlet for my frustrations and talking to me about anything and everything when I was waiting for things to finish in the lab.

I would not be here today if not for my high school teacher, Laura Akesson. Thank you for being cool enough on the first day of biomedical engineering class that I didn’t switch to another elective, and thank you for showing me what biomedical engineering could be and sparking my interest in the field. I must also thank Dr. Gerald Miller from Virginia Commonwealth University, who came and spoke to our class one day and encouraged me that even though I didn’t like chemistry, I could still become a biomedical engineer. Thank you for making my four years at VCU challenging, entertaining, and fulfilling. I would also like to thank Dr. Jennifer Wayne from Virginia Commonwealth University, who taught me biomechanics and who gave me wonderful advice about graduate school.

Thank you to my father, Dave Arble, for supporting me 100% along the way. Thank you for driving 8 hours and back to move me in and out of my apartment. Thank you for always believing in me without question, and making me step outside of my comfort zone sometimes. Thanks to my mother, Dr. Fran Arble, who has always worked
hard to provide for me and has helped me in so many ways. Thank you for talking sense into me when I needed it.

Special thanks to my sister, Dr. Deanna Arble, for giving me a path to follow and always giving me good advice, even if I didn’t always take it. Thank you for talking to me when I needed it and cheering me up when I was down. I’d also like to thank my brother-in-law, Dr. Ignacio Rivero Covelo, for his unique opinions on all things American and for helping us start some interesting family traditions. Thanks to both of you for convincing me that it was okay to be the Wolowitz of the family.

Last but not least, I’d like to thank Daniel Kolmer, who put up with me through this whole process even when it was extremely difficult for both of us. Thanks for supporting me and believing in me.
TABLE OF CONTENTS

List of Tables and Figures........................................................................................................ iii

Chapter 1 – Introduction ........................................................................................................ 1
  1.1 Background ..................................................................................................................... 1
  1.2 Research Objectives ...................................................................................................... 2
  1.3 Organization of Text ...................................................................................................... 3

Chapter 2 – Review of Literature .......................................................................................... 4

Chapter 3 – The LG/J Murine Strain Exhibits Near-Normal Tendon Biomechanical Properties Following a Full-Length Central Patellar Tendon Defect ........................................................................................................ 11
  3.1 Introduction ................................................................................................................... 11
  3.2 Methods ........................................................................................................................ 12
  3.3 Results ............................................................................................................................ 14
  3.4 Discussion ...................................................................................................................... 23

Chapter 4 – Discussion and Conclusions ............................................................................. 26
  4.1 Further Directions .......................................................................................................... 27

References ............................................................................................................................ 29
LIST OF TABLES AND FIGURES

Figure 1. Average Failure Curves for LG/J, MRL/MpJ, and C57/B6 ...................... 16
Figure 2. Average Failure Curves for LG/J, Native through 8 Weeks .................... 17
Figure 3. Gross morphology of the MRL/MpJ and LG/J PT repair tissue .......... 18
Table 1. Mechanical Properties for LG/J, MRL/MpJ, and C57/B6 ...................... 20
Figure 4. Ultimate Load as Percent of Native ................................................. 21
Figure 5. Linear Stiffness as Percent of Native ............................................... 21
Figure 6. Maximum Stress as Percent of Native ................................................ 22
Figure 7. Elastic Modulus as Percent of Native ............................................. 22
Chapter 1 – Introduction

1.1 Background

Musculoskeletal injuries are extremely prevalent in the United States, accounting for 99 million ambulatory care visits and $197.1 billion in direct medical costs from 2009 – 2011. Studies have indicated tendon and ligament injuries account for 30% of musculoskeletal related health care visits. As a tendon ages, it exhibits decreased tensile strength and lower stiffness, which increases the likelihood of tendon injury. As the population continues to age, tendon injuries will continue to account for a large proportion of medical visits in the United States.

Overuse and trauma are the main types of tendon injury. Rotator cuff tendons, which typically tear due to overuse as patients age, are the most commonly injured tendons in the body, with a prevalence of over 80% in the elderly. In addition, a study found that rotator cuff tears are present in 23% of asymptomatic patients, indicating that the problem is more widespread than physician visits show. On the other hand, tendons are often victims of traumatic injuries. Flexor tendons are commonly lacerated – 54.8% of patients with small hand lacerations and 92.5% of patients with deep lacerations exhibit flexor tendon injuries.

Both overuse and traumatic injuries can require medical intervention. For many overuse injuries, rest and modifying daily activities are sufficient – studies have found that two weeks rest can ease two weeks of overuse. Surgical treatments are often employed for traumatic flexor tendon injuries, as well as rotator cuff injuries. Grafts can also be used as replacements when the tendon cannot be surgically repaired.
In the last few years, our laboratory has set out to create functional tissue engineering benchmarks. The research described in this thesis is part of an effort to understand and to identify the genes and pathways important to tendon healing. Once the integral pieces of tendon healing have been identified, methods to increase their presence after tendon injury can be developed, allowing for increased healing and better outcomes after surgery. In addition, tissue engineering strategies can be developed using similar techniques to allow for the generation of tendons for use in grafts.

