I, Andrea L. Lalley, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Biomedical Engineering.

It is entitled:
Identifying and Evaluating Novel Biological Targets to Improve Musculoskeletal Tissue Engineering Strategies

Student's name: Andrea L. Lalley

This work and its defense approved by:

Committee chair: Jason Shearn, Ph.D.
Committee member: Keith Kenter, M.D.
Committee member: Rulang Jiang, Ph.D.
Committee member: Jeffrey Johnson, Ph.D.
Identifying and Evaluating Novel Biological Targets to Improve Musculoskeletal Tissue Engineering Strategies

A dissertation submitted to the
Division of Research and Advanced Studies of the University of Cincinnati
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY (Ph.D.)

in the Biomedical Engineering Program
of the College of Engineering and Applied Science

2014

by

Andrea L. Lalley

B.S. University of Cincinnati
Cincinnati, OH, 2010

Committee Chair: Jason T. Shearn, Ph.D.
Abstract

Tendon and ligament injuries pose a significant socioeconomic burden to the United States. Tissue engineering seeks to design tissue substitutes that are better equipped to improve the healing process. Previous work shows a benefit to this approach; however, we have not produced native biological and mechanical properties. To design effective tissue substitutes, we need to investigate successful tendon tissue formation. This dissertation is focused on identifying and evaluating biological targets using regenerative models and normal development to guide tissue engineering strategies.

We proposed a strategy for identifying, prioritizing, and evaluating potential biological success criteria for improving tendon healing and repair and determined that successful tendon formation and function relies on scleraxis-expressing cells, well-aligned collagen, and a tendon-bone insertion site. To expand our potential biological design criteria, we planned to investigate normal development and regenerative models, such as the MRL/MpJ.

To validate the MRL/MpJ as a model of regenerative-like healing, we evaluated its response to a central patellar tendon injury and found improved mechanical outcomes at 8 weeks post surgery compared to wildtype. This response was not a result of a p21 deficiency.

We then evaluated the RNA transcriptome at early post-surgical time points using RNA-Seq and found the MRL/MpJ decreases activation of immune-related pathways while expressing developmental transcription factors.

While our laboratory focuses on tendon, we also acknowledge the importance of translating our findings to other tissue systems. We thus investigated the MRL/MpJ’s responses to a critical-size femoral osteotomy. While the MRL/MpJ did not show improved biomechanical
outcomes, increased expression of bone remodeling markers was observed up to 5 weeks post injury, suggesting a prolonged healing response.

To assess the role of mohawk homeobox in tendon maturation, we investigated tendon morphology and biomechanical properties using a mohawk homeobox knock-out strain. Mkx-/tendons were significantly smaller than wildtype, displayed abnormal collagen color/vibrancy, and displayed a 33% and 31% reduction in linear stiffness and modulus, respectively.

Finally, we investigated a mouse model of collagenase-induced tendon injury. This allows for comparison among multiple tendon injury models to understand mechanistic differences in the natural healing responses. Injecting 15000IU-20000IU collagenase into the patellar tendon resulted in decreased mechanical properties at 2 weeks, with a return to normal levels by 5 weeks.

Future work should focus on connecting normal development and regenerative processes, identifying the role of the immune response in tendon healing, and evaluating additional models of regeneration to strengthen our tissue engineering approaches.
Acknowledgements

I have been unbelievably fortunate to work in a research group during my time as a graduate student that fosters teamwork and collaboration. While I am listed as the author of this dissertation, each person listed in this section has contributed in some way, and for that, I am extremely thankful.

First and foremost, I must thank my adviser, Dr. Jason Shearn. Dr. Shearn recruited me to pursue a graduate degree while I was an undergraduate student at UC, and I am extremely thankful that he did. He has guided me to become the scientist I am today, allowing me to work independently to investigate and solve problems, but always there to help me along the way. Thank you for your patience, your kindness, and for building my confidence as a researcher. I am forever grateful.

I would like to thank Dr. Rulang Jiang for serving on my dissertation committee and for collaborating with our lab group to strengthen our biologic approaches to tissue engineering. I believe our research is much stronger with you as a contributor.

I would like to thank Dr. Jeffrey Johnson for serving on my dissertation committee and for conveying to me the importance of being an educator to future generations of engineers and scientists.

I would like to thank Dr. Keith Kenter for serving on my dissertation and for providing valuable clinical perspective that I have incorporated into my own research strategy. I sincerely appreciate your efforts to encourage orthopaedic residents to partake in basic science studies, as it greatly improves the quality of our research.

I must extend gratitude to Dr. David Butler for his mentorship over the years, especially in regards to experimental design and ‘seeing the big picture’.
I also must extend a most sincere thank you to my friend and mentor, Dr. Nat Dyment. He has taught me the importance of questioning the norm and has always been there to brainstorm new ideas and approaches. His constant support has been a true blessing.

I would especially like to thank Cindi Gooch who has been my go-to person in the lab, helping me to coordinate studies, brainstorm experiments, and determine the most efficient way to get things accomplished in the research environment. She has also become a close friend and someone I can always rely on for a laugh.

A huge thank you to my friends and colleagues, Steve Gilday, Andrew Breidenbach, and Rebecca Spatholt. Each of them not only provided technical and experimental assistance on many occasions, but made the whole PhD experience much more fun and enjoyable.

I must also thank Dr. Marc Galloway for his contributions to our research and his kindness during my early days as a graduate student. Thanks to Dr. Nami Kazemi, Dr. Chris Casstevens, Dr. Steven Dailey, and Dr. Brian Grawe for providing surgical expertise for each of the murine studies in this dissertation and adding valuable clinical perspective during experiment development.

I would also like to thank Linda Moeller, Lori Beth Derenski, Mike Sanderson, Michelle Montoya, Chris Bauer, and Shelley Tipton. I would not have made it through graduate school without their help and guidance.

I would like to extend thanks to my high school biology teacher, Mrs. Barb Rehn, who encouraged me to obtain an undergraduate degree in biomedical engineering. Taking her class convinced me to pursue a career in science.

Finally, my family and friends have provided limitless love and support during my time as a graduate student. I must extend a sincere amount of love and gratitude to my parents, Jeff
and Teresa, who have supported me in countless ways throughout my life and instilled in me the importance of dedication, a strong work ethic, and a positive attitude. Thanks to my grandfather, Fred, who has been a constant supporter during my life, and, of course, thanks to my brothers, Jacob and Eric, who make each day a little more interesting and a lot more entertaining. Thank you all so very much.
Organization of Dissertation

This dissertation is composed of 9 chapters including Chapter 1 – Background, Rationale, and Specific Aims, Chapter 2 – Literature Review, Chapter 3 through Chapter 8 – Scientific Manuscripts, and Chapter 9 – Conclusions and Recommendations.

The first chapter provides a brief background and rationale for this dissertation. The specific aims and hypotheses for each of the scientific studies are also presented. Chapter two provides a thorough review of the literature with respect to tendon structure/function, natural tendon healing and repair, normal tendon development, and regenerative models.

Chapter three through chapter eight constitute the scientific manuscripts. Chapter three outlines the need for developing biological success criteria and a strategy to identify, prioritize, and evaluate potential criteria. Chapter four evaluates the utility of the MRL/MpJ murine strain as a regenerative model with respect to tendon healing. Chapter five analyzes the RNA transcriptomes from three mouse strains to characterize the early tendon healing response and identify potential pathways and targets for future study. Chapter six investigates the MRL/MpJ’s healing response to a critical-size femoral osteotomy. Chapter seven evaluates the role of mohawk homeobox with respect to tendon maturation in the adult animal. Chapter eight focuses on developing a more clinically relevant model of tendon injury through the use of collagenase.

To conclude, chapter nine outlines major findings from this dissertation, remaining gaps in knowledge, along with recommendations for future directions.
Table of Contents

Abstract ............................................................................................................................................i
Acknowledgements.............................................................................................................................iv
Organization of Dissertation ...............................................................................................................vii
List of Tables ........................................................................................................................................1
List of Figures .......................................................................................................................................2
Chapter 1. Background, Rationale, and Research Objectives .........................................................3
Chapter 2. Literature Review .............................................................................................................11
Chapter 3. Functional Tissue Engineering of Tendon: Establishing Biological Success Criteria for Improving Tendon Repair ....36
  Abstract .........................................................................................................................................37
  Introduction ......................................................................................................................................38
  Using FTE Principles to Improve Tendon Repair ..........................................................................38
  Need for Developing Biological Success Criteria ...........................................................................40
  Strategy to Develop Biological Success Criteria ...........................................................................41
  Comparing Normal Development and Natural Healing to Select Biological Success Criteria ..........43
  Discussion .......................................................................................................................................49
  Conclusion .....................................................................................................................................51
Acknowledgements ..............................................................................................................................52
Chapter 4. Improved Biomechanical and Biological Outcomes in the MRL/MpJ Murine Strain Following a Full-Length Patellar Tendon Injury .................................................................53
  Abstract .........................................................................................................................................54
  Introduction ......................................................................................................................................55
  Results ............................................................................................................................................57
  Discussion .......................................................................................................................................70
  Materials and Methods ...................................................................................................................74
Acknowledgements ..............................................................................................................................81
Chapter 5. Comparing and Contrasting the Temporal Healing RNA Expression Profiles Following Injury Among the C57BL/6, LG/J, and MRL/MpJ Murine Strains ..................................................................82
  Abstract .........................................................................................................................................83
  Introduction ......................................................................................................................................84
  Results ............................................................................................................................................85
  Discussion .......................................................................................................................................91
  Materials and Methods ....................................................................................................................96
Acknowledgements ............................................................................................................................100
Chapter 6. The MRL/MpJ Does Not Recover Native Mechanical Properties Following a Critical-Size Femoral Osteotomy........................................123
  Abstract ........................................................................................................124
  Introduction ..................................................................................................125
  Results .........................................................................................................126
  Discussion ....................................................................................................134
  Materials and Methods ...........................................................................136
  Acknowledgements ..................................................................................138

Chapter 7. Loss of Mohawk Homeobox Function Produces Morphological Abnormalities and Biomechanical Deficiencies in the Adult Murine Patellar Tendon.................................................................143
  Abstract ......................................................................................................144
  Introduction ................................................................................................145
  Results .........................................................................................................147
  Discussion ....................................................................................................152
  Materials and Methods ...........................................................................155
  Acknowledgements ..................................................................................157

Chapter 8. Developing a Murine Model of Collagenase-Induced Patellar Tendon Injury...............................................................158
  Abstract ......................................................................................................159
  Introduction ................................................................................................160
  Results .........................................................................................................162
  Discussion ....................................................................................................166
  Materials and Methods ...........................................................................168
  Acknowledgements ..................................................................................172

Chapter 9. Conclusions and Recommendations........................................173

Bibliography ..............................................................................................186
List of Tables

Table 1. Delta C_T values for genes for C57BL/6 and MRL/MpJ native PT

Table 2. Structural and material properties for C57BL/6 and MRL/MpJ native PT

Table 3. Delta Ct values for genes measured in C57BL/6 and MRL/MpJ PT defect

Table 4. Structural and material properties for C57BL/6 and MRL/MpJ defect and contralateral sham tissues following surgery compared to age-matched native PT

Table 5. Experimental design – C57BL/6 and MRL/MpJ full-length, full-thickness central PT defects were compared for histology, gene expression, and biomechanics

Table 6. Gene symbols and gene expression assay ID numbers

Table 7. (+2, +2) GO terms and p values (Native)

Table 8. (-2, -2) GO terms and p values (Native)

Table 9. (+2, +2) GO terms and p values (Day 3)

Table 10. (-2, -2) GO terms and p values (Day 3)

Table 11. (+2, +2) GO terms and p values (Day 7)

Table 12. (-2, -2) GO terms and p values (Day 7)

Table 13. (+2, +2) GO terms and p values (Day 14)

Table 14. (-2, -2) GO terms and p values (Day 14)

Table 15. (+2, +2) GO terms and p values (C57BL/6)

Table 16. (-2, -2) GO terms and p values (C57BL/6)

Table 17. (+2, +2) GO terms and p values (LG/J)

Table 18. (-2, -2) GO terms and p values (LG/J)

Table 19. (+2, +2) GO terms and p values (MRL/MpJ)

Table 20. (-2, -2) GO terms and p values (MRL/MpJ)

Table 21. Elevated transcription factor expression at 3 and 7 days for MRL/MpJ

Table 22. Decreased transcription factor expression at 3 and 7 days for MRL/MpJ

Table 23. Gene symbols and gene expression assay ID numbers

Table 24. Mechanical properties for C57BL/6 and MRL/MpJ native femur

Table 25. Mechanical parameters for defect, sham and age-matched native controls following critical size femoral osteotomy

Table 26. Experimental design comparing C57BL/6 and MRL/MpJ mechanical and biological measures following critical-size femoral osteotomy

Table 27. Patellar tendon dimensions for Mkx+/- and Mkx-/- mouse strains

Table 28. Structural and material properties for Mkx+/+ and Mkx-/- patellar tendon properties

Table 29. Rupture rates following patellar tendon collagenase injections at 24 hours, 2 weeks, and 5 weeks post injury

Table 30. Structural outcomes following 15000IU and 20000IU collagenase injections at 24 hours, 2 weeks, and 5 weeks post injury

Table 31. Experimental design for collagenase injections evaluated biomechanically at 24 hours, 2 weeks, and 5 weeks post injection
List of Figures

Fig. 1: Dissertation outline.................................................................10
Fig. 2: The hierarchical nature of tendon..................................................13
Fig. 3: Tendon healing process following acute injury...............................20
Fig. 4: A model of blastema formation and maturation in the zebrafish caudal fin...30
Fig. 5: Using tissue engineering to improve tendon repair in a rabbit PT defect model........40
Fig. 6: Establish mechanical and biological success criteria for assessing TE tendon repairs......................................................................................................................................................42
Fig. 7: Biological success criteria for tendon tissue engineering......................48
Fig. 8: Load-displacement curves for native 20-week old C57BL/6 and MRL/MpJ PT ..........59
Fig. 9: Cellular proliferation following tendon injury in C57BL/6 and MRL/MpJ................63
Fig. 10: Temporal expression of p21 following tendon injury in C57BL/6 and MRL/MpJ.......64
Fig. 11: Average load-displacement failure curves for repair tissue at 2, 5, and 8 weeks post-injury.................................................................67
Fig. 12: Mechanical properties of defect and sham tendon tissues as a percent of native........68
Fig. 13: F4/80 staining of C57BL/6 and MRL/MpJ at 3, 7, and 14 days post injury........69
Fig. 14: Images of native PT, PT defect, and PT repair tissue at 5 weeks post injury........76
Fig. 15: Plot clustering analysis for differential pathway activation-strain comparison.........101
Fig. 16: Plot clustering analysis for differential pathway activation-time comparison........110
Fig. 17: qPCR validation of RNASeq data................................................99
Fig. 18: Summary of clustering comparisons.............................................119
Fig. 19: Gross comparisons of native C57BL/6 and MRL/MpJ femurs..................127
Fig. 20: X-ray comparisons between C57BL/6 and MRL/MpJ for native tissue, 2 weeks post osteotomy, and 5 weeks post osteotomy........................................................129
Fig. 21: Cryohistology for C57BL/6 and MRL/MpJ native tissue and 2-, 5-weeks post osteotomy..........................................................................................................................................................133
Fig. 22: Surgical equipment for creating the femoral osteotomy.....................139
Fig. 23: A critical-size femoral osteotomy created at surgery.........................140
Fig. 24: Gross observations of Mkx+/- and Mkx-/- patellar tendon and surrounding tissue.........................................................................................................................147
Fig. 25: Load-displacement failure curves for native 20-week old C57BL/6 and Mkx+/- PT.................................................................................................................................149
Fig. 26: Load-displacement and stress-strain failure curves for 20-week old Mkx+/- and Mkx-/- PT.........................................................................................................................150
Fig. 27: Two distinct phenotypes for Mkx-/- PT............................................151
Fig. 28: Histology for Mkx+/- PT.................................................................152
Fig. 29: Gross PT morphology following collagenase injection....................162
Fig. 30: Normalized structural properties following collagenase injection at 24 hours......164
Fig. 31: Normalized structural properties following 15000IU and 20000IU collagenase injections.................................................................................................................................166
Fig. 32: Flowchart including major research findings, gaps in knowledge, and future directions.................................................................180
Chapter 1

Background, Rationale, and Research Objectives

Prevalence of Musculoskeletal Injuries

Musculoskeletal injuries, particularly to ligaments and tendons, pose a large burden on the United States socioeconomic climate, with 110 million patients presenting with an injury in 2008 costing nearly $30 billion annually\(^1\). The incidence of musculoskeletal injuries is expected to increase as the population continues to age\(^2\). Overuse tendon injuries affecting the Achilles tendon, patellar tendon, and extensor carpi radialis brevis accounted for 7% of all musculoskeletal-related doctor visits in 2002 alone\(^3\). Further, the rotator cuff presents one of the most commonly chronically injured musculoskeletal structures with 5 million physician visits being attributed to cuff tendons between 1998-2004 \(^4\).

Bone diseases and fractures also greatly impact the United States population. Approximately 7.9 million patients are affected by osseous fractures in the United States annually\(^5\) with 1.5 million resulting from an underlying bone disease\(^6\). The most common cause of fracture is a result of osteoporosis; however, metastatic bone diseases also cause debilitating injuries due to tumor resection\(^7\).

Given the prevalence of musculoskeletal injuries and the wide variety of affected individuals, developing effective strategies to improve treatment and tissue repair is important. Depending on the type of musculoskeletal injury, current treatment options include no surgical intervention by modifying daily activity levels\(^8\), surgically repairing the damaged tissue\(^9,10\), or replacing the damaged tissue using autografts or allografts\(^11,12\). Repairing the damaged tissue directly is often complicated by the quality of the tissue, while surgical autograft and allograft
replacements present problems in terms of donor site morbidity or elicitation of an immunological response, respectively\textsuperscript{13, 14}. There has been some success using these approaches; however, concerns remain as long-term conditions, such as osteoarthritis, continue to present complications\textsuperscript{15, 16}. Our laboratory is dedicated to identifying new therapeutic strategies to improve musculoskeletal healing using tissue engineering approaches.

**Background and Rationale**

Our laboratory seeks to design tissue-engineered constructs guided by the principles of functional tissue engineering to improve tendon and ligament healing. This paradigm emphasizes the importance of first understanding the normal physiologic loading environment of a musculoskeletal tissue and then designing replacements or augmentations that can accommodate these forces with a safety factor\textsuperscript{17}. Through a series of optimization studies over the last 15 years investigating construct material, cell number, construct length, and in vitro preconditioning protocols, we have produced positive results\textsuperscript{18-24}. By seeding autologous rabbit mesenchymal progenitor cells on a bovine-derived collagen gel, preconditioning the construct in vitro by delivering a tensile stimulus over a two-week period, and implanting the construct into a central third patellar tendon defect produced significantly improved mechanical properties compared to natural healing at twelve weeks post implantation, reaching 32\% of normal failure force, 50\% beyond previously recorded forces for activities of daily living (as recorded in the rabbit patellar tendon)\textsuperscript{25}. These results suggest our approach has merit; however, further improvements require understanding not only the normal mechanical loading environment, but also the biological processes regulating normal tendon function and healing.

Unfortunately, the tendon natural healing process fails to restore the native mechanical properties of tendon tissue following injury\textsuperscript{18, 26, 27}. As we attempt to design more effective
tissue engineered augmentations, investigating successful models of tendon tissue formation presents an opportunity to identify shortcomings associated with natural healing and design therapeutics to improve the process. Similar in concept to establishing mechanical design criteria for tendon, the musculoskeletal field also needs to identify and evaluate biological design criteria to further improve tendon healing\textsuperscript{28}.

**Specific Aims and Hypotheses**

Given the shortcomings associated with natural tendon healing, evaluating a successful model of tendon healing (i.e. regenerative-like healing) presents an opportunity to identify novel molecular targets for investigation and modulation in our tissue engineering strategies. Thus, the first aim of my dissertation is to evaluate the utility of the Murphy Roths murine strain (MRL/MpJ), a model previously shown to exhibit regenerative-like responses to injury in other tissue types, as a model of regenerative-like healing following a full-length patellar tendon injury.

We developed a set of hypotheses for this aim based on preliminary data and our expected results.

**Hypothesis One:** The MRL/MpJ will show improved biomechanical outcomes following injury compared to the C57BL/6.

**Hypothesis Two:** The MRL/MpJ will exhibit increased expression levels for tendon-related markers.

**Hypothesis Three:** The MRL/MpJ healing response will show decreased expression of the cyclin-dependent kinase inhibitor p21 compared to the C57BL/6 at early time points post-injury.
Driving tissue-engineered repairs down a regenerative-like healing pathway as opposed to a natural healing pathway may prove to be key in restoring normal tissue structure and function. In addition to characterizing the biomechanical and biological responses, we were also interested in evaluating the entire MRL/MpJ tendon healing RNA transcriptome. RNAseq technology produces a comprehensive view of the entire RNA transcriptome of a tissue sample, aiding in the detection of genes that may be uniquely expressed (or not expressed) in the MRL/MpJ during healing. For the second part of aim one, we sought to identify factors that are differentially expressed among the MRL/MpJ, LG/J, and C57BL/6 mouse strains in response to a central third patellar tendon injury. The identified pathways represent potential targets for modulation in our future tissue engineering work. We generated the following set of hypotheses for this aim.

**Hypothesis Four:** The MRL/MpJ strain will express increased levels of genes implicated in normal development compared to the C57BL/6 strain following patellar tendon injury.

**Hypothesis Five:** The MRL/MpJ strain will show decreased expression of genes involved in the immune response compared to C57BL/6 strain following patellar injury.

While the focus of our laboratory has been to improve tendon tissue engineering strategies, we realize the importance of translating our approaches and findings to other injury and animal models, with the ultimate goal of developing clinically applicable therapies. To this end, we have applied our approach of investigating regenerative healing to another injury model system: a murine critical-size femoral osteotomy. While the majority of bone fractures heal sufficiently with proper fixation and stabilization, non-union outcomes can occur following a severe fracture or tumor resection. We have previously shown that critical-size (1.6mm) murine osseous defects fail to heal sufficiently following injury, as indicated by a failure to bridge the
created defect\textsuperscript{29}. In aim two of this dissertation, we chose to investigate the MRL/MpJ healing response to a critical size femoral injury by evaluating biomechanical and biological outcome measures.

**Hypothesis Six:** The MRL/MpJ strain will exhibit improved biomechanical outcomes following the creation of a critical-size (1.6mm) femoral osteotomy compared to the C57BL/6 mouse.

**Hypothesis Seven:** The MRL/MpJ strain will show increased levels of bone metabolizing enzymes at time points post-injury compared to the C57BL/6 mouse.

Regenerative healing is one approach to investigating successful tissue formation following injury; however, it continues to be debated whether the MRL/MpJ is a model of true ‘regeneration’. An alternative strategy to investigate successful tissue formation is normal development. Over the past several years, our laboratory has collaborated with Cincinnati Children’s Hospital Medical Center’s Developmental Biology group (Dr. Chris Wylie and Dr. Rulang Jiang) to identify novel biological targets from normal tendon development to investigate with tissue engineering approaches. This collaboration has yielded exciting results\textsuperscript{30, 31}. As developmental findings continue, it is our job as tissue engineers to evaluate these targets and the role they play in the adult animal, both in maturation and healing. Thus, in aim 3, we propose to evaluate the role of Mohawk Homeobox (Mkx), a transcription factor identified to play a key role in successful tendon development, by knocking out Mkx expression and determining the biomechanical and morphological outcomes in the adult animal.

**Hypothesis Eight:** Loss of mohawk homeobox gene function will produce decreased patellar tendon mechanical properties in a 20-week old mouse compared to an age-matched heterozygous littermate.
Hypothesis Nine: Loss of mohawk homeobox gene function will lead to gross morphological and histological patellar tendon abnormalities including discoloration, lack of collagen vibrancy, and decreased size.

We have utilized the central-third patellar tendon defect model to investigate tendon healing because it is easily reproducible and allows for consistent treatment evaluation; however it is not clinically relevant. Therefore, in the second part of this aim, we sought to develop a murine model of collagenase induced tendon injury that more closely resembles common clinical presentations. This will allow us to investigate multiple injury types (‘acute’ vs. ‘chronic’), evaluate the effects of different injury types on the natural healing process (i.e. does an ‘acute’ injury heal differently than a ‘chronic’ injury?), and determine how potential therapeutic molecules might induce differential responses in contrasting injury model systems.

Hypothesis Ten: A single patellar tendon collagenase injection will produce decreases in patellar tendon biomechanical properties in the short term with a return to near normal biomechanical properties at extended time points following injection.

Summary

The proposed research promises to first characterize the regenerative properties of the MRL/MpJ mouse as it relates to musculoskeletal healing and to identify activated pathways that are contributing to this healing phenotype. Work in Aims 1 and 2 will evaluate the utility of using the MRL/MpJ as a model of musculoskeletal regeneration. Data collected through RNA-Seq analysis of the repairing tendon will help us as we move forward in identifying pathways that may have therapeutic potential.

Work in Aim 3 seeks to evaluate an identified target from normal development with respect to adult maturation and healing. While we have successfully identified several molecules
of interest from normal developmental processes, we are still developing evaluation tools to ascertain the importance of these molecules in the adult. Data collected through this aim will lay the foundation for future work involving target evaluation in a more clinically relevant tendon injury model.

As a whole, this work is significant because it has the potential to greatly impact the field of regenerative medicine and is translatable to other injury models and species. Investigating regenerative healing and normal development together to identify and evaluate new markers of interest to focus tissue engineering strategies is an advantageous approach strengthening the impact of potential research findings (Fig. 1).
Fig. 1: My doctoral research seeks to identify and evaluate novel biological targets from a regenerative-like healing model and normal development. To begin, we will characterize the MRL/MpJ murine strain as a model of regenerative-like healing in our patellar tendon defect injury model and our critical-size femoral defect injury model. Second, we will sequence the RNA transcriptome of tendon healing tissue using three murine strains at early time points to identify differentially expressed pathways and transcription factors. This information will aid in identifying molecules and pathways to focus future tissue engineering work. Finally, we will evaluate the role of a marker identified from normal development, mohawk homeobox, and the role it plays in adult patellar tendon maturation. Preliminary work in establishing a murine model of collagenase-induced tendon injury will lay the foundation for future work investigating multiple models of tendon healing and repair strategies. Overall, this work seeks to identify and evaluate successful pathways to drive our tissue-engineered repairs down as opposed to natural healing pathways.
Chapter 2

Literature Review

The musculoskeletal system consists of bones, tendons, ligaments, cartilage, fascia, and muscles providing the framework for body movement and protection of inner organs. Injuries to the musculoskeletal system are prevalent and designing successful therapies continues to present a challenge to the orthopaedic community.

Tendons connect muscle to bone and their primary function is to transmit forces generated in muscle to bone producing skeletal movement. Found throughout the body, including, but not limited to, the supraspinatus tendon within the rotator cuff, the flexor tendons of the hand, and the patellar tendon located just distal to the patella, tendons resist large tensile forces to stabilize joints. The focus of this dissertation will be primarily on the patellar tendon, an extrasynovial tendon that functions to transmit forces generated in the quadriceps muscle to the tibia. Anatomically, the patellar tendon connects the patella to the tibia; however, it is also semi-continuous with the quadriceps tendon and therefore can be characterized as a tendon.

Bones allow for movement and protection while also maintaining mineral homeostasis. Bones can be categorized as long, short, irregular, or flat depending on shape. The femur is the longest and strongest bone in the body, found in the proximal region of the lower extremity articulating between the pelvic bone and the patella and tibia at the distal end. While the primary focus of this literature review is on tendon, a brief section will review bone structure, function, and healing, providing foundation for a study investigating osseous natural healing presented in Chapter 5.
i. **Tendon Structure and Function**

Tendons can be classified as either intrasynovial or extrasynovial\(^{34}\). Intrasynovial tendons are encased by a synovial sheath, which releases synovial fluid, functioning to reduce friction during gliding motions\(^{34}\). These tendons can be found in regions where fine positional movements are required, such as the flexor tendons of the hand\(^{35}\). Extrasynovial tendons, such as the Achilles tendon and the supraspinatus tendon in the rotator cuff, do not have a synovial sheath resulting in a higher resistance to gliding motions\(^{34}\). Overall tendon structure can be quite different, dependent on the location and functional roles they possess; however, commonalities exist in the basic structure including the extracellular matrix composition, proteoglycan and glycoprotein content, and cellular composition.

Tendons are hierarchical tissues composed of primarily type I collagen, the most predominant fibrillar collagen found throughout the body\(^{36}\). Kastelic et al. describe the hierarchical organization of collagen beginning at the molecular level with tropocollagen\(^{36}\). Five tropocollagen molecules bind together to form a microfibril. Several microfibrils form a subfibril, and together, many subfibrils form a fibril. Collagen fibrils join together to form a tendon fascicle. These fascicles are separated by the endotenon, a network of criss-crossed collagen fibers that functions to hold the larger collagen fascicles together forming the tendon proper (Fig. 2). The paratenon is a connective tissue structure that encases the tendon proper to decrease frictional forces that arise during movement\(^{37}\). The epitenon is yet another layer of reticular connective tissue which is continuous with both the endotenon and the paratenon and further functions to protect the tendon against micro-damage\(^{37}\).
Fig. 2: The hierarchical nature of tendon. Tendon organization begins at the molecular level where glycine, proline, and hydroxyproline predominate to form triple helical tropocollagen molecules, which aggregate together to form fibrils. Multiple fibrils form a fiber. These fibers are organized into fascicles encased by the endotenon. Several fascicles encased by the epitenon form the tendon proper. The hierarchical structure imparts mechanical strength to the tissue. Reproduced from Wang 2006.