1.2 Research Objectives

Our laboratory has previously studied the patellar tendon healing of MRL/MpJ and C57/B6 mice and discovered that the MRL/MpJ strain displayed improved biomechanical outcomes after a central-third patellar tendon defect was introduced\textsuperscript{12}. With the goal of better understanding the biological markers and pathways involved in tendon healing, our laboratory decided to expand the data set to potentially narrow down the possible pathways that yield improved healing. Knowing the genetic makeup of the MRL/MpJ strain, we chose to evaluate the biomechanical properties of the LG/J murine strain which makes up 75% of the MRL/MpJ mouse genome\textsuperscript{13}. The information obtained from research into the LG/J strain could provide us with useful information regarding which portion of the MRL/MpJ genome to further analyze as indicators of increased healing potential. We hypothesized that the LG/J strain would heal just as well, if not better, than the MRL/MpJ strain, narrowing the genetic scope to 75% of the MRL/MpJ genome.

The purpose of this research was to characterize tendon healing of the LG/J murine strain. This was achieved by a) measuring the biomechanical properties of the LG/J patellar tendon after injury and comparing to normal LG/J patellar tendons, and b)
observing the histological changes in the LG/J patellar tendons after injury and comparing to normal LG/J patellar tendons. After the biomechanical properties for the LG/J strain were obtained, they were also compared to existing data for the MRL/MpJ and C57/B6 strains to determine the relationship between the healing characteristics of all three strains.

1.3 Organization of Text

Chapter 2 examines the current research on tendon healing in healer vs. non-healer mice and the genetic differences between the C57/B6, MRL, and LG/J murine strains.

Chapter 3 presents a manuscript intended for publication describing the biomechanical tests performed on the LG/J patellar tendon, their results, and how they compare to C57/B6 and MRL murine strains.

Chapter 4 discusses the study as a whole. Future directions for this research are also discussed in addition to how this study will impact the fields of mammalian regeneration, musculoskeletal biomechanics, and tissue engineering.
Chapter 2 – Literature Review

Tendons are collagenous structures that transmit loads from muscle to bone, allowing for movement. Tendons are made up of mostly type I collagen (65-80%) and elastin (1-2%) in a proteoglycan matrix, and are organized in a hierarchical manner beginning with the collagen fibrils and working up to the tendon as a whole\textsuperscript{14}. Tropocollagen molecules crosslink to form collagen molecules, which join together and create the microfibrils\textsuperscript{14,15}. These microfibrils join together to create the collagen fibril, the smallest visible unit in the tendon\textsuperscript{14,15}. Collagen fibrils join together to create the collagen fiber, the orientation of which depends on the forces the tendon experiences\textsuperscript{14,15,16,17}. For example, the patellar tendon experiences forces along the long axis, and in turn collagen fibers are oriented along the long axis\textsuperscript{17}. Collagen fibers combine to create primary fiber bundles or subfascicles, which combine to form secondary fiber bundles or fascicles, and finally fascicles combine to form the tertiary fiber bundles\textsuperscript{14}. The tertiary fiber bundles join together, forming the tendon as a whole, which is surrounded by the epitenon\textsuperscript{14}. Fiber diameter and collagen concentration varies, as does the fibril diameter and fibril concentration in a collagen fiber\textsuperscript{14}. This structure creates the unique properties of tendons, allowing them to transmit large forces from muscle to bone while still retaining shape and function over a long period of time.

Tendons have fairly unique biomechanics properties. Tendons display a high stiffness during stress-strain tests in addition to behaving viscoelastically\textsuperscript{18}. Tendon viscoelasticity arises from the interaction of collagen fibers and proteoglycans as the tendon strains and water flows in and out\textsuperscript{18}. These properties give rise to the unique stress-strain curve observed when tendons undergo tensile testing\textsuperscript{19}. The curve first
exhibits a toe region where the collagen crimp expands; the crimp is fully lost in the linear region, where stiffness is obtained, of the curve\textsuperscript{19}. The tendon then begins to fail, no longer exhibiting linearity on the curve, until the tendon reaches its ultimate load\textsuperscript{15}. Human patellar tendons have been found to have an ultimate tensile strength in the range of 53.6 – 64.7 MPa and a stiffness between 504 – 660 MPa\textsuperscript{20,21}. Murine patellar tendons exhibit an ultimate strength of around 4.5 N and an elastic modulus between 105 and 140 MPa\textsuperscript{12,22}.

Tendons are divided into two main types: intrasynovial and extrasynovial. Intrasynovial tendons are contained in a tendon sheath, which often helps to guide the tendon, such as is the case for the flexor tendons of the hand\textsuperscript{8}. The surface of intrasynovial tendons is called the epitenon, which is incredibly smooth to assist with gliding during movement\textsuperscript{23}. Extrasynovial tendons are not contained within a tendon sheath and as a result have greater blood supply than intrasynovial tendons\textsuperscript{24}. Extrasynovial tendons are surrounded by paratenon, which is rougher than epitenon and causes increased friction\textsuperscript{23}. However, research has shown that the paratenon can help in reducing friction if under the proper biological conditions, such as if an extrasynovial tendon is used as a graft for an intrasynovial tendon\textsuperscript{25}. This allows extrasynovial tendons to be used when intrasynovial tendons are unable to be obtained without excess negative outcomes.