These well-aligned type I collagen fibrils give tendon its inherent tensile strength. The collagen fibrils possess a characteristic ‘crimp’ pattern, which is thought to provide additional protection during early stages of tendon loading aiding in the transmission and/or absorption of large tensile forces. Upon the application of forces above 2% strain, the collagen fibers assume a fully parallel alignment and begin to stretch under elastic deformation. As strains exceed 4%,
plastic deformation occurs as collagen fibers begin to fail eventually leading to total tissue failure at strains in the range of 8-10%\textsuperscript{41}. Another aspect of tendon that makes it uniquely equipped to handle large tensile forces is the bimodal distribution of both large (100 to 150 nm) and small (40 to 75 nm) diameter collagen fibrils\textsuperscript{42,43}. It is thought that the large collagen fibrils function to resist tensile loads while the small collagen fibrils improve interfibrillar binding, elasticity, and withstand creep\textsuperscript{44}.

In addition to collagen type I, tendon is composed of additional minor collagens. Collagen type III, thought to be an immature form of collagen typically co-localizes with collagen type I containing tissues, often exhibits increased expression levels in response to tendon injury\textsuperscript{45}. Collagen type X, a short-chained collagen, is involved in the calcification process within the heterotrophic region of the tendon-to-bone insertion site\textsuperscript{46}. Together with collagen type I, these collagens impart mechanical strength to further resist tensile loading.

Along with collagens, proteoglycans and glycoproteins can be found functioning to modulate collagen expression and organization, along with interacting with the resident cell population\textsuperscript{37}. Glycoproteins are a broad class of proteins containing an oligosaccharide side chain and perform a number of different functions\textsuperscript{37}. In tendon, tenascin-C is a glycoprotein that activates following injury or excessive loading events and is thought promote cell proliferation and migration\textsuperscript{47}. Small leucine-rich proteoglycans (decorin, lumican, biglycan, and fibromodulin) are involved in the collagen fibrillogenesis process throughout development and healing to regulate the collagen fibril diameter size and alignment\textsuperscript{48}. Decorin has been shown to be vital to regulating collagen fibril diameter. Loss of decorin function results in abnormally shaped collagen fibrils, disproportionate distribution of collagen fibril diameters, and decreased mechanical properties of mature tendon tissue\textsuperscript{49}.
The primary cell type in tendon is a tendon fibroblast, also known as a tenocyte. Tenocytes are spindle-shaped cells situated along collagen fibers, oriented along the axis of tension\textsuperscript{39}. These cells interact with one another, the surrounding extracellular matrix, and signaling molecules to produce collagen, collagen cross-links, and matrix-organizing proteins\textsuperscript{50}. Tendon tissue is characterized as having a low cell to extracellular matrix ratio; however, this can vary within different tendon types and regions along the length of the tendon\textsuperscript{50}.

Significant work has been done to better understand the molecular expression profile that maintains the tenogenic nature of the tenocyte. To date, there are no proteins known to be tendon-specific. Many studies characterizing this cell type have experimented in vitro with reports of phenotypic drift occurring in culture, making it difficult to identify the true nature of this cell\textsuperscript{51, 52}. It has been shown that nearly all tenocytes express the basic helix-loop-helix transcription factor, scleraxis (Scx)\textsuperscript{53}. This transcription factor plays a role throughout early tendon development and differentiation and is believed to modulate collagen type I expression into adulthood\textsuperscript{53}. Scott et al. created bio-artificial tendons and applied a cyclic load over a three week period finding increased scleraxis and collagen type I expression compared to non-stimulated controls, suggesting scleraxis expression can be modulated through mechanical loading events\textsuperscript{54}. Mohawk homeobox (Mkx) is another transcription factor identified from normal tendon development and is believed to function in tenocyte maturation and/or collagen fibril diameter regulation\textsuperscript{55, 56}. While these two transcription factors are expressed during tendon development and maturation, they are not specific to tendon tissue and can be found expressed in tissues that experience significant mechanical loading and collagen production\textsuperscript{57, 58}. Identifying factors that are uniquely expressed in tendon tissue continues to be an area of great interest in the tendon field.
Further, tenocytes are mechanosensitive, in that they are capable of responding to alterations in the local loading environment and then modulating expression of necessary factors to accommodate these changes through a process termed mechanotransduction\textsuperscript{39, 59}. This involves interactions among extracellular matrix proteins, cell surface receptors, internal actin cytoskeleton, and internal transcriptional and translational machinery which function to modify the surrounding environment in response to loading alterations\textsuperscript{59, 60}. Normal physiologic loads maintain the homeostatic tendon environment; however, abnormal loading events, either in excess or less than normal, can contribute to tendon injury\textsuperscript{61, 62}. Understanding the normal tendon loading environment, and how it becomes altered in the event of injury, is important for designing tissue replacements and augmentations.

\textit{ii. Mechanical Properties of Tendon}

Tendon tissue is characterized as being viscoelastic, exhibiting both viscous and elastic properties under deformation. It is hypothesized that the viscoelastic nature of tendon is a result of the interactions among collagens, proteoglycans, glycoproteins, and water\textsuperscript{63}. This categorization has several mechanical implications for tendon that have been well characterized over the years: 1. A viscoelastic material is sensitive to the rate at which load is applied and will respond differently dependent on this applied rate\textsuperscript{64}, 2. When a constant strain is applied to tendon tissue, the stored energy within the tissue will dissipate over time, a phenomenon known as creep\textsuperscript{65}, 3. When the tissue is repeatedly loaded and unloaded with small, sub-failure load levels, energy is lost in the form of heat producing hysteresis, whereby the tissue lengthens and internal stresses are dissipated\textsuperscript{66}.

A tendon’s primary function is to transmit large forces from muscle to bone. To accommodate these forces, tendons possess large tensile strength. To better understand the
tendon’s mechanical environment, investigators have implemented a variety of non-invasive
techniques to estimate the normal in vivo force levels for individual tendons during walking,
jumping, and running exercises in human subjects\textsuperscript{67-70}. There is large variability in the load
levels different tendons typically withstand during activities of daily living due to the tendon’s
location and function within the body\textsuperscript{69}. For example, the Achilles tendon, the largest tendon in
the human body, connects the gastrocnemius and soleus muscles to the calcaneus. It has been
estimated the Achilles tendon withstands loads up to eight times body weight during running and
jumping activities with load levels ranging from 3100N-5330N\textsuperscript{67}. Ultrasonography imaging of
the patellar tendon during isometric contractions to maximal exertion levels in male college
students indicates stiffness values ranging from approximately 2000 N/mm – 2700 N/mm
dependent on the rate at which the tissue was loaded\textsuperscript{68}.

Tendon failure properties can also vary significantly depending on the tissue size, subject
age, and rate at which it is loaded. Failure testing of human cadaveric flexor tendons (flexor
digitorum profundus and flexor digitorum superficialis) reveals tensile failure loads of
1175±245N and 1296±319N, respectively, indicating the inherent strength of a relatively small
connective tissue\textsuperscript{71}. Human cadaveric Achilles tendons fail at approximately 5098±1199N
(n=18) with recorded elastic modulus of 819±208 MPa\textsuperscript{72}, while the patellar tendon had an elastic
modulus of 582±244 MPa\textsuperscript{73}. Clearly, understanding the normal loading environment and
structural and material properties of tendons aides tissue engineers in designing effective tissue
replacements and augmentations. A significant amount of work has been done over the last three
decades to fully characterize the mechanical properties of tendon and ligament tissues. This
work has laid the foundation for establishing a set of mechanical design criteria to guide tissue
engineering strategies; however, more recent work seeks to better understand the biological
mechanisms regulating tendon development, natural tendon healing, and repair strategies to 
further improve engineered approaches.

iii. Mechanisms of Tendon Injury and Healing

It has been estimated that approximately 110 million patients presented with a 
musculoskeletal injury in 2008, with a large portion of these presentations attributed to tendon 
and ligament injuries\(^1\). These injuries affect a broad range of individuals with varying degrees of 
severity that can be characterized as either acute or chronic\(^74\). Tendon injuries can result from 
abnormal, excessive loading events, isolated to a single event for an acute injury or through 
repetitive loading over an extended period of time for a chronic injury\(^74\). Acute tendon injuries, 
or ruptures, are generally more likely to occur in younger populations, often resulting from an 
abnormal acceleration/deceleration event\(^75\). Previous findings suggest that an acute tendon 
injury may actually be a result of an underlying chronic condition; however, this continues to be 
debated\(^76\). Overuse, chronic tendon injuries affecting the Achilles tendon, patellar tendon, and 
extensor carpi radialis brevis accounted for 7% of all musculoskeletal-related doctor visits in 
2002\(^3\). The rotator cuff is one of the most chronically injured musculoskeletal structures with 5 
million physician visits between 1998-2004\(^4\). These types of injuries traditionally affect older 
individuals as degenerative processes begin to materialize, contributing to decreased collagen 
and elastin production, reduced collagen fibril diameters and collagen cross-linking\(^77\).

In the event of an acute tendon injury, the natural healing response can be characterized 
in three-stages (Fig.3)\(^74, 78, 79\): inflammation, matrix production, and matrix organization and 
maturation. The inflammatory phase begins immediately following injury with an influx of 
inflammatory cells, inflammatory mediators, and immune cells to the injury site. Over the 
following twenty-four to forty-eight hours, a fibrin clot forms to stabilize the damaged tissue
while neutrophils, erythrocytes, and macrophages function to remove damaged tissue and eliminate sources of infection. These inflammatory cells also release chemotactic molecules to recruit collagen-producing fibroblasts from the surrounding paratenon and retinaculum and elicit neovascularization processes. Once the injury site is stabilized, the matrix production phase initiates with increased cellular proliferation, increased collagen types I and III synthesis, along with matrix-associated protein production, including tenomodulin, fibromodulin, and tenascin-C. This phase lasts anywhere from one to three weeks following the initial inflammatory phase, although the matrix produced during this phase is poorly organized. The final phase, matrix maturation, overlaps with the second phase as the extracellular matrix produced becomes organized through collagen turnover, formation of collagen cross-links, and collagen realignment.
Fig. 3: Tendon healing process following acute injury. Immediately following injury, inflammatory and immunological processes dominate the healing response. This phase is followed by cellular recruitment, proliferation, and matrix production. In the following weeks and months, the matrix material aligns, collagen cross-links form, and the tensile strength of the tissue increases, although never reaching native mechanical properties. Reproduced from Leadbetter 1992\textsuperscript{79}

While some aspects of the healing process have been characterized, many unknowns remain. Debate continues as to whether intrinsic, extrinsic, or a combination of both types of healing are responsible for driving the process. Intrinsic healing theory suggests that healing originates from the injured tendon itself, with recruitment of different cell types from the para-, epi-, and endotenon, along with resident tendon cell repairing the damage\textsuperscript{80}. Extrinsic healing is accomplished through recruitment of proliferating fibroblasts and immature cells, along with other factors circulating in the blood supply, to the injury site\textsuperscript{81}. In all likelihood, it is a combination of processes that ultimately lead to the repair; however, identifying each process’ contribution to the repair could help to more efficiently guide therapeutic development\textsuperscript{78, 82}.  

20
Furthermore, it is still unknown why a tendon fails to heal to normal mechanical and biological levels. Many hypotheses exist. Some postulate the poor vascular supply to tendons prevents required nutrients, oxygen, and healing cell types from entering the injury site\textsuperscript{78, 83}. Others suggest it is mechanical loading issue – either a result of too much or too little force being applied to the damaged tissue disrupting the complex loading distributions to surrounding tissues\textsuperscript{61, 84-87}. And still, others hypothesize poor healing results from the absence or inadequate expression of required chemical cues to correctly coordinate the reformation of the extracellular matrix. Throughout the healing process, a large number of growth factor families are expressed to regulate the healing process, including transforming growth factor βs (TGFβ), insulin-like growth factors (IGF), fibroblast growth factors (FGF), and platelet-derived growth factors (PDGF)\textsuperscript{88-90}. Perhaps tendon does not heal to normal levels because these factors are improperly expressed and require therapeutic intervention to improve healing outcomes. Again, it is likely that each of these play a role and new approaches must be implemented to fully characterize the shortcomings associated with natural healing.

Despite the body’s best efforts, the natural healing process does not fully restore the damaged tissue to uninjured morphological or biomechanical properties with the repair tissue exhibiting disorganized matrix alignment characteristic of scar\textsuperscript{74}. Previous work shows mechanical properties failing to reach normal levels up to eight weeks, twenty-six weeks, and twelve months following mouse and rabbit patellar tendon injuries, and sheep Achilles tendon injury, respectively\textsuperscript{18, 26, 27}. The poor natural healing outcomes often necessitate a reduction of daily activity levels, physical therapy, and/or surgical intervention.
iv. Tendon Repair

Current clinical treatment for tendon and ligament injuries fall into one of the following categories: non-operative, repair of the damaged tissue, or complete replacement of the damaged tissue with a graft\textsuperscript{11, 91}.

Taking a non-operative approach to a tendon rupture is dependent on the location and severity of the injury. For an Achilles tendon rupture, a distance of 10mm or less between the ruptured tendon ends with the ankle in neutral position qualifies for non-operative treatment\textsuperscript{92}. This approach requires the joint to be stabilized using a brace or cast preventing further tissue damage\textsuperscript{93}. Physical therapy is prescribed approximately 3 weeks following injury to improve the healing response, increase joint mobility, and prevent adhesion formation\textsuperscript{92, 94}. Non-operative treatment is traditionally advocated for older patients whose health may be compromised by surgery or sedentary individuals who do not require full tendon strength\textsuperscript{92, 93, 95}. Unfortunately, non-operative healing leads to decreased tissue mechanical properties and an increased rate of re-injury compared to surgical intervention. In a study investigating non-operative versus operative treatment of a subcutaneous Achilles tendon rupture, it was found that non-operative outcomes at 3-5 years following injury showed decreased gastrocnemius and soleus muscle size coupled with impaired muscle function (decreased range of joint motion, decreased mechanical strength)\textsuperscript{95}. A prospective study comparing the re-rupture rates between non-operative and operative treatment of Achilles tendon rupture articles published between 1959 and 1997 found operative treatment produced superior repair outcomes with 2.8\% re-rupture rate compared to 11.7\% for non-operative treatment\textsuperscript{96}.

Direct surgical repair of the damaged tissue ends is an approach employed when the quality of the injured tissue is of sufficient quantity and quality; however, this approach tends to
produce inconsistent surgical outcomes. For example, direct repair of the rotator cuff results in failure rates of 11%-95% evaluated two years post repair\textsuperscript{10, 97}. The Achilles tendon and flexor tendons of the hand show improved surgical outcomes with direct repair; however, variability remains a concern\textsuperscript{9, 98}. Often, ruptures occur in degenerative tendons making direct repair difficult as the native tissue is of poor quality. Removing the damaged tissue and transplanting a graft tissue is commonly used when direct repair is not possible.