Two types of tendon injury are common: chronic and acute. Chronic tendon injury is known as tendinopathy, which often involves tendon pain and inflammation\textsuperscript{2}. Tendinopathy occurs due to many factors, two of which include aging and overuse\textsuperscript{26}. Acute tendon injury involves a rupture to the tendon, either partial or full, resulting in
decreased range of motion of affected joints. Tendons are extremely important structures, however after injury they are unable to repair to normal properties, often healing only to 33% of normal. Tendons undergo the same basic healing cascade as all tissues in the body: inflammation, proliferation, and remodeling. The inflammation period only lasts a few days, during which red and white blood cells, in addition to platelets, enter the injury, bringing growth factors and other chemicals to the tendon. In a study performed on canine flexor tendons, Gelberman and colleagues found that a fibrin clot forms at the rupture site and that the epitenon thickens next to the injury due to recruited tenocytes. In ruptures that occur at the tissue enthesis, a collagen scar is formed by tendon and bone fibroblasts instead of a fibrin clot. In the proliferation stage, regulatory macrophages release growth factors and help resolve inflammation.

In addition, the recruited tenocytes begin to synthesize a collagen matrix of mostly type III collagen to repair the injury. Injured tendons display an increased production of type III collagen compared to normal tendons, which consist of only about 5% type III collagen. Type III collagen fibers are thinner than type I collagen fibers, and an altered ratio of type III to type I collagen alters the biomechanics of the tissue as a whole. The presence of excess type III collagen lowers the ultimate load the tendon can carry, which in turn increases the likelihood of rerupture, or rupture in the case of tendinopathic tendons. During the remodeling stage, tenocytes ramp up production of type I collagen and the fibers of the extracellular matrix move from a more random orientation to a more aligned orientation. Although the matrix becomes more aligned, it does not become perfectly aligned, leading to scar formation and decreased mechanical properties.
Three main approaches to tendon injury exist: non-operative natural healing, surgical repair, and surgical replacement\textsuperscript{8,25,29}. For rotator cuff injuries, the first line of treatment is simple rest while also limiting shoulder motions that cause the patient pain\textsuperscript{34}. In addition, patients often undergo a physical therapy regimen to regain any lost range of motion from the injury\textsuperscript{34,35}. Oral non-steroidal anti-inflammatory drugs (NSAIDs) are often prescribed to patients to temporarily relieve rotator cuff pain until stretching or physical therapy alleviates the pain\textsuperscript{35,36}. Boudreault and colleagues performed a literature evaluation and found that NSAIDs have short term benefits for patients with rotator cuff injury but injectable corticosteroids are no more successful than oral NSAIDs\textsuperscript{36}. Non-operative methods for Achilles tendon rupture have been evaluated in a multicenter study to be no different in regards to complications than operative methods of repair\textsuperscript{37}. However, patients who undergo surgery to repair a ruptured Achilles tendon have a stronger tendon after healing than patients who do not have surgery\textsuperscript{37}.

Non-operative Achilles tendon healing involves wearing a cast to immobilize the tendon and allow it to heal naturally\textsuperscript{37}. Early motion is often introduced by replacing the cast with an immobilizer that can be removed to allow for the patient to perform small movements involving the Achilles tendon\textsuperscript{38}. Other methods involve applying a second cast after 4 weeks allowing for full weight-bearing but no ankle movement\textsuperscript{39}.

Surgical repair is another common treatment for tendon injury. Surgical repair of the Achilles tendon allows the surgeon to introduce tension to the tendon, resulting in a better collagen fiber orientation and therefore tensile strength\textsuperscript{40}. Various methods exist for Achilles tendon repair, with the methods broken down into two groups: augmented repairs and non-augmented repairs\textsuperscript{40}. Augmented repairs require the use of extra material
to reinforce the tendon, such as part of the gastrocnemius muscle, whereas non-augmented repairs only use sutures to repair the tendon. Studies have found that non-augmented repairs have similar results to augmented repairs in terms of reruptures, infections, pain, stiffness, and range of motion; however, non-augmented repairs have significantly shorter surgical duration which indicates non-augmented repairs may be superior. While literature evaluations by Popovic have found that surgical repair of the Achilles tendon results in a lower re-reputation rate, this is contradicted by the literature evaluation performed by Willits, which found the re-rupture rates to be equal in both groups; however, both authors agree the rate of complications are higher for surgical repairs. Rotator cuff repair is a common surgery performed, with an increase in incidence of 238% from 1995 – 2009 in New York state. Surgery can be performed either arthroscopically or open, and studies have found similar failure results for both methods. Surgical rotator cuff repair results in significantly decreased pain and increased range of motion in the shoulder, with 86% percent of patients reporting no pain after surgery and 94% of patients reported improved range of motion. A study comparing rotator cuff repair to non-surgical physical therapy found that not only did surgical repair yield better results, but that a significant amount of patients in the non-operative group required rotator cuff repair during the 5 year evaluation period.