Grafting is the third approach when non-operative and direct repair methods are deemed inadequate. Grafts can be either autografts (tissues harvested from the patient’s own body) or allografts (tissues harvested from cadavers or animals). Autograft and allograft tissue replacements are often complicated by donor site morbidity and immuno-incompatibility between donor and host, respectively\textsuperscript{13, 14}. While these approaches often return the patient to pre-injury activity levels in the short-term, long-term chronic impairments result. Anterior cruciate ligament reconstructions with patellar tendon autografts produced satisfactory ligament stability in the short term; however, patient follow-up at thirteen years post grafting showed 75% of patients exhibited degenerative symptoms characterized by increased joint laxity and increased likelihood of graft rupture\textsuperscript{99}. Other studies show similar long-term outcomes suggesting current grafting approaches are not adequate to maintain overall joint structure\textsuperscript{15, 16}. As mentioned previously, individual tendons can be quite different depending on their location, loading environment, and matrix composition/orientation. Using, for example, a patellar tendon to replace an intrasynovial ligament such as the anterior cruciate ligament, may not be the best approach when one considers the structural and compositional differences between these two structures.
In summary, the shortcomings associated with natural tendon healing, coupled with the poor long-term outcomes of traditional surgical interventions, necessitate alternative strategies to improve tendon and ligament healing. Clinical approaches often seek to repair or replace the damaged tissues; however, long-term outcomes result in degenerative symptoms and associated pain. Identifying therapies to augment the healing process using tissue-engineered strategies has been a method advocated by many in the orthopedic community.

v. Tissue Engineering

Tissue engineering is an approach that has been employed over the last three decades for designing biological and/or synthetic tissue replacements or augmentations to enhance the healing process of an injured tissue. This approach combines cells, biologic and/or synthetic materials, chemical supplementation, and engineering principles to create what is commonly referred to as a tissue engineered construct, or TEC. A wide variety of tissue-engineered substitutes have been created to treat tissue damage and loss, including therapies for skin, heart, bone, and cornea, although with varying degrees of success. Engineered cartilage and skin substitutes have received Food and Drug Administration approval for human therapy; however, clinical use has produced inconsistent outcomes in terms of repair improvement. Developing a ‘one-size-fits-all’ product proves to be a problem as each human injury is unique, with a range of confounding variables including patient age, activity level, weight, height, along with injury type, severity, and location. Refining current tissue engineering techniques to eliminate outcome variability and developing sophisticated therapies to address more complex tissue systems and confounding variables continue to be the focus of laboratories throughout the world.
In the context of musculoskeletal tissue engineering, understanding the importance of maintaining, and in the case of injury, reestablishing, normal mechanical loading profiles for musculoskeletal function has led to the development of functional tissue engineering\textsuperscript{108}. This sub-field of tissue engineering seeks to incorporate biomechanical aspects into the tissue engineering design process, which has produced a set of mechanical success criteria as described by Butler et al.\textsuperscript{109}. These criteria outline the need to understand normal in vivo loading parameters and design tissue engineered therapies to first exceed measured peak in vivo forces, and secondly, match normal tangent stiffness up to peak in vivo force levels with a safety factor\textsuperscript{109}. These criteria laid the foundation for assessing successful in vitro tissue engineered construct creation and in vivo repair outcome.

Previous tissue engineering studies using autologous mesenchymal progenitor cells (MPCs) seeded in a collagen scaffold, mechanically pre-conditioned in vitro, and implanted into a rabbit central patellar tendon defect showed moderate success. The tendon repair tissue matched normal patellar tendon up to 32% of normal failure force, 50% beyond peak in vivo forces measured for activities of daily living, by twelve weeks post injury\textsuperscript{101}. While these results show promise, serial fiber failures initiated beyond 32% of normal failure force. Exposing this repair tissue to more strenuous activity levels (i.e. 40% of normal failure force as measured in the goat PT\textsuperscript{110}) could potentially lead to failure. Fan et al designed an anterior cruciate ligament (ACL) replacement composed of mesenchymal stem cells seeded on a knitted silk scaffold, which was implanted following the excision of a rabbit native ACL. Mechanical testing completed twenty-four weeks following implantation showed that the tissue-engineered ligament reached only 18% of normal maximum load (24.59 ± 1.64N compared to 131.82 ± 17.64N)\textsuperscript{111}.  

25
While these results generally show improvements compared to natural healing, there is still significant room for advancement.

Future success will depend on not only recapitulating native tendon mechanical properties, but also better understanding the biological properties and the molecular mechanisms that function to produce the mechanical outcomes. Knowing this information will aid tissue engineers in selecting appropriate cell types, construct material, and in vitro pre-conditioning protocols to improve current design strategies. The field has come a long way in the last several years with the advent of sophisticated sequencing tools and bioinformatics; however, there is still a need to identify and characterize successful models of tendon formation to help guide tissue engineering strategies. Several approaches have been advocated seeking to identify successful models of tissue formation including regenerative healing and normal development$^{30,112}$.

vi. Overview of Bone Structure, Function, and Healing

The skeletal system provides the overall framework for the body providing movement, protection, and mineral homeostasis. The focus of this section will be on the structure, function, and healing capacity of the long bones of the body, particularly the femur. The femur consists of a long diaphysis with two epiphyses situated on both the proximal and distal ends for articulation between the pelvic bone at the proximal end and the patella and tibia at the distal end.

Bone is composed of organic and inorganic components making up 40% and 60% of its dry weight, respectively$^{32}$. The organic portion consists of collagen, proteoglycans, and non-collagenous proteins, particularly osteopontin, osteocalcin, and osteonectin$^{113}$. Of the organic components, 85-90% consists of collagen, primarily type I collagen, along with type III to a lesser degree, providing elasticity and tensile strength. Of the non-collagenous proteins, osteocalcin, the most prevalent, functions to promote mineralization$^{113}$. The inorganic portion of
bone is primarily hydroxyapatite, a form of calcium apatite, which imparts compressive integrity to bone tissue\textsuperscript{114}. The large inorganic component provides mechanical strength to the tissue; however, unlike tendon, it is brittle. Subsequently, bone is stronger in compression than in tension. The femur exhibits nearly double the strength in compression compared to tension with failure occurring at approximately 12,000lb./sq.in. in tension and 20,000lb./sq.in. in compression\textsuperscript{33}.

The cellular component of bone consists of osteoblasts, osteoclasts, and osteocytes. These cells function to maintain normal tissue physiology through a process of constant remodeling\textsuperscript{32}. Osteoblasts produce matrix material and function in the matrix mineralization process\textsuperscript{114} and osteoclasts resorb old bone allowing osteoblasts to produce new matrix\textsuperscript{114}. Osteocytes are differentiated osteoblasts found embedded in the calcified tissue connected to one another via canaliculi sensing mechanical and chemical changes in the extracellular matrix and communicating this information to osteoblasts and osteoclasts through paracrine signaling\textsuperscript{114}.

Approximately 7.9 million patients are affected by osseous fractures in the United States annually\textsuperscript{5}. Typically, bone heals sufficiently if the fracture is properly fixed and mechanically stabilized. However, approximately 10% of fractures exhibit impaired bone healing producing delayed union or nonunion\textsuperscript{5}. There are several strategies for treating a nonunion; however, a variety of risk factors, the type of injury, and a patient’s predisposition make selecting a specific therapy difficult and often, the defect fails to heal completely\textsuperscript{5,115}. Investigating animal models of non-union osseous healing presents an opportunity to identify shortcomings associated with the process and develop therapeutic strategies.
vii. Regenerative Healing Models

Regeneration is defined as the ‘homeostatic ability to maintain tissue structure in the face of normal cell turnover or loss of tissue damaged by trauma or disease’\textsuperscript{116}. The phenomenon of regeneration has been observed dating back to the time of the ancient Greeks as illustrated by Aristotle’s observations of regenerating lizard and snake tails\textsuperscript{117}. During the 18\textsuperscript{th} century, scientists, such as Rene-Antoine Ferchault de Reaumur and Lazzao Spallanzani, observed and experimented with a number of regenerative models, including salamanders, hydra, snails, and earthworms\textsuperscript{118}. With the advent of more sophisticated technologies in the 20\textsuperscript{th} and 21\textsuperscript{st} centuries, scientists have continued to investigate these species, at the molecular and cellular levels to identify the source of the regenerative phenotype.

Successful tissue regeneration is relatively limited in the vertebrate kingdom; however, some models do exist. The urodele has long been studied since it exhibits complete limb regeneration following injury via a scar-free mechanism. In the event of urodele injury, an epithelial cell layer provides initial stabilization of the injury site and closes the wound\textsuperscript{119}. Next, through recruitment of resident stem cells or de-differentiation of cells localized at the injury site, a mass of undifferentiated, proliferating cells form a blastema\textsuperscript{119}. The \textit{Hoxa} and \textit{Hoxd} genes, already expressed at basal levels within uninjured limbs, increase expression levels to choreograph the placement of cells and alignment of the redeveloping limb, similar to the normal developmental processes observed across species\textsuperscript{119, 120}. The zebrafish exhibits a regenerative response to a caudal fin injury\textsuperscript{121}. Similar to the urodele response to limb amputation, the zebrafish responds the insult by first stabilizing the injury and then forming a blastema. The blastema consists of hyperproliferative, undifferentiated cells that grows outward, allowing cells to differentiate and produce and organize matrix as the new tissue begins to form over time.
There is debate as to the source of the cells that contribute to the healing response in regenerative systems\textsuperscript{122}. One theory suggests the concept of dedifferentiation. Dedifferentiation is a process whereby resident, fully differentiated cells respond to an injury by reverting to a highly proliferative, undifferentiated state to repair the damaged tissue\textsuperscript{123}. Another potential source of the reparative cells is resident stem cells. These cell types become activated in the event of injury and proliferate to form a large pool of progenitor cells that can respond and repair the damaged tissue\textsuperscript{123}. Recent evidence suggests that both theories may hold true. Sandoval-Guzman et al used the Cre-loxP system to show that the Notophthalmus viridescens (newt) and Ambystoma mexicanum (axolotl), both members of the salamander family, implemented different processes for cellular recruitment to create the repair blastema following a muscle injury. The newt was found to undergo myofiber dedifferentiation, while a PAX7+ cell population (muscle satellite cells) provided the healing response in the axolotl\textsuperscript{123}. Of course, these results could be specific to the type of tissue that was injured or the severity of the injury; nevertheless, these data suggest that several mechanisms have the capacity to successfully regenerate damaged tissue in different animal species.
Fig. 4: A model of blastema formation and maturation in the zebrafish caudal fin. Following injury, cells stabilize the wound by forming an epithelium. By 24 hours, the blastema forms consisting of a mass of proliferating, undifferentiated cells. It is still unknown if the blastema forms through resident stem/progenitor cell recruitment or via dedifferentiation. By 48 hours, the blastema continues to move outward, as cells proximal to the blastema begin to differentiate and produce and organize the extracellular matrix. After 72 hours, the blastema begins to decrease in size and proliferative capacity as the tissue completes the tissue growth phase. Muscle segment homeobox b (msxb) is a homeodomain transcription factor believed to regulate cellular proliferation within the blastema. Reproduced from Nechiporuk 2002.

Regenerative processes also exist in mammalian species. Annual deer antler regeneration is a process that has long been investigated as a mammalian model of regeneration. Each year, mature male deer shed their antlers and then regrow a new set by way of a blastema and subsequent cartilage and bone formation. Parathyroid-related peptide molecule and retinoic acid. Digit tip regrowth has been observed in both murine and juvenile humans also through the formation of a blastema believed to be regulated by the Msh homeobox 1 (Msx1) transcription factor, a factor associated with normal developmental processes and other models.
of regeneration. Regeneration has been studied for hundreds of years because of the unlimited potential it has to revolutionize therapeutic approaches to human injury and disease. Now, with the advent of numerous murine models and extensive molecular tools, we are poised to fully characterize the regenerative process. With this in mind, identifying a murine model of regenerative-like healing could provide a useful strategy for investigating regenerative pathways to guide tissue engineering strategies for future clinical utility.

The Murphy Roths Large (MRL/MpJ) murine strain has been identified as a model of successful mammalian tissue regeneration following injury. This mouse strain is a result of a series of crosses between the C57BL/6J, C3H/HeDi, AKR/J strains followed by backcrosses to the LG/J strain, with the LG/J strain accounting for 75% of the genetic background. Originally created to investigate mechanisms regulating the autoimmune disorder of systemic lupus erythematosis, this mouse was found to repair through-and-through ear hole punches via the development of a blastema, similar in structure and characteristics of blastemas identified in normal development and in the urodele injury response. The repair process also functioned to reform hair follicles, sebaceous glands, and well-aligned collagen creating, as concluded by the authors, fully regenerated skin tissue. In addition to ear hole closures, studies have shown regenerative-type responses in other tissue types including heart, articular cartilage, spinal cord, and cornea, although the degree of regeneration varies. In some cases, the MRL/MpJ repair response is no better than wild type. This would suggest the response may be dependent on the type or severity of the induced injury; however, protocol differences may confound the results. Additionally, characterizing the healing process in other tissue systems, such as tendon and bone, two of the most commonly injured musculoskeletal structures, could further aid in clarifying the possible mechanism.
Through genetic analysis, mutations were found to be distributed over 20 different loci on 7 chromosomes, implying that multiple factors contribute to the healing phenotype. This has led to difficulties in elucidating the mechanisms regulating this process with no definite pathway identified as being responsible.

**viii. Potential Mechanisms of Mammalian Regeneration**

Reduced expression of the cyclin dependent kinase inhibitor, p21, has been looked to as a potential contributor to the MRL/MpJ healing phenotype. This molecule is a downstream effector of the tumor suppressor, p53, and acts at the G1 checkpoint to prevent cell cycle progression to S phase in the event of DNA damage. Previous studies in the MRL/MpJ show decreased p21 expression, significantly below normal levels, resulting in the accumulation of cells in the G2/M phase. In the event of injury, these cells actively participate in the repair process. Additionally, when an ear punch is created in a p21-/- mouse, it is capable of repairing the injury via a scar-free mechanism similar to that of the MRL/MpJ, while a p53-/- exhibits healing no different from wild type. This indicates the regenerative capacity may lie in a mutation within an alternative regulator of p21, such as the TGF-β1/SMAD2,3 pathway or be a result of an entirely different genetic mutation.

Further studies investigating mechanisms regulating the MRL/MpJ repair process show that cells contributing to the healing response express the embryonic stem cell markers Nanog, Islet-1, and Sox-2. This may suggest that the healing mechanism is initiated by a ‘reactivation’ of the resident cells into an undifferentiated state along with enhanced cellular recruitment to the injury site. Questions remain as to how these markers become activated in the response. Others advocate that the improved response results from a shortened or decreased inflammatory/immunological response following injury. Researchers speculate that
inflammatory cells, particularly neutrophils, slow the reepithelialization and the process of matrix remodeling\textsuperscript{105}.

A better understanding is needed to not only identify the genetic differences that exist between the MRL/MpJ and wild type injury responses, but the downstream effects of these differences with respect to cellular proliferation, cellular recruitment, and matrix production and organization remain unknown. Interestingly, many of the pathways activated during regenerative healing are also activated during normal development\textsuperscript{143, 144}. Investigating normal tendon developmental processes offers yet another approach to identifying pathways for modulation in tissue engineering approaches.

\textit{ix. Normal Tendon Development}

A major focus of the musculoskeletal field over the last decade has been to characterize the mechanisms that regulate normal development throughout embryogenesis and early postnatal growth to aid in target identification for modulation in natural healing scenarios and tissue engineering therapies\textsuperscript{30, 112, 145, 146}.

Normal tendon development serves as a ‘positive control’ for tendon formation, occurring in a seamless orchestration of cell signaling and matrix production. In general, tendon development is driven by increased cellular proliferation and spatial patterning followed by subsequent cellular differentiation and matrix production\textsuperscript{147}. At 10.5 days post conception, the basic helix-loop-helix transcription factor scleraxis (Scx) is observed and co-localized with Sox9, an HMG-box containing transcription factor present in chondrogenic cells, in the mesenchymal tissue\textsuperscript{53, 148}. As development continues with limb bud initiation at 11.5 days post conception, Scx and Sox9 continue to be co-expressed\textsuperscript{148}. Once the joint condenses, Scx expression localizes with the joint, while Sox9 expression coincides with the cartilage that will eventually form
bone\textsuperscript{148}. As the tendon condenses at approximately 13.5 days post conception, Scx is relegated to the space between Sox9-expressing cartilage/bone precursors and MyoD-expressing muscle precursors\textsuperscript{148}. Markers important for growth and maturation, including collagen type I, fibromodulin, and tenomodulin, are expressed throughout the body of the tendon through late embryogenesis and early post-natal time points, while tenascin-C, biglycan, and tenomodulin become localized at the tendon-to-bone insertion site as time progresses\textsuperscript{147}. The fibroblast growth factor (FGF) signaling pathway, vital to initiating tendon development\textsuperscript{149} is also highly involved in regulating the process\textsuperscript{147}.

Mohawk homeobox (Mkx) is another transcription factor identified as a key regulator for successful tendon tissue formation during embryonic and early post-natal developmental stages\textsuperscript{55-57}. Members of the homeobox superfamily of genes function to pattern and spatially organize the embryo during development\textsuperscript{57}. Mkx contains three amino acids between helix I and helix II of the homeodomain, so it is further classified as a three-amino-acid loop extension (TALE) member of the atypical homeobox genes\textsuperscript{57}. Mkx expression is observed beginning approximately nine days post conception and can be found localized to the testis cords of the developing male, early chondrogenic mesenchymal cells of the skeleton, the kidney, and the immature tendon cells of the limbs and tail\textsuperscript{57}. In early tendon tissue, Mkx expression co-localizes with Scx expression; however, it seems they act independently of one another to regulate tendon cell differentiation and maturation\textsuperscript{55, 57}. Loss of Mkx gene expression resulted in irregular collagen fibril sizes and distribution, abnormal tendon sheath formation, and decreased expression of collagen type I, fibromodulin, and tenomodulin evaluated up to twenty-one days following birth\textsuperscript{55}. Gross inspection showed abnormal collagen color and vibrancy\textsuperscript{55}. It is
unknown how loss of Mkx function impacts long-term tendon mechanical properties and biological function in the adult animal.

While normal tendon development presents us with a superior model for understanding tendon formation, the environments that exist in an injury model and in a developing embryo are quite different, limiting us in the comparisons we can make. Developing approaches to translate findings from normal development to the adult animal, particularly with respect to healing, presents a challenge for the tissue engineering community.

x. Summary

The purpose of this literature review is to illustrate the need to identify and characterize novel biological targets for manipulation in tissue engineering applications by investigating successful models of tissue formation, namely regenerative healing models and normal developmental processes. This information will allow tissue engineers to make better-informed decisions for design parameters and improve the ultimate repair outcome.
Chapter 3

Functional Tissue Engineering of Tendon: Establishing Biological Success Criteria for Improving Tendon Repair

Andrew P. Breidenbach Ph.D.\textsuperscript{a,1}, Steven D. Gilday B.S.\textsuperscript{a,b,1}, Andrea L. Lalley B.S.\textsuperscript{a,1}, Nathaniel A Dyment, Ph.D.\textsuperscript{c}, Cynthia Gooch, B.S.\textsuperscript{a}, Jason T. Shearn, Ph.D.\textsuperscript{a}, David L. Butler, Ph.D.\textsuperscript{a}

\textsuperscript{a}Biomedical Engineering Program, College of Engineering and Applied Science, University of Cincinnati, Cincinnati, Ohio

\textsuperscript{b}Medical Scientist Training Program, College of Medicine, University of Cincinnati, Cincinnati, Ohio

\textsuperscript{c}Department of Reconstructive Sciences, College of Dental Medicine, University of Connecticut Health Center, Farmington, Connecticut

\textsuperscript{1}These authors contributed equally to this manuscript.

\textsuperscript{1}This manuscript is currently \textit{in press} in the Journal of Biomechanics.
Abstract

Improving tendon repair using Functional Tissue Engineering (FTE) principles has been the focus of our laboratory over the last decade. Although our primary goals were initially focused only on mechanical outcomes, we are now carefully assessing the biological properties of our tissue-engineered tendon repairs so as to link biological influences with mechanics. However, given the complexities of tendon development and healing, it remains challenging to determine which aspects of tendon biology are the most important to focus on in the context of tissue engineering. To address this problem, we have formalized a strategy to identify, prioritize, and evaluate potential biological success criteria for tendon repair. We have defined numerous biological properties of normal tendon relative to cellular phenotype, extracellular matrix and tissue ultra-structure that we would like to reproduce in our tissue-engineered repairs and prioritized these biological criteria by examining their relative importance during both normal development and natural tendon healing. Here, we propose three specific biological criteria which we believe are essential for normal tendon function: (1) scleraxis-expressing cells; (2) well-organized and axially-aligned collagen fibrils having bimodal diameter distribution; and (3) a specialized tendon-to-bone insertion site. Moving forward, these biological success criteria will be used in conjunction with our already established mechanical success criteria to evaluate the effectiveness of our tissue-engineered tendon repairs.
1. Introduction

Tendon and ligament injuries continue to burden the U.S. population and economy, affecting over 110 million patients \(^1\) and costing an estimated $30 billion annually \(^{150}\). Repairing these injuries remains a challenge, often resulting in long-term impairments, such as chronic tendinopathy and osteoarthritis \(^{151}\). Tissue engineering represents a novel approach to potentially improve tendon and ligament repair outcomes by combining cells, biomaterials, and in vitro preconditioning to prepare tissue-engineered constructs (TECs) for in vivo implantation. Taking this concept a step further, functional tissue engineering (FTE) establishes the importance of biomechanical aspects of the design process by focusing on how soft and/or hard tissues are normally loaded during activities of daily living (ADLs) \(^{17,108}\). By incorporating these concepts, the field is poised to more effectively design repairs that meet the demands of the in vivo setting. If we are to design truly successful repairs, we must understand both the mechanical and biological in vivo environments influencing the TEC following implantation. Building on the FTE paradigm to establish mechanical success criteria, we now seek to establish biological success criteria to further improve the evaluation of tissue-engineered repairs for clinical translation.

2. Using FTE principles to improve tendon repair

Since FTE was first described in 2000 \(^{17}\), our laboratory has been designing tissue-engineered constructs to improve tendon healing following injury with the ultimate goal of clinical translation. Tendons contain compositionally and structurally distinct, but mechanically interconnected, regions regulated by the normal loading environment and the location within the body \(^{50,152}\). When tendons are not repaired after injury, the natural tendon healing process often fails to restore normal mechanical properties, leading to increased rates of re-injury \(^{153}\) as well as
tendinopathy and osteoarthritis in the long-term. The standard of care for many acute tendon injuries is surgical repair, but post-operative clinical outcomes have been variable. As such, the concept of developing a tissue-engineered repair to augment the healing process remains an attractive alternative.

Guided by FTE principles, we established two primary mechanical success criteria to evaluate the effectiveness of our tissue-engineered constructs (composed of autologous, mesenchymal progenitor cells seeded in collagen scaffolds) to repair a rabbit central patellar tendon (PT) defect.

2.1. Exceeding peak in vivo forces

One aspect of FTE is the importance of characterizing the normal mechanical properties of native tissue for benchmarking engineered constructs and in vivo repairs. We measured peak in vivo forces in the rabbit and goat, finding that tendons experience quite different levels of peak in vivo force (IVF). In the rabbit Achilles', flexor digitorum profundus, and patellar tendons, in vivo loads range from 11% to 28% of a tissue's failure force during moderate ADLs. However, in the goat model, the percentage approaches 40% of tensile failure load. These results suggested that TECs must be designed to accommodate the differences between species and tissue sites.

2.2. Matching normal tangent stiffness up to peak IVFs with a safety factor

Using the rabbit central PT defect model, we concluded that any TEC repair must match the tangent stiffness of normal PT up to the peak IVFs but also incorporate a safety factor to account for potential overloading during more strenuous activities. Working towards this goal, we strategically improved our repairs by optimizing the cell density, scaffold material, and mechanical preconditioning of the TEC in culture before implantation. Not only did we
generate 12-week repairs of the central PT that exceeded peak IVFs recorded in this model, but we also matched the tangent stiffness of the normal tendon up to 50% beyond peak IVFs (Fig. 5).

![Graph showing mechanical properties of different repair methods including natural healing (NH), Sponge-Cell-Stimulated (SCS), Gel-Sponge-Cell (GSC), Sponge-Cell (SC), Gel-Sponge (GS), with NH showing significantly inferior biomechanical outcomes.](image)

**Fig. 5:** Our laboratory's iterative efforts to improve tendon repair in a rabbit central patellar tendon defect model using a functional tissue engineering approach. Natural healing (NH) resulted in significantly inferior biomechanical outcomes when compared to the normal uninjured tendon (N). Over the next decade, we developed tissue-engineered constructs (TECs) for augmenting tendon healing by incorporating cells, collagen scaffolds and in vitro tensile stimulation. Our best TEC repair (denoted as SCS) consisted of a collagen sponge, a mesenchymal stem cell population, and in vitro tensile stimulation, achieving 32% of normal patellar tendon failure force and exceeding tangent stiffness for activities of daily living. Adapted with permission from[101].

3. Need for developing biological success criteria

While previous studies have shown our TECs produce an improved mechanical repair outcome compared to natural healing, we still have not fully restored normal tendon structure and function in the following two ways:
Repair tissue does not match tangent stiffness up to at least 40% of failure force, which corresponds to the highest measured in vivo loads we have recorded \(^{110}\). While matching tangent stiffness up to 32% of failure force is 50% above the peak IVFs recorded in the rabbit PT, there is still room for improvement and focusing on mechanics alone may be insufficient to reach this goal.

*We do not adequately understand the biological mechanisms which lead to the successful repairs seen previously.* There are several questions that still remain which may help explain these results. Specifically, what was the cell phenotype at the time of harvest, after preconditioning in culture, and following repair? How was the matrix assembled during the repair process and how does its composition change over time? How do interactions between cells, matrix and the local mechanical environment lead to regional differences in tissue properties? These are all questions that would not only help explain these results but also provide potential predictive success criteria for future experiments.

Ultimately, tendon repairs must be able to withstand in vivo mechanical forces to be truly considered a clinical success. Successful mechanical outcomes are, in turn, a function of an ordered sequence of appropriate biological processes. Therefore, we believe it is critical for the field to begin establishing biological parameters which lead to mechanical and clinical benchmarks of successful repair. To address these shortcomings, we propose adapting the FTE paradigm to establish biological success criteria.

4. Strategy to develop biological success criteria

We have previously discussed how our lab has used FTE principles to create mechanical success criteria by (1) measuring normal in vivo tendon forces, (2) selecting mechanical parameters based on sub-failure tendon mechanics, and (3) prioritizing a subset of these
parameters to establish mechanical success criteria for functional assessment of our tissue-engineered repairs. We can now use this same approach to generate biological success criteria for tendon repair. First, we must define what constitutes the normal biological properties of tendon. Second, we need to identify biological parameters from the numerous available candidates that are critical to tendon function. Finally, we should assess how deviations in these biological parameters affect mechanical outcomes in order to prioritize which biological properties are crucial for successful tendon repair (Fig. 6).

**Fig. 6:** Our strategy to establish mechanical and biological success criteria for functional assessment of tissue-engineered tendon repairs. Building on the FTE paradigm we used to develop mechanical design criteria, we now seek to establish biological design criteria to more fully characterize these repairs. Our strategy is to investigate the normal biological processes responsible for tendon development and maturation, identify and categorize the biological parameters that may be important, and finally, assess how these chosen biological parameters affect the mechanical outcomes of tissue repair. This approach can be applied to other tissue systems and injury models to begin developing connections between the biological processes and the mechanical outcomes.
Mature tendon cells (tenocytes) express necessary transcription factors and signaling ligands to maintain their phenotype and synthesize extracellular matrix proteins which form the scaffolding for the living tendon tissue. The cellular phenotype and extracellular matrix composition varies along the tendon length, generally dividing the tissue into a myotendinous junction, tendon midsSUBSTANCE and enthesis 160, 161, thereby producing regional variations in mechanical properties 162-164. Such compositional variation necessitates that we define normal tendon biological parameters based upon the (1) cellular phenotype, (2) extracellular matrix, and (3) regionalization of these two parameters within the tissue ultra-structure.

Understanding tendon development is critical to defining what makes a “tendon a tendon” and allows for the identification of markers which are required for proper tendon formation and potentially necessary for successful repair following injury. While many potential biological properties could be used to establish success criteria for tendon repair, prioritizing these criteria is essential to improving the efficiency and effectiveness of the process. Our lab has been using a strategy of comparing normal tendon development to natural healing to identify potential targets to modulate in future repair studies. Here we introduce three biological success criteria relative to the repair’s cellular phenotype, extracellular matrix and tendon ultra-structure, which we believe are essential for normal tendon function (Fig. 7).

5. Comparing normal development and natural healing to select biological design criteria

5.1. Cellular phenotype: scleraxis-expressing cells

The developmental biology field has begun to identify mechanisms leading to normal tendon development, namely transcription factors which aid in characterizing cell phenotype and function to regulate the expression of other important tendon genes, such as extracellular matrix proteins. Scleraxis 53, 165-167, mohawk homeobox 55, 56 and early growth response 1 168, 169 are
three of the more extensively studied tenogenic transcription factors. All have been shown to be important regulators of normal tendon development and maturation, as loss of expression of these markers results in phenotypes ranging from severely impaired tendon function in load-bearing tendons 166 to abnormalities in collagen fibrillogenesis and impaired biomechanical properties 55, 56, 168, 169. Moreover, spatial variations in the expression of these transcription factors can alter the development of the tendon enthesis. For example, a chondrogenic-tenogenic progenitor pool of cells resides at the enthesis during embryonic development. These cells dually express scleraxis (Scx) and the chondrogenic transcription factor SRY-box containing gene 9 (Sox9), which give rise to the fibrocartilage in the enthesis 170, 171. Scx-expressing progenitors are required for enthesis formation, as Scx knockout mice exhibit impaired bone tuberosity formation 172. Furthermore, conditionally knocking out Sox9 or disrupting chondrogenic signaling pathways in Scx-expressing cells also results in defective enthesis formation in limb tendons 31, 171.