The third and final option for the treatment of tendon injury is replacement of the tendon with a graft, either autografts or allografts. Autografts must be harvested from the patient and result in a second wound site that must be healed, increasing the likelihood of complications, but are readily available in patients without previous tendon injuries. Allografts are transplanted tendons from a cadaver, which are decellularized before
implantation, however disease transmission and rejection remain a risk\textsuperscript{49,50}. Tendon allografts are most commonly used to repair injured ligaments, however they are occasionally used to replace injured tendons when repair is not possible and autograft replacements cannot be obtained, such as chronic patellar tendon ruptures\textsuperscript{50,51}. Autograft repair of the Achilles tendon with the semitendinosus tendon resulted in good patient outcomes and no re-ruptures\textsuperscript{52}. Additionally, flexor tendons in the hand are often replaced with autografts. Extrasynovial grafts, such as the plantaris tendon, or intrasynovial grafts, such as the flexor digitorum longus, may be used\textsuperscript{54}. Success rates for both types of grafts for flexor tendon replacement were similar, with patients experiencing no pain and no less than 50% recovery in the joint\textsuperscript{54,55}.

In 1998, the Murphy Roths Large (MRL) murine strain was found to demonstrate regenerative properties when ear hole punches for identification closed within 4 weeks\textsuperscript{13}. The hole punches healed in a scarless manner due to formation of a blastema with normal epithelial and fibroblast proliferation and the formation of an ordered, not disordered, extracellular matrix\textsuperscript{13}. The MRL mouse has displayed regeneration in various other tissues, such as peripheral nerves, heart tissue, corneas, and articular cartilage\textsuperscript{56-59}. MRL mouse peripheral nerves regenerated in ear hole punches through the blastema, and hair follicles that regenerated in this area appeared to be innervated by the nerves, indicating true nerve regeneration\textsuperscript{56}. The MRL strain has also demonstrated improved articular cartilage healing over normal strains, replacing the defect with hyaline cartilage instead of a fibrous mass\textsuperscript{59}. To assess the MRL strain’s ability to regenerate tendon, our laboratory induced a full-length patellar tendon defect and assessed mechanical properties after healing\textsuperscript{12}. The study found that the MRL strain did show improved healing over the
C57/B6 strain: at 8 weeks, the MRL strain achieved 81% of normal ultimate load and 77% of normal linear stiffness\(^{12}\).

The MRL murine strain is made up genetically of 75% LG/J, 12.6% AKR, 12.1% C3H, and 0.3% C57/B6 strains\(^{13}\). This has prompted the question: does the MRL strain get it’s regenerative properties from the majority of it’s genetic makeup, the LG/J strain, or did it obtain these properties from a random mutation along the crosses? Studies have found that the LG/J murine strain does exhibit regenerative properties in tissues such as articular cartilage and ear-hole punches\(^{59-64}\). Blankenhorn and colleagues attempted to identify the gene (or genes) responsible for the increased healing in both the MRL and LG/J strains; their research identified 23 potential genes involved in healing, with further study required to determine which genes actually play a part\(^{62}\). Since the LG/J strain appears to heal as well or better than the MRL strain, our laboratory has decided to investigate the use of the LG/J strain as a model of tendon regeneration.
Chapter 3 – The LG/J Murine Strain Exhibits Near-Normal Tendon Biomechanical Properties Following a Full-Length Central Patellar Tendon Defect

3.1 INTRODUCTION

Musculoskeletal injuries are a frequent cause of health care visits in the United States, amounting to 99 million visits to ambulatory care facilities from 2009 to 2011\(^1\). Studies have suggested that tendon and ligament injuries account for 30% of musculoskeletal health care visits\(^2\). Due to the aging population, tendon and ligament injuries will only become more prevalent in the United States, resulting in higher direct medical costs and lost productivity. Tendon and ligament injuries do not heal well, and current treatments have limited long-term success with increased likelihoods of rerupture and progression of osteoarthritis\(^65\). Better methods for connective tissue healing and repair would improve treatment outcomes and reduce medical costs and lost productivity. Our laboratory seeks to better understand adult tendon healing, which can be used to create new treatment strategies to improve clinical outcomes.

Our laboratory has previously investigated the use of the Murphy Roths Large (MRL/MpJ) murine strain as a potential tendon regenerator\(^12\). The MRL/MpJ strain was found to fully regenerate ear-hole punches, including hair follicles, through the formation of a blastema\(^13\). This discovery prompted the study of the MRL/MpJ strain’s regenerative properties in other tissues, and studies have found regeneration in peripheral nerves, heart tissue, and articular cartilage\(^56,58,59\). Currently, there is no consensus on what causes the MRL/MpJ strain to demonstrate regenerative properties. 36 genes have been identified as potential genes involved in scarless wound healing, however since the genes may act together or alone, determining what makes the MRL/MpJ a regenerative healer is difficult\(^66\).
The MRL/MpJ mouse is made up genetically of 75% LG/J, 12.6% AKR, 12.1% C3H, and 0.3% C57/B6 strains. The LG/J mouse has been linked with increased healing of certain tissues, such as articular cartilage and ear-hole punches, however no research has been made into tendon healing. 23 genes have been identified as potentially involved with increased LG/J healing. We sought to determine the tendon healing properties of the LG/J mouse in comparison to the MRL/MpJ mouse in an effort to narrow the potential responsible genes. This will allow laboratories to more effectively examine genes potentially linked to tendon regeneration. We hypothesized that the LG/J strain would exhibit similar biomechanical outcomes to the MRL/MpJ strain, with significant improvements over the C57/B6 strain.