Of the aforementioned markers, Scx appears to be the most critical regulator of tendon formation known to date. Scx expression defines tendon progenitors during limb condensation 53, 165, and it continues to be expressed in mature tenocytes (Fig. 7). It regulates expression of other tenogenic markers including type I collagen 173, tenascin-C 53, 174, 175, and tenomodulin 175. Its expression during development is so critical that knocking it out results in disorganized and reduced collagen content and a loss of function in many axial and limb tendons 166. However, its role in tendon healing and repair are only beginning to be clarified.

Scleraxis expression during tendon healing exhibits distinct spatiotemporal patterns. Although Scx remains slightly down-regulated during early stages of tendon natural healing (compared to normal tendon) 176-178, its expression increases in the tendon callus during later
stages of healing \textsuperscript{178}. Furthermore, mechanical loading of the healing tendon during late-stage remodeling can further increase Scx expression \textsuperscript{177}. Interestingly, Scx expression during healing appears to originate from a paratenon source of progenitors \textsuperscript{176}. These cells migrate to the wound site then express Scx as the tenascin-rich provisional matrix transitions to a collagenous matrix. While direct correlations with biomechanical properties have yet to be made, increased Scx expression during remodeling appears to be commensurate with increased biomechanical properties \textsuperscript{26,178}.

Scx expression is necessary, but certainly not sufficient, for the formation of mechanically functional tendon. Although Scx has a role in tendon development and healing, it is also up-regulated in the fibrotic response of many other tissues \textsuperscript{179-181}. As fibrotic scar is generally characterized by disorganized collagen assembly, biological success criteria should include an organized and competent extracellular matrix to give rise to improved tendon mechanical properties.

5.2. Extracellular matrix: collagen organization and alignment

Tendons are collagenous tissues composed predominantly of collagen type I \textsuperscript{182}, along with lesser amounts of collagen types III \textsuperscript{183}, V \textsuperscript{184,185}, VI \textsuperscript{186,187} and X \textsuperscript{188}, and additional matrix proteins including proteoglycans, such as decorin \textsuperscript{49} and lumican \textsuperscript{189}. Hierarchical in nature, the tendon matrix is composed of collagen microfibrils and fibrils, which aggregate to form fibers. Given their highly organized structure, tendons exhibit high tensile strength, resisting loading in the axial direction, vital to normal tendon function in the body \textsuperscript{60,190}.

The complex process through which collagen fibrils are processed, assembled, and organized is known as collagen fibrillogenesis \textsuperscript{191,192}. Collagen fibrillogenesis is highly regulated during tendon development, involving the interaction of integrins, collagens, and collagen
binding proteins. Early in development, small diameter fibrils predominate. As the tendon grows and begins to experience loading, collagen fibrils become longer and larger in diameter, resulting in a matrix that assumes a bimodal distribution of both large ($100–150$ nm) and small ($40–75$ nm) collagen fibrils to form the mature tendon. The larger diameter fibrils are thought to resist tensile loading and the smaller diameter fibrils to negate creep and improve fibril binding strength. Proteoglycans, such as decorin, biglycan, fibromodulin, and lumican, also play a key role in collagen fibrillogenesis by regulating collagen fibril interactions and ultimately tendon mechanics.

Researchers disagree about why bimodal fibril distributions are not restored after injury. Some attribute the inferior mechanical properties to an altered collagen fibrillogenesis process, with a predominance of small diameter fibrils that never form large diameter fibrils. Others contend it may not be the size of the fibrils, but their total number and/or packing density that ultimately affects the mechanical outcome. Regardless, natural tendon healing typically results in scar tissue formation, consisting of small collagen fibrils that are not oriented along the direction of loading. Further complicating the healing process and rehabilitation is the formation of adhesions between tendons and other structures. Tendon adhesions generally affect intrasynovial flexor tendons, such as in the finger, and can reduce joint range of motion, often leading to pain and discomfort.

Given that tendons are highly loaded structures vital to skeletal and joint movement, understanding their matrix composition is vital to developing effective tissue-engineered repairs. While an aligned collagen matrix is necessary for normal mechanical function, the composition of the matrix varies along the length of the tendon, producing regional variations in mechanical properties. Therefore, as we develop biological success criteria for tendon repair, we must also...
consider regional differences in the tissue ultra-structure.

5.3. Tissue ultra-structure: zonal fibrocartilaginous enthesis

Tendons provide the physical linkages between muscle and bone and serve to transmit muscular forces to the skeleton. Thus, a functional tendon actually consists of three specialized tissue regions: the myotendinous junction, the tendon midsubstance, and the tendon-to-bone insertion site (enthesis).

The enthesis is particularly critical for proper mechanical function because it facilitates force transmission between the compliant tendon and the much stiffer bone while also ameliorating potentially damaging stress concentrations that would otherwise accumulate at this interface. Uninjured entheses exhibit gradations in cell phenotype, biochemical composition, matrix organization, and mineral distribution along their length. The enthesis contains rounded fibrochondrocytes and large amounts of type II and X collagen as well as proteoglycans and glycoproteins such as aggrecan, biglycan, and tenascin C. Due to differences in collagen fiber organization and crimp pattern, local strains near the insertion are often 2–3 times greater than in the tendon midsubstance under sub-failure tensile loads.

As discussed previously, the transcription factors Scx and Sox9 appear to be critical for enthesis formation. Recent evidence also indicates that Indian hedgehog (IHH) signaling is involved in enthesis differentiation, and knocking out this pathway in Scx-expressing cells during development results in morphologic and biomechanical deficits that persist into adulthood. However, even if the correct biological cues are in place, experiments using botox to inhibit muscular contraction have shown that muscle loading is also required for normal enthesis formation. In fact, the presence of mature fibrocartilage at the enthesis is correlated with increased compressive loads in this region, indicating that both biologic and mechanical

47
factors are important players in enthesis development and maturation.

Unfortunately, once damaged, the complex structure of the enthesis is not regenerated during natural healing. Deposition of disorganized scar tissue at the healing tendon–bone interface and the absence of a morphologically normal fibrocartilage transition region results in altered biomechanical properties and premature failures at the enthesis. Thus, restoring the fibrocartilaginous interface between tendon and bone represents our third biological success criterion for tissue-engineered tendon repair.

**Fig. 7:** Biological success criteria include (1) scleraxis-expressing cells situated between (2) densely-packed collagen fibers aligned along the axis of tension and (3) a zonal enthesis with unmineralized and mineralized fibrocartilage. Toluidine blue staining (A, C, and G) and ScxGFP fluorescence (B, D, and H) in a 4-week old murine patellar tendon depict highly aligned tendon fibroblasts within the tendon midsubstance and stacked, rounded cells within the zonal insertion site that are predominately ScxGFP+. Two photon images of tendon midsubstance (E and F) and enthesis (I and J) depict highly-aligned, densely-packed collagen fibers (second harmonic generation signal in blue and gray) within the tendon midsubstance that extend through the enthesis into the underlying bone. The tidemark (red dotted line) indicates the junction between the unmineralized and mineralized fibrocartilage. Scale bars = 100 μm.
6. Discussion

Establishing a set of biological success criteria to accompany the mechanical design goals of tendon repair would greatly benefit both the clinical and tissue engineering communities. The idea of creating biological design standards for engineered tissues is not new, but linking structure to function in biological systems remains a significant challenge. This is certainly true in the case of tendon healing and repair as the complexity of these processes makes it difficult to parse out which biological criteria make the largest contributions to tendon mechanics and long-term repair outcomes. To address this problem, we have defined aspects of normal tendon biology relative to cellular phenotype, extracellular matrix and tissue ultra-structure that we would like to reproduce in our tissue-engineered repairs. We are prioritizing these biological criteria by examining their role and relative importance during natural tendon formation and tendon healing. In future tissue engineering studies, we will assess whether achieving these chosen biological criteria improves repair biomechanics.

Although straightforward in principle, this strategy does have challenges. (1) Many aspects of normal tendon biology are not well understood with other biological markers of normal tendon function still waiting to be discovered. Even very fundamental questions such as “what defines a tenocyte?” are still being debated. (2) Tendons are dynamic tissues whose biologic proper-ties depend on anatomical and mechanical cues, which adds a layer of complexity to our strategy. (3) From a clinical perspective, patient factors such as age, gender, disease, and activity level affect normal tendon properties, making it even more difficult to establish a set of biological success criteria that would be broadly applicable. (4) Prioritizing biological criteria is currently a rather subjective process since few published studies have linked a particular biological criterion with a functional mechanical outcome. We chose to
compare and contrast normal tendon development (a model of successful tendon formation) with inadequate natural healing. We reasoned that biological criteria present during normal development but absent in natural healing might be effective initial targets to modulate during repair. However, this approach to prioritizing biological success criteria is predicated on the assumption that successful tendon healing should spatially and temporally resemble normal tendon development, which may not be the case.

While our approach represents one possible strategy for establishing a set of biological success criteria for tendon repair, other approaches should be investigated. (1) Recent evidence indicates that some animal models, such as the Murphy Roth's Large (MRL) mouse, display “super healing” capabilities 126. Uncovering biological differences between super healers and normal healers may reveal new biological criteria that significantly affect tendon repair. (2) Studies investigating the underlying genetic and/or structural differences between patients with good and poor surgical outcomes could identify biological criteria most highly correlated with clinical success.

Establishing clear linkages between biology and mechanics in tissue-engineered tendon repairs will require well-designed and appropriately controlled studies that isolate specific biological criteria and quantify their effects on mechanical repair outcomes. However, such studies are inherently difficult because biological criteria are often qualitative in nature and may be difficult to measure. Biological criteria also vary widely across both spatial and temporal scales. Small, early changes in one biological criterion (for example, expression of a certain transcription factor) may result in drastic changes in mechanics later on, but these correlations are hard to detect. Furthermore, biological processes are often interrelated and many compensatory mechanisms are activated if normal biology is altered in any way, making it
difficult to isolate the effects of a single factor. Finally, establishing biological and mechanical homology across species is challenging but will be required if novel tendon repair strategies are to be translated towards the clinic. This is a particularly difficult task for functional tissue engineers, since investigators use different injury models and assess different mechanical repair outcomes at different time points.

7. Conclusion

The tissue engineering field needs to develop better strategies and adopt more unified approaches for the identification, prioritization, and evaluation of biological success criteria for tissue repair. Our laboratory has developed a general strategy in which we: (1) identify and categorize biological parameters of normal tendon based on cellular phenotype, extracellular matrix and tissue ultra-structure; (2) select a subset of biological parameters by examining their relative importance during both normal development and natural healing; and (3) prioritize these parameters by experimentally assessing whether they affect mechanical outcomes in a tissue engineering scenario. This paradigm, although presented here only in the context of tendon repair, could be applied to any load-bearing tissue in the body. We have begun using this strategy to select specific biological success criteria critical to normal tendon function. In our view, a successful tendon repair must not only meet the stated mechanical design limits but also exhibit (1) scleraxis-expressing cells embedded within (2) a well-organized and axially-aligned collagen matrix that is (3) securely attached to bone via a fibrocartilaginous enthesis as a protection against long term failure. Future tissue engineering studies linking these biological success criteria with improved in vivo mechanical outcomes will be critical for validating the utility of this approach. Ultimately, understanding which biological criteria are most predictive of
successful mechanical outcomes will expedite and streamline the tissue engineering process and result in improved treatments for tendon injuries.

Acknowledgments

The authors would like to acknowledge funding from NIH grants R01-AR056943-05, T32-GM063483, R01-AR54713, R01-AR052374, and T90-DE021989 and the NSF IGERT training program 333377. The authors would like to thank Drs. Cy Frank (U. of Calgary), Richard Brand (OREF) and Jack Lewis (U. of Minnesota) for stimulating discussion regarding the strategy proposed in this paper. The authors also want to recognize the many other collaborators over the past 36 years who have contributed to aspects of this work. These individuals include bioengineering colleagues at the University of Cincinnati (Drs. Hani Awad, Aditya Chaubey, Kumar Chokalingam, John Cummings, Matthew Dressler, Edward Grood, Bala Haridas, John Holden, Shawn Hunter, Natalia Juncosa-Melvin, Sanjit Nirmalananandhan, Frank Noyes, and Donald Stouffer, as well as David Glos, Matthew Harris, and John West); veterinary and human surgery colleagues in Cincinnati (Drs. Greg Boivin, Chris Casstevens, Marc Galloway, Brian Grawe, Michael Greiwe, Samer Hasan, Namdar Kazemi, Keith Kenter, and Donna Korvick); researchers at Cincinnati Children's Hospital (Lindsey Aschbacher-Smith, Jane Florer, Chris Frede, and Drs. Chia-Feng Liu, Richard Wenstrup, Rulang Jiang, Han Liu, and Christopher Wylie); bioengineering, biological, and design collaborators and co-authors in the musculoskeletal field (Ms. Mary Beth Privitera and Drs. Al Banes, Arnold Caplan, David Fink, Steve Goldstein, Steve Gordon, Farsh Guilak, Peter Maye, Van Mow, David Mooney, Heather Powell, David Rowe, Jeff Ruberti, Ronen Schweitzer, Randall Young, and Savio Woo).
Chapter 4

Improved Biomechanical and Biological Outcomes in the MRL/MpJ Murine Strain Following a Full-Length Patellar Tendon Injury

Andrea L. Lalley, B.S.\textsuperscript{a}, Nathaniel A. Dyment, Ph.D.\textsuperscript{b}, Namdar Kazemi, M.D.\textsuperscript{c}, Keith Kenter, M.D.\textsuperscript{c}, Cindi Gooch, B.S.\textsuperscript{a}, David L. Butler, Ph.D.\textsuperscript{a}, Jason T. Shearn, Ph.D.\textsuperscript{a}

\textsuperscript{a}Biomedical Engineering Program, College of Engineering and Applied Science, University of Cincinnati, Cincinnati, Ohio

\textsuperscript{b}Department of Reconstructive Sciences, College of Dental Medicine, University of Connecticut Health Center, Farmington, Connecticut

\textsuperscript{c}Department of Orthopaedic Surgery, College of Medicine, University of Cincinnati, Cincinnati, Ohio

\textsuperscript{1}This manuscript is currently in preparation for the Journal of Orthopaedic Research
ABSTRACT

Musculoskeletal injuries affect a large portion of the U.S. population and current clinical treatment strategies fail to restore long-term native tissue structure and function. Tissue engineering is an approach being used to improve the tendon healing process; however, the field still needs to establish biological benchmarks to assess the effectiveness of tissue-engineered structures. Investigating regenerative models, such as the MRL/MpJ, offers the ability to understand successful models of healing and apply these findings to tissue-engineered therapies. This study seeks to evaluate the MRL/MpJ’s healing response following a central patellar tendon injury compared to a wildtype control. Gene expression and histology were assessed at 3, 7, and 14 days following injury and mechanical properties were measured at 2, 5, and 8 weeks. Native patellar tendon biological and mechanical properties were not different between the strains. Following injury, the MRL/MpJ displayed increased structural and material properties between 5 and 8 weeks; however, tenogenic expression patterns were not different between the strains at early time points. Furthermore, expression of the cyclin-dependent kinase inhibitor, p21, was not different between the strains, suggesting an alternative mechanism may be responsible for the healing process. Future studies will investigate the collagen structure and alignment at 5 and 8 weeks and further characterize the healing transcriptome at both early and later time points to elucidate the mechanisms driving the MRL/MpJ’s improved healing process.
INTRODUCTION

Musculoskeletal injuries, particularly to tendons and ligaments, are frequent and present a large burden on the U.S. economy\(^1\),\(^{150}\). While current treatment strategies often accelerate a patient’s return to pre-injury activity levels through direct repair or replacement of the damaged tissue, long-term outcomes lead to increased rates of re-injury or the development of chronic conditions, such as osteoarthritis\(^15\),\(^{229}\). Developing new therapeutic strategies is needed to improve the shortcomings associated with current clinical practices.