3.2 METHODS

Experimental Design:

Patellar tendon dimensions along with structural and material properties were assessed after a full-length, full thickness central PT injury in 16-week male LG/J mice. Breeding pairs (stock number: 000675) were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in-house. Biomechanical data was obtained after 2, 5, and 8 (n= 8–10 per time point) weeks of natural healing post surgery. Comparisons were made to uninjured PTs from 16-week LG/J mice (n=12). Biomechanical data was compared to data previously obtained in our laboratory for C57/B6 and MRL/MpJ strains.

Surgical Procedure:

A central-third patellar tendon defect was created using a previously described surgical technique. The University of Cincinnati Institutional Animal Care and Use Committee approved all procedures prior to the study beginning. Mice were anesthetized
using 3% isoflurane by inhalation and both hindlimbs were shaved and disinfected with 70% alcohol and betadine solutions. An incision was created in the skin to expose the patellar tendon, and a Jeweler’s forceps was inserted under the tendon to isolate it from surrounding tissues and apply tension. A 23-guage needle was used to create an incision on the lateral edge of the central-third of the tendon, and a modified Jeweler’s forceps was inserted in the incision and pushed up to create the medial edge of the defect. A scalpel was used to remove the central-third defect from the tendon and to disrupt the bony surface of the tibia. Incisions were closed using 5-0 prolene (Ethicon, Somerville, NJ) and mice were allowed unrestricted cage activity after verifying the anesthesia wore off appropriately. Mice were euthanized using carbon dioxide asphyxiation and secondary cervical dislocation.

Biomechanical Testing:

All samples were stored at -20°C until the day of testing, when they were removed and thawed at room temperature. The limbs were dissected and the gross morphological properties of the patellar tendon were observed. Muscle and surrounding tissue were removed from the tibia, and the outer two thirds of the patellar tendon were cut away, leaving only the central third. The patella-patellar tendon-tibia complex was then dissected out and the tibia was potted in PMMA (Dentsply International, York, PA) and secured with a staple to prevent slippage inside a grip that could be mounted to the TestResources 100R system (TestResources, Shakopee, MN). The patella was placed in a specially designed grip to prevent slippage during the test. 0.9% PBS at 37°C was poured into the tank to mimic the in vivo environment. A 0.02 N preload was applied to the tendon and tendon thickness was measured using digital photography. Optical
measurements were obtained using ImageJ (Version 2.0.0). The tendon was preconditioned for 25 cycles between 0% and 1% strain and failed in uniaxial tension at 0.1% of total tendon length/second. Load and displacement were recorded during the test by the TestResources system. Ultimate load, failure displacement, stress, and strain were also recorded, and linear stiffness and elastic modulus were calculated from the load-displacement and stress-strain curves.

Statistical Analysis:

Native LG/J tendon dimensions and mechanical properties were compared to native C57/B6 and MRL/MpJ properties using independent Welch’s two-sample t-tests. A one-way ANOVA with Tukey’s Honest Significant Difference was used to test the significance of healing at each time point compared to native LG/J properties. Welch’s two-sample t-tests were used to compare mechanical properties between strains at the same time points. All comparisons were made using a p value of 0.01. All statistical testing was performed using R (version 3.2.3).

3.3 RESULTS

Native Patellar Tendon Properties

LG/J cut PT widths (0.60±0.07) were not statistically different from the C56/B6 strain (0.58±0.03, p=0.4106) or the MRL/MpJ strain (0.55±0.05, p=0.05327). LG/J cut PT lengths (3.04±0.21) were also not statistically different from either strain (C57/B6: 2.95±0.10, p=0.1559; MRL/MpJ: 3.08±0.11, p=0.6374). The LG/J PT thickness (0.66±0.17) was significantly higher than both the C57/B6 strain (0.47±0.06, p=0.002236) and the MRL/MpJ strain (0.45±0.03, p=0.001089). Additionally, the LG/J
PT cross-sectional area (0.40±0.12) was significantly higher than both the C57/B6 strain (0.27±0.04, \(p=0.003143\)) and the MRL/MpJ strain (0.25±0.03, \(p=0.0009434\)).

LG/J ultimate load (4.30±1.50 N) was not significantly different from either the C57/B6 strain (4.44±0.75 N, \(p=0.7685\)) or the MRL/MpJ strain (4.31±1.53 N, \(p=0.9855\)). Linear stiffness also did not differ significantly between the LG/J strain (10.88±2.34 N/mm) and the C57/B6 strain (9.76±1.3 N/mm, \(p=0.1576\)) or the MRL/MpJ strain (9.31±1.7 N/mm, \(p=0.07845\)). The maximum stress experienced by the LG/J strain (11.49±4.00 MPa) was significantly different from the C57/B6 strain (16.20±2.72 MPa, \(p=0.002728\)), however it was not significantly different from the MRL/MpJ strain (17.85±6.72 MPa, \(p=0.0149\)). The LG/J elastic modulus (86.00±16.58 MPa) was not significantly different from the C57/B6 strain (105.30±20.11 MPa, \(p=0.01306\)), however it was significantly different from the MRL/MpJ strain (130.98±28.03 MPa, \(p=0.00028\)).
LG/J Mechanical Properties Return to Near Normal Values by 8 Weeks

At 5 and 8 weeks post surgery, the LG/J PT healed to 84% and 97% of native ultimate load and demonstrated no significant difference from native PT tissue (3.65±0.79 N vs. 4.30±1.50 N, p=0.639; 4.17±1.20 N vs. 4.30±1.50 N, p=0.995).

Additionally, at 5 and 8 weeks post surgery, the LG/J PT healed to 75% and 97% of native linear stiffness and demonstrated no significant difference from native PT tissue (8.18±1.69 N/mm vs. 10.88±2.34 N/mm, p=0.102; 10.52±3.40 N/mm vs. 10.88±2.34 N/mm, p=0.989). The elastic modulus returned to 92% of native at 8 weeks post surgery.
and showed no significant difference from native tissue (78.62±38.69 MPa vs. 86.00±16.57 MPa, \(p=0.931\)). Maximum stress returned to 86% of native at 8 weeks post surgery and showed no significant difference from native tissue (9.90±4.45 MPa vs. 11.49±4.00 MPa, \(p=0.823\)). Morphologically, the LG/J tissue was whiter and exhibited a smaller visual defect and 5 and 8 weeks compared to the MRL/MpJ tissue (see Figure 3). By 8 weeks, the LG/J tendon appears almost normal with the exception of the small gap in the center.

![Figure 2. Average failure curves for LG/J repaired tendon tissue and 2, 5, and 8 weeks post surgery. At two weeks, the repair is significantly weaker than native, however at 5 and 8 weeks there is no significant difference between the repair tissues and native. The](image-url)
8-week curve overlaps the native curve at some points, such as failure, and exhibits a similar slope. Error bars represent SD.

Figure 3. Gross morphology of the MRL/MpJ and LG/J PT repair tissue at 5 and 8 weeks post surgery.
The LG/J ultimate load was not statistically different from the MRL/MpJ strain or the C57/B6 strain at 2 weeks, but was statistically increased over both the MRL/MpJ and the C57/B6 at 5 weeks (3.65±0.74 N vs. 1.96±0.26 N, \(p=0.001573\); 3.65±0.74 N vs. 1.87±0.55 N, \(p=0.0001792\)). Additionally, at 8 weeks the LG/J ultimate load was significantly increased compared to the C57/B6 strain (4.17±1.16 N vs. 1.82±0.66 N, \(p=0.0005678\)). There was no significant difference in maximum stress between either the LG/J and MRL/MpJ or C57/B6 strains at any time points post surgery.

The LG/J linear stiffness was not statistically different from the MRL/MpJ strain or the C57/B6 strain at 2 weeks, however at 5 and 8 weeks the LG/J exhibited increased stiffness over the MRL/MpJ strain, despite not being significantly different at the chosen confidence level (8.18±1.69 N/mm vs. 4.06±1.40 N/mm, \(p=0.02413\); 10.52±3.40 N/mm vs. 7.21±1.00 N/mm, \(p=0.02831\)). At 5 and 8 weeks, the LG/J strain linear stiffness was significantly increased from the C57/B6 strain (8.18±1.69 N/mm vs. 4.74±0.79 N/mm, \(p=0.0004097\); 10.52±3.40 N/mm vs. 5.72±0.85 N/mm, \(p=0.004802\)). There was no significant different in elastic modulus between either the LG/J and MRL/MpJ or C57/B6 strains at any time points post surgery.

The LG/J strain experienced an increase in cross-sectional area at 2 weeks compared to native, however the different was not statistically significant (0.60±0.35 vs. 0.39±0.12, \(p=0.9241\)). The cross-sectional area decreased from the 2-week time point at 5 and 8 weeks, however remained slightly elevated over native values – these values were also not statistically significant with \(p\) values of 0.2641 and 0.3136, respectively.
Table 1. Mechanical Properties for C57/B6, MRL/MpJ, and LG/J Native and Defects

(mean±SD)