To better design effective treatment options for tendon and ligament injuries, investigating the natural tendon healing process offers insight towards identifying inadequacies associated with the body’s response. Unfortunately, the natural tendon healing process produces scar tissue, shown to be biomechanically inferior to native, uninjured tissue up to one year post-injury in a sheep Achilles tendon rupture model\(^27\), 26 weeks post-injury in a rabbit patellar tendon (PT) model\(^18\), and 8 weeks post-injury in a murine PT defect model\(^26\).

Tissue engineering has proved to be a promising approach towards improving the tendon healing process; however, previous attempts have focused on meeting the mechanical demands of the tissue as established by the functional tissue engineering paradigm\(^17\). While these results show promise, the tissue engineering field needs to establish biological success criteria to benchmark future therapies as described in Chapter 3. By investigating successful models of tendon healing, we can begin to identify novel biological pathways regulating the process. Understanding modes of adult, scar-free healing may provide valuable information that can be used to stimulate natural healing processes to achieve successful tissue formation following injury.
Tissue regeneration is limited in the mammalian kingdom; however, the Murphy Roths Large (MRL/MpJ) murine strain has been identified as a potential model of successful mammalian healing following injury. Originally created to investigate systemic lupus erythematosus, this mouse was found to repair identifying ear hole punches via the development of a blastema and subsequent reformation of sebaceous glands, hair follicles, and aligned collagen[126]. Further studies have shown regenerative-like healing in other tissues including cornea[105], heart[128], spinal cord[131, 230], and articular cartilage[129], although with varying degrees of healing. In some cases, the MRL/MpJ healing proved no better than the wild type response[132, 133], suggesting the response may be dependent on the type or severity of the induced injury. Currently, the field is divided on what is driving the MRL/MpJ healing phenotype. Through genetic analysis, mutations were found to be distributed over 20 different loci on 7 chromosomes[135], suggesting multiple factors may be involved in the phenotype making it difficult to isolate one particular pathway or target of interest for study.

Several investigators suggest the phenotype may be a result of a mutation causing decreased expression of the cyclin-dependent kinase inhibitor, p21[136-138]. This molecule is a downstream effector of the tumor suppressor, p53, acting at the G1 checkpoint to prevent cell cycle progression to S phase in the event of DNA damage[137]. Several studies investigating the MRL/MpJ healing process have shown p21 to be expressed below normal levels both in cells derived from non-injured tissue and in cells participating throughout the in vivo healing process[136-138]. Despite this work, other researchers suggest the healing capacity may not be a result of a p21 deficiency at all, rather a combination of decreased inflammatory and immunological responses[105] or retention of embryonic-like molecular features[141]. It could be that different molecular pathways are responsible for the healing phenotypes for varying tissue types;
however, more investigation is needed to identify the actual mechanism driving the MRL/MpJ healing process.

Given the poor natural healing outcomes following tendon injury, investigating the MRL/MpJ healing response offers the opportunity to characterize a superior form of tendon healing. Driving tissue-engineered repairs down a regenerative-like pathway, as opposed to a natural healing pathway, may be key in restoring native tissue structure and function. Thus, the objective of this study was to evaluate the healing potential of the MRL/MpJ murine strain in response to a full-length, full-thickness central PT injury compared to C57BL/6 controls based on biomechanical, biological, and morphological outcome measures. We hypothesized the MRL/MpJ would exhibit 1) increased expression of tendon-related genes, 2) decreased expression of p21, and 3) improved biomechanical outcomes at time points post-injury compared to the C57BL/6 strain.

RESULTS

Native Patellar Tendon Properties of C57BL/6 versus MRL/MpJ

We first compared the mechanical and biological properties of native C57BL/6 and MRL/MpJ patellar tendon tissue to determine any innate differences. Gross observations showed similar overall PT appearance between the two strains including well-defined PT medial and lateral borders, characteristic white collagen color and vibrancy, and comparable PT dimensions. The MRL/MpJ had significantly larger intact PT width (1.22±0.26 vs. 1.00±0.11, p=0.009) and length (3.07±0.11 vs. 2.95±0.11, p=0.013) compared to the C57BL/6; however, the tested MRL/MpJ and C57BL/6 PT widths (0.55±0.05 vs. 0.58±0.03, p=0.052) and thicknesses (0.45±0.03 vs. 0.47±0.06, p=0.364) were not different between the two strains and subsequently, there were no significant differences observed for cross-sectional area (p=0.065).
To determine any underlying genetic differences related to the tenogenic phenotype between the two strains, we performed qPCR to assess expression of a number of tendon-related genes, including transcription factors and extracellular matrix collagens, proteoglycans, and glycoproteins. Based on findings reported in the literature, we also evaluated expression of the cyclin-dependent kinase inhibitor, p21, and Myc, a known inhibitor of p21.

Overall, the genetic results showed minimal differences among expression levels of the measured genes of interest (Table 1). Cₜ values were reported normalized to 18S. The MRL/MpJ tissue showed slightly elevated collagen type I expression compared to C57BL/6 (p=0.048) with no other tenogenic markers showing significant expression differences (p>0.05). Interestingly, p21 expression between the two strains was not different (p=0.422).

Table 1. Delta Cₜ values for genes of interest for C57BL/6 and MRL/MpJ native patellar tendon normalized to 18S (mean±SD)

<table>
<thead>
<tr>
<th>Gene</th>
<th>C57BL/6 Native</th>
<th>MRL/MpJ Native</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col1a1</td>
<td>8.4±0.6</td>
<td>9.7±0.6</td>
</tr>
<tr>
<td>Col3a1</td>
<td>11.4±1.1</td>
<td>12.0±0.8</td>
</tr>
<tr>
<td>Dcn</td>
<td>10.4±0.4</td>
<td>11.3±0.5</td>
</tr>
<tr>
<td>Egr1</td>
<td>19.2±1.0</td>
<td>20.1±0.1</td>
</tr>
<tr>
<td>Fmod</td>
<td>12.2±0.1</td>
<td>13.4±0.7</td>
</tr>
<tr>
<td>Mkx</td>
<td>17.8±0.4</td>
<td>18.6±0.7</td>
</tr>
<tr>
<td>Scx</td>
<td>16.1±0.1</td>
<td>16.3±0.4</td>
</tr>
<tr>
<td>Tnc</td>
<td>15.7±1.9</td>
<td>17.4±1.5</td>
</tr>
<tr>
<td>Tnmd</td>
<td>14.4±0.6</td>
<td>14.8±0.9</td>
</tr>
<tr>
<td>Myc</td>
<td>18.8±0.5</td>
<td>19.2±0.2</td>
</tr>
<tr>
<td>p21</td>
<td>17.5±0.6</td>
<td>17.9±0.3</td>
</tr>
</tbody>
</table>

*significantly different than C57BL/6 native PT

Structural and material properties were recorded following tensile failure testing. There were no significant differences observed for ultimate load (p=0.781), linear stiffness (p=0.582), maximum stress (p=0.409), or elastic modulus (p=0.052) as shown in Table 2 and Fig. 8.

Table 2. Structural and material properties for C57BL/6 and MRL/MpJ native patellar tendon (mean±SD)

<table>
<thead>
<tr>
<th>Property</th>
<th>C57BL/6 Native</th>
<th>MRL/MpJ Native</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-Sectional Area (mm²)</td>
<td>0.28±0.04</td>
<td>0.25±0.03</td>
</tr>
<tr>
<td>Ultimate Load (N)</td>
<td>4.44±0.75</td>
<td>4.31±1.46</td>
</tr>
<tr>
<td>Linear Stiffness (N/mm)</td>
<td>9.76±1.30</td>
<td>10.22±2.65</td>
</tr>
<tr>
<td>Max Stress (Mpa)</td>
<td>16.20±2.72</td>
<td>17.85±6.40</td>
</tr>
<tr>
<td>Modulus (Mpa)</td>
<td>105.30±20.11</td>
<td>126.96±30.93</td>
</tr>
</tbody>
</table>
Fig. 8: Load-displacement failure curves for native 20-week old C57BL/6 and MRL/MpJ patellar tendon. There were no significant differences in structural properties between the two strains (p>0.05). Error bars represent SEM.

In summary, the C57BL/6 and MRL/MpJ native tendon tissues were similar in tissue size and morphology, structural and material properties, and showed comparable tenogenic expression profiles. Further, p21 expression was not different between the two strains. We next conducted an experiment to evaluate the capacity of the MRL/MpJ tendon healing process by creating full-length, full-thickness central PT defects.

**Tenogenic Expression Profiles are Not Different Between MRL/MpJ and C57BL/6 at Early Post-Surgical Time Points**

The strain of the mouse did not have an effect on gene expression (p>0.05); however, time did significantly affect expression (p<0.05). There were no statistical differences between either strain for the measured genes of interest at any time point. Delta C_T values are reported normalized to 18S expression (Table 3).
Collagens

For the C57BL/6 strain, Col1a1 expression peaked at day 7 but returned to normal levels by day 14. Col3a1 expression levels were elevated at 3, 7, and 14 days following injury, peaking at day 7. The MRL/MpJ showed a similar trend for collagen expression levels; however, Col1a1 expression remained elevated at day 14.

Proteoglycans and Glycoproteins

At day 3, Dcn, Fmod, and Tnmd expression were decreased in both strains compared to their respective native controls (p<0.05). By day 7, Dcn, Fmod, and Tnmd expression returned to normal levels in the MRL/MpJ. In the C57BL/6, Dcn expression was no different than normal at day 7, but was decreased at day 14. Fmod expression was decreased at day 7, but returned to normal levels by day 14, while Tnmd expression returned to normal levels at day 7. Tenascin-C expression was elevated in the MRL/MpJ (p<0.05) at all time points, but only at day 7 for C57BL/6.

Transcription Factors

Egr1 expression was not altered following injury in either strain (p>0.05). Mkx and Scx expression levels were similar in both strains, showing decreased expression at day 3 (p<0.05), but a return to normal levels by day 7.

p21 Mechanism

p21 showed increased expression for both strains at the day 3 timepoint (p<0.05) and remained elevated in the MRL/MpJ strain at all time points, while returning to normal levels in the C57BL/6 by day 7. Myc is a basic helix-loop-helix leucine-zipper transcription factor known to function as a natural inhibitor of p21\textsuperscript{140} and exhibited similar expression levels between the
two strains with increased expression at 3 and 7 days post injury and a return to normal expression levels by day 14.
Table 3: Delta Ct values for genes of interest measured in C57BL/6 and MRL/MpJ defect tissue normalized to 18S

<table>
<thead>
<tr>
<th></th>
<th>Col1a1</th>
<th>Col3a1</th>
<th>Dcn</th>
<th>Egr1</th>
<th>Fmod</th>
<th>Mkx</th>
<th>Scx</th>
<th>Tnc</th>
<th>Tnmd</th>
<th>Myc</th>
<th>p21</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C57BL/6</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>8.4±0.6</td>
<td>11.4±1.1</td>
<td>10.4±0.4</td>
<td>19.2±1.0</td>
<td>12.2±0.1</td>
<td>17.8±0.4</td>
<td>16.1±0.1</td>
<td>15.7±1.9</td>
<td>14.4±0.6</td>
<td>18.8±0.5</td>
<td>17.5±0.6</td>
</tr>
<tr>
<td>D3 Defect</td>
<td>8.5±0.1</td>
<td>10.1±0.1a</td>
<td>12.7±0.3a</td>
<td>18.9±0.6</td>
<td>17.8±0.9a</td>
<td>21.7±0.9a</td>
<td>17.5±0.8a</td>
<td>14.3±0.2</td>
<td>18.3±0.9a</td>
<td>17.2±0.3a</td>
<td>16.1±0.4a</td>
</tr>
<tr>
<td>D7 Defect</td>
<td>6.5±0.5a</td>
<td>8.4±0.5a</td>
<td>11.4±0.8</td>
<td>19.2±1.0</td>
<td>14.3±1.2a</td>
<td>19.0±1.5</td>
<td>15.5±0.7</td>
<td>13.6±0.7a</td>
<td>14.4±1.2</td>
<td>17.5±0.8a</td>
<td>16.7±0.6</td>
</tr>
<tr>
<td>D14 Defect</td>
<td>7.4±1.5</td>
<td>9.9±0.8a</td>
<td>11.3±0.5a</td>
<td>19.1±0.78</td>
<td>13.4±1.4</td>
<td>19.1±0.9</td>
<td>16.2±1.5</td>
<td>15.3±1.6</td>
<td>13.8±1.7</td>
<td>19.6±1.2</td>
<td>17.3±0.6</td>
</tr>
<tr>
<td><strong>MRL/MpJ</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>9.7±0.6</td>
<td>12.0±0.8</td>
<td>11.3±0.5</td>
<td>20.1±0.1</td>
<td>13.4±0.7a</td>
<td>18.6±0.7</td>
<td>16.3±0.4</td>
<td>17.4±1.5</td>
<td>14.8±0.9</td>
<td>19.2±0.2</td>
<td>17.9±0.3</td>
</tr>
<tr>
<td>D3 Defect</td>
<td>8.6±0.2</td>
<td>10.0±0.3a</td>
<td>13.8±0.5a</td>
<td>19.8±1.2</td>
<td>18.7±1.8a</td>
<td>21.8±0.9a</td>
<td>18.1±1.0a</td>
<td>13.7±0.4a</td>
<td>19.1±1.8a</td>
<td>17.1±0.4a</td>
<td>16.3±0.1a</td>
</tr>
<tr>
<td>D7 Defect</td>
<td>7.0±0.7a</td>
<td>8.3±0.8a</td>
<td>11.9±1.2</td>
<td>18.9±0.9</td>
<td>14.7±1.1</td>
<td>19.2±1.3</td>
<td>16.1±0.8</td>
<td>13.3±0.7a</td>
<td>14.1±1.0</td>
<td>17.7±0.7a</td>
<td>16.5±0.9a</td>
</tr>
<tr>
<td>D14 Defect</td>
<td>7.4±0.5a</td>
<td>9.0±0.8a</td>
<td>11.9±0.9</td>
<td>18.6±1.2</td>
<td>13.4±0.5</td>
<td>18.9±0.7</td>
<td>16.4±0.5</td>
<td>14.6±0.6a</td>
<td>13.1±0.7</td>
<td>18.7±1.0</td>
<td>16.7±0.8a</td>
</tr>
</tbody>
</table>

*a*significantly different than respective native PT (p<0.05)
MRL/MpJ Repair Tissue Shows Increased Cellular Proliferation Compared to C57BL/6

But Is Not a Result of a p21 Deficiency

At 3 days post injury, proliferating cells were present within the tendon healing region, with no differences in proliferation between the two strains (p=0.41). At 7 and 14 days following injury, the MRL/MpJ showed increased cellular proliferation (p=0.002 and p=0.037, respectively) compared to the C57BL6, reaching 14.1% and 4.8%, respectively (Fig. 9).