<table>
<thead>
<tr>
<th></th>
<th>Cross-Sectional Area (mm²)</th>
<th>Ultimate Load (N)</th>
<th>Linear Stiffness (N/mm)</th>
<th>Max Stress (MPa)</th>
<th>Modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C57/B6</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native PT (n=14)</td>
<td>0.28±0.04</td>
<td>4.44±0.75</td>
<td>9.76±1.30</td>
<td>16.20±2.72</td>
<td>105.30±20.11</td>
</tr>
<tr>
<td>2-week Defect (n=8)</td>
<td>0.38±0.07</td>
<td>2.10±0.25</td>
<td>4.50±0.66</td>
<td>5.80±0.72</td>
<td>39.03±6.45</td>
</tr>
<tr>
<td>5-week Defect (n=9)</td>
<td>0.31±0.06</td>
<td>1.87±0.55</td>
<td>4.74±0.79</td>
<td>6.31±2.19</td>
<td>49.53±15.12</td>
</tr>
<tr>
<td>8-week Defect (n=7)</td>
<td>0.25±0.03</td>
<td>1.82±0.66</td>
<td>5.72±0.85</td>
<td>7.24±2.47</td>
<td>67.63±11.62</td>
</tr>
<tr>
<td><strong>MRL/MpJ</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native PT (n=11)</td>
<td>0.25±0.03</td>
<td>4.31±1.53</td>
<td>9.31±1.99</td>
<td>17.85±6.72</td>
<td>130.96±39.99</td>
</tr>
<tr>
<td>2-week Defect (n=8)</td>
<td>0.54±0.12</td>
<td>2.91±1.15</td>
<td>6.50±1.43</td>
<td>5.33±1.64</td>
<td>39.37±7.27</td>
</tr>
<tr>
<td>5-week Defect (n=10)</td>
<td>0.39±0.07</td>
<td>1.96±0.29</td>
<td>4.06±1.40</td>
<td>5.12±1.31</td>
<td>38.44±12.87</td>
</tr>
<tr>
<td>8-week Defect (n=10)</td>
<td>0.31±0.08</td>
<td>3.48±0.92</td>
<td>7.21±1.00</td>
<td>11.33±3.73</td>
<td>74.49±20.67</td>
</tr>
<tr>
<td><strong>LG/J</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native PT (n=12)</td>
<td>0.39±0.12³</td>
<td>4.30±1.46</td>
<td>10.88±2.34</td>
<td>11.49±4.00²</td>
<td>86.00±16.58³</td>
</tr>
<tr>
<td>2-week Defect (n=10)</td>
<td>0.60±0.35</td>
<td>2.47±1.02⁴</td>
<td>5.66±2.38²</td>
<td>5.85±4.74</td>
<td>43.92±32.54⁴</td>
</tr>
<tr>
<td>5-week Defect (n=8)</td>
<td>0.45±0.09</td>
<td>3.65±0.74³⁴</td>
<td>8.18±1.69²</td>
<td>8.40±2.67</td>
<td>57.25±15.61</td>
</tr>
<tr>
<td>8-week Defect (n=8)</td>
<td>0.46±0.16</td>
<td>4.17±1.16³</td>
<td>10.52±3.40³</td>
<td>9.90±4.45</td>
<td>78.61±38.69</td>
</tr>
</tbody>
</table>

³Significantly different from respective native  ⁴Significantly different from C57/B6
²Significantly different from MRL/MpJ
Figure 4. Comparison of the percentage of native ultimate load between LG/J, MRL/MpJ, and C57/B6 strains. Error bars represent SD.

Figure 5. Comparison of the percentage of native linear stiffness between LG/J, MRL/MpJ, and C57/B6 strains. Error bars represent SD.
Figure 6. Comparison of the percentage of native maximum stress between LG/J, MRL/MpJ, and C57/B6 strains. Error bars represent SD.

Figure 7. Comparison of the percentage of native elastic modulus between LG/J, MRL/MpJ, and C57/B6 strains. Error bars represent SD.
3.4 DISCUSSION

The goal of this study was to examine the mechanical properties of the LG/J murine patellar tendon before and after injury to determine if the strain exhibited increased healing over wild-type and superhealer mice. In general, most of the physical and mechanical properties of native LG/J tendons were not significantly different from either the C57/B6 or MRL/MpJ native tendons. One main difference between the three strains was found – tendon thickness. The LG/J strain exhibited a significantly higher native tendon thickness than both the C57/B6 and MRL/MpJ strains, which in turn resulted in a significantly higher cross-sectional area for the LG/J strain over both the C57/B6 and MRL/MpJ strains. Due to the relationship between cross-sectional area and stress, and stress and elastic modulus, this also decreased the maximum stress and elastic modulus seen by the native LG/J tendons compared to the C57/B6 and MRL/MpJ.

Since there are no difference between native ultimate loads and linear stiffness, comparisons can easily be made between the tissues at various time points post surgery. However, it is much more difficult to make these comparisons for ultimate stress and elastic modulus for each strain, since differences do exist in the native tissues. While the LG/J tissue does seem to display a superior healing curve for these two properties, it is difficult to say for sure if the healing is in fact superior to the MRL/MpJ and C57/B6. Although this is an issue, and may result in biased reporting of the healing capabilities of the LG/J strain, the most important properties to focus on are currently the ultimate load and the linear stiffness. This is due to the fact that structural properties are favored over material properties in a clinical setting – when searching for a tendon graft, a clinician focuses mostly on if the graft will withstand the maximum forces applied to it.
After injury, the LG/J tendons only differed significantly from native properties at
the 2-week time point. By 8 weeks, the LG/J tendons reached 97% of normal for both
ultimate load and linear stiffness, exhibiting essentially normal failure curves, compared
to 81% of native ultimate load and 77% of native linear stiffness in the MRL/MpJ at 8
weeks. Although the MRL/MpJ had higher values for ultimate load and linear stiffness at
2 weeks compared to the LG/J (yet not statistically significant), the MRL/MpJ’s healing
response appears to drop off slightly after the 2-week point, resulting in decreases in
ultimate load and linear stiffness at 5 weeks. The LG/J, on the other hand, has a slightly
slower healing response from injury to 2 weeks post injury, but it does not exhibit any
decreases in healing and instead improves from 2 weeks to 5 weeks and again from 5
weeks to 8 weeks. This suggests that there may be different healing pathways in the two
strains that activate at different times post-injury, and that genes located in the 25% of the
genome the MRL/MpJ does not share with the LG/J may be responsible for the LG/J’s
increased healing ability. Overall, the results support our hypothesis that the LG/J strain
heals as well as the MRL/MpJ strain, and supplies evidence for the LG/J strain returning
to normal properties by 8 weeks.

This study does have limitations. One, the defect tissue was not clearly delineated
from the tendon struts, so removing the struts and leaving only the defect tissue was
difficult. Normal tendon tissue may have remained in some samples, leading to potential
higher results. Two, our excisional model of tendon injury is not clinical relevant. Most
tendon injuries are a result of a chronic condition called tendinopathy, which is linked to
aging and excessive exercise. Tendinopathy, and it’s associated degeneration, may also
be the cause of ruptures that don’t have an identifiable traumatic cause. Three,
patellar tendon dimensions were treated as if they were uniform throughout the tendon; since studies have shown that tendons do not exhibit a uniform cross-sectional area throughout their length, this can result in incorrect material property calculations\textsuperscript{69}.

Future studies will seek to analyze the transcriptome and proteome of the LG/J strain to better understand the healing process. A more complete understanding of this strain’s healing ability will aid in creating functional tissue engineering benchmarks and solutions for tendon injuries, as well as potential clinical treatments to stimulate the body’s natural tendon healing.
Chapter 4 – Discussion and Conclusions

The MRL/MpJ murine strain has been extremely coveted in research, since it displays regenerative properties in various tissues. After our laboratory investigated the use of the MRL/MpJ strain as a model of tendon regeneration, we were not satisfied with the MRL/MpJ’s healing ability and sought a better model. This led us to investigating the healing properties of the LG/J murine strain, which genetically makes up 75% of the MRL/MpJ mouse. We theorized that if the LG/J strain exhibited better healing properties than the MRL/MpJ strain, we could narrow future genetic studies to just the 25% of the LG/J genome that is not shared with the MRL/MpJ strain, and potentially pinpoint the gene or genes responsible for increased healing and regeneration.

After creating a full-length, full-thickness patellar tendon defect in 16 week old LG/J mice, we allowed the mice to undergo natural healing for 2, 5, and 8 weeks. We found that the LG/J strain exhibits a steady increase in mechanical properties at each time point, reaching near-normal values by 8 weeks. This is in contrast to the MRL/MpJ strain, which actually exhibits a decline in mechanical properties between weeks 2 and 5, and only healed to 81% of normal ultimate load by 8 weeks. Interestingly, the MRL/MpJ does exhibit ultimate load and linear stiffness at 2 weeks compared to the LG/J strain. This raises many questions. What causes the decrease in MRL/MpJ mechanical properties from 2 to 5 weeks? Can this process be stopped? Do the same genes and pathways act on both the MRL/MpJ and the LG/J strains? Is the slight difference in ultimate loads and linear stiffnesses between the two strains at 2 weeks clinically relevant? If so, can we stimulate pathways in a mouse to yield the increased healing from injury to 2 weeks that the MRL/MpJ strain exhibits, but then allow the LG/J healing to continue from 2 to 8
weeks? While the end goal of tendon healing research is to discover methods to return an injured tendon to normal properties, perhaps a more clinically relevant model would be increased healing immediately after injury to aid in preventing reruptures.

**4.1 Further Directions**

The next step for this research is to examine the transcriptome and the proteome of the LG/J and MRL/MpJ strains to pinpoint what RNAs and proteins are present in the healing tendon at various time points. With this information, we may be able to shed light on some of the questions this research has brought up. The results from these further studies will aid geneticists in determining which genes are causing the increased healing response in both the MRL/MpJ and the LG/J mouse. Finding these regenerative genes will be a huge asset to the regenerative medicine community, as these genes can be activated or bred into laboratory animals to study other types of regeneration. Additionally, if the regenerative genes are found to be dormant in other strains, or even other species, perhaps methods can be devised to activate these genes after injury, allowing the body to more effectively heal itself and leading to the decreased need for surgical intervention following injury.

These studies will be an asset not just to those in the regenerative medicine community, but also to those in the tissue engineering community. A tissue engineered repair will not work inside the body if the body does not integrate the repair to surrounding tissue. A better understanding of the healing process for various tissues will allow engineers to design these repairs to better integrate with the body, resulting in positive long-term outcomes. Engineers may also utilize the genes responsible for regenerative healing into tissue engineered repairs directly. One of the goals of tissue
engineering is to create patient-specific repairs after a wound occurs – for example, growing a patellar tendon in the laboratory after a patient has ruptured their patellar tendon. Understanding which genes stimulate regenerative healing may be helpful in creating these patient-specific repairs, as these genes may be able to be activated in the cells as they are seeded onto a scaffold.

Although many of these prospects are far off, this research may provide an important stepping stone in the process of creating tissue engineered tissues and regenerative medicine therapies. Continued investigation into the LG/J and MRL/MpJ murine strains is necessary to answer old and new questions in both fields.
References