Fig. 9: The MRL/MpJ shows increased cellular proliferation compared to C57BL/6 (p<0.05) at 7 and 14 days in the tendon healing region as measured by the number of EdU positive cells normalized to total cell number. The images are representative of the defect region for C57BL/6 and MRL/MpJ 7 days following injury (DAPI-Blue, EdU-Yellow). S: Strut, D: Defect. Error bars indicate SD.

The cyclin-dependent kinase inhibitor, p21, functions at the G1 checkpoint to prevent cell cycle progression in the event of DNA damage. Previous findings reported in the literature suggest a mutation resulting in reduced expression of p21 may be a contributor to the healing phenotype. We measured p21 expression following injury in the C57BL/6 and MRL/MpJ at 3,
7, and 14 days post injury. As discussed previously, p21 expression was not different between the C57BL/6 and MRL/MpJ native tissues. Interestingly, there was no difference in p21 expression between the two strains at any time point investigated post injury (Fig. 10). Both strains displayed increased expression of p21 3 days following injury (p<0.05); however, expression levels were not different than native tissue at 7 or 14 days for either strain.

Fig. 10: The C57BL/6 and MRL/MpJ show similar temporal expression of the cell cycle regulator, p21, following tendon injury. At day 3, both the C57BL/6 and MRL/MpJ showed significantly increased expression of p21 compared to respective native controls (p<0.05).

The MRL/MpJ Healing Process Produces Improved Structural and Material Properties Following Tendon Injury Between 5 and 8 Weeks

Murine strain, time post surgery, and surgical treatment each significantly affected cross-sectional area, ultimate load, and linear stiffness, while only time post surgery and surgical treatment significantly affected ultimate stress and modulus (p<0.05; Table 4, Fig. 11 A and B). With respect to structural properties, both C57BL/6 and MRL/MpJ displayed reduced values compared to respective native tissue for ultimate load and linear stiffness at 2 and 5 weeks.
(p<0.05); however the MRL/MpJ displayed increased linear stiffness compared to C57BL/6 at the 2 week time point (6.50 ± 1.43N vs. 4.50 ± 0.66N, p=0.042). By 8 weeks, the MRL/MpJ had achieved 81% and 77% of native ultimate load (3.48 ± 0.92N vs. 4.31 ± 1.53N, p=0.078) and linear stiffness (7.21 ± 1.00N/mm vs. 9.31 ± 1.99N/mm, p=0.001), respectively, while the C57BL/6 reached only 41% and 59% of native ultimate load (1.82 ± 0.66N vs. 4.44 ± 0.75N, p<0.001) and linear stiffness (5.72 ± 0.85N vs. 9.76 ± 1.30N, p<0.001), respectively. Furthermore, the MRL/MpJ showed significant increases in ultimate load and linear stiffness parameters between 5 and 8 weeks (p<0.05) and were significantly greater than C57BL/6 values at the 8 week time point (p<0.05; 3.48 ± 0.92N vs. 1.82 ± 0.66N and 7.21 ± 1.00N/mm vs. 5.72 ± 0.85N/mm, respectively). The C57BL/6 structural properties plateaued, failing to improve beyond the 2 week time point (Table 4, Fig. 11, Figs.12a, b).

With respect to material properties, both the C57BL/6 and MRL/MpJ strains showed reduced values compared to respective native tissue for ultimate stress and modulus at 2, 5, and 8 weeks (p<0.05; Table 4, Fig. 11, Figs. 12c, d). While the C57BL/6 reached a plateau following the 2 week time point (p>0.05), the MRL/MpJ showed improved ultimate stress and modulus between 5 and 8 weeks (p<0.05).
Table 4: Structural and material properties for C57BL/6 and MRL/MpJ defect and contralateral sham tissues following surgery compared to age-matched native PT (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>Ultimate Stress (MPa)</th>
<th>Modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C57/BL6</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&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.10±0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.50±0.66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.80±0.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.03±6.45&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 week Sham (n=8)</td>
<td>0.28±0.02</td>
<td>4.98±1.08</td>
<td>9.61±0.83</td>
<td>18.48±7.57</td>
<td>109.57±30.11</td>
</tr>
<tr>
<td>5 week Defect (n=9)</td>
<td>0.31±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.87±0.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.74±0.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.31±2.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>49.53±15.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5 week Sham (n=9)</td>
<td>0.28±0.05</td>
<td>4.45±1.17</td>
<td>9.73±2.08</td>
<td>16.23±4.49</td>
<td>109.11±28.12</td>
</tr>
<tr>
<td>8 week Defect (n=7)</td>
<td>0.25±0.03</td>
<td>1.82±0.66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.72±0.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.24±2.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>67.63±11.62&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>8 week Sham (n=7)</td>
<td>0.25±0.03</td>
<td>4.99±0.97</td>
<td>10.61±2.20</td>
<td>20.37±3.64</td>
<td>129.45±29.26</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&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>2.91±1.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.50±1.43&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>5.33±1.64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.37±7.27&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 week Sham (n=8)</td>
<td>0.42±0.07</td>
<td>7.18±1.65</td>
<td>12.06±2.47</td>
<td>17.01±4.44</td>
<td>93.20±20.92</td>
</tr>
<tr>
<td>5 week Defect (n=10)</td>
<td>0.39±0.07&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>1.96±0.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.06±1.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.12±1.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.44±12.87</td>
</tr>
<tr>
<td>5 week Sham (n=10)</td>
<td>0.38±0.05</td>
<td>5.11±1.25</td>
<td>11.06±3.47</td>
<td>13.82±3.29</td>
<td>98.50±27.74</td>
</tr>
<tr>
<td>8 week Defect (n=10)</td>
<td>0.31±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.48±0.92&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.21±1.00&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>11.33±3.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>74.49±20.67&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>8 week Sham (n=10)</td>
<td>0.29±0.07</td>
<td>6.42±1.30</td>
<td>13.02±1.91</td>
<td>23.39±6.77</td>
<td>153.00±46.73</td>
</tr>
</tbody>
</table>

<sup>a</sup>significantly different than respective native PT (p<0.05), <sup>b</sup>significantly different to C57BL/6 defect at corresponding time point (p<0.05)
Fig. 11: Average load-displacement failure curves for repair tissue at 2, 5, and 8 weeks for the C57BL/6 and MRL/MpJ strains. (a) The C57BL/6 healing tissue was significantly inferior to native tissue in terms of structural properties at all time points following injury ($p<0.05$). (b) The MRL/MpJ healing tissue was significantly less than native tissue at 2 and 5 weeks; however, by 8 weeks, the MRL/MpJ reached native values with respect to ultimate load ($3.48 \pm 0.92\text{N}$ vs. $4.31 \pm 1.53\text{N}$, $p>0.05$). Error bars indicate SEM.
Fig. 12: Structural and material properties of defect and sham tendon tissues plotted as a percent of native for (a) ultimate load, (b) linear stiffness, (c) ultimate stress, and (d) modulus. The MRL/MpJ repair tissue exhibited improved structural and material properties between 5 and 8 weeks post surgery, while the C57BL/6 failed to display significant improvements beyond the 2 week time point. Error bars indicate SD (*significantly different with respect to time for MRL/MpJ sham tissue (p<0.05); #significantly different with respect to time for MRL/MpJ defect tissue (p<0.05).

The MRL/MpJ Shows A Reduced Immunological Response Following Injury

The MRL/MpJ mouse was originally developed to investigate systemic lupus erythematosus, an autoimmune disorder; however, it was found that the reparative capability of this animal following an ear hole punch was superior to natural healing. Previous reports suggest the MRL/MpJ exhibits abnormal immunological and inflammatory responses to injury, which has been looked to as a contributing factor to the improved healing phenotype\textsuperscript{105}. We evaluated macrophage infiltration following injury by staining for F4/80, a membrane protein found on
macrophages and microglial cells. Native C57BL/6 and MRL/MpJ tissues showed no differences in expression (Fig. 13a-b); however, compared to the C57BL/6 at corresponding time points post injury, the MRL/MpJ macrophage concentration appears to be decreased at 3, 7, and 14 days (Fig. 13). Both strains show increased macrophage presence following injury; however it was heightened in the C57BL/6, especially at the day 7 timepoint (Fig. 13e-f).

**Fig. 13:** The MRL/MpJ strain shows a decreased immune response following tendon injury. F4/80 staining showed no differences between the C56BL/6 and MRL/MpJ native tissue (a,b); however by 3 days, infiltrating macrophages are present in the paratenon, struts, fat pad, and healing region of the C57BL/6 (c) while the MRL/MpJ response is predominant in the paratenon only (d). By 7 days, macrophage infiltration peaks in the C57BL/6, but remains reduced in the MRL/MpJ (e, f). At day 14, the response has reduced in both strains, concentrated to the paratenon and fat pad (g, h). In image h, S-Strut and D-Defect.
DISCUSSION

The objective of this study was to evaluate the utility of the MRL/MpJ as a murine model of regenerative-like healing following a full-length, full-thickness central patellar tendon injury based on mechanical, histological, and biological response measures. We initially compared the native patellar tendon tissues for both the MRL/MpJ and C57BL/6 and found no differences with respect to mechanical properties or gene expression for tendon-related markers or markers in the p21 pathway. This was of particular interest because previous studies have suggested abnormal regulation of p21 is a major contributor to the healing phenotype\textsuperscript{138, 231}. Following full-length, full-thickness PT injury, the MRL/MpJ displayed enhanced cellular proliferation at 7 and 14 days; however, there were no differences in tendon-related gene expression profiles at early postsurgical time points. Further, p21 expression was not different at 3, 7, or 14 days between the strains. While the mechanical outcomes showed little difference between the two strains at 2 and 5 weeks, the MRL/MpJ showed significant improvements to structural properties between 5 and 8 weeks post injury, reaching 81% and 77% of native ultimate load and linear stiffness, respectively.

Our results suggest that the initial response to tendon injury in each of these strains is similar with respect to overall healing progression and expression of tenogenic markers (measured at 3, 7, and 14 days post injury). The differences in the healing processes did not manifest until later time points when it was observed that the MRL/MpJ showed improvements in structural and material properties between 5 and 8 weeks while the C57BL/6 reached a plateau at 2 weeks. This suggests the MRL/MpJ healing process extends beyond the 2-week time point, with continued matrix production and remodeling occurring. Studies have shown that the accumulation of tendon mechanical properties during early post-natal development takes several
months, resulting from increases in collagen content, fibril diameter mean, and proteoglycan and glycoprotein concentration\textsuperscript{42, 43, 232}. The MRL/MpJ healing process could be progressing in much the same way.

The differences in cellular proliferation and immune cell infiltration at early time points, coupled with improved mechanical outcomes at later time points, indicate an alternative mechanism is likely regulating the MRL/MpJ healing process. Questions remain as to the molecular pathways that drive the MRL/MpJ’s healing response. Answering these questions has been difficult because there has been a broad range of healing outcomes observed in the MRL/MpJ following injury in a number of tissue systems\textsuperscript{105, 129, 132, 231, 233}. Previous studies propose the MRL/MpJ healing phenotype may be a result of decreased expression of the cyclin-dependent kinase inhibitor, p21\textsuperscript{138, 231}. It is postulated that a mutation causing abnormal p21 expression produces an accumulation of cells in the G2/M phase of the cell cycle, resulting in increased cellular proliferation despite cellular DNA damage\textsuperscript{138, 231}. In the event of an injury, these cells have been shown to actively participate in the repair process\textsuperscript{138}. Further, studies investigating a p21 knockdown strain suggest a similar healing capacity to the MRL/MpJ\textsuperscript{139}. In this study, we observed increased cellular proliferation in the MRL/MpJ compared to wildtype at 7 and 14 days; however, p21 expression was \textit{increased} compared to native expression in the MRL/MpJ at these time points, and not different compared to p21 expression in the C57BL/6 at any time point. This indicates the regenerative capacity may lie in an alternative pathway unrelated to the p21 mechanism or it could be that mechanisms regulating the healing phenotype may differ depending on the type of injury and/or injury location\textsuperscript{134, 234}.

Findings from several studies investigating the MRL/MpJ mouse point to decreased inflammatory and immunological responses following injury that may be driving the healing
phenotype\textsuperscript{105, 235, 236}. In this study, we compared F4/80 expression, a membrane protein that demarcates macrophages and microglial cells, in the healing tissue between the two strains and found the MRL/MpJ showed decreased expression, suggesting the immune response may be dampened compared to the wildtype response. Based on previous reports showing improved skin repair in neutrophil-depleted mice, Ueno et al investigated corneal healing in both the MRL/MpJ strain and neutrophil-depleted mice\textsuperscript{105}. They postulated infiltrating neutrophils might slow the reepithelialization process, leading to poor healing outcomes in the wildtype animal. Both the MRL/MpJ and neutrophil-depleted mice showed enhanced reepithelialization and healing outcomes compared to wildtype; however, the MRL/MpJ’s response was accelerated compared to the neutrophil-depleted mice, suggesting the phenotype is not solely a result of a decreased immunological response\textsuperscript{105}.

Others suggest the healing phenotype may result from a retention of embryonic-like features characteristic of normal development\textsuperscript{141}. Naviaux et al found MRL/MpJ expressed the stem-cell related marker, Nanog, in uninjured heart tissue followed by a 10-fold increase in response to cryoinjury with negligible expression measured in the wildtype for both conditions. Similar expression patterns were observed between the two strains for Islet-1 and Sox2, two other pluripotential markers. Increased expression of these markers in the MRL/MpJ strain was coupled with increased aerobic glycolytic energy metabolism and decreased reactive oxidative species, processes synonymous with developmental features\textsuperscript{141}. We measured expression of scleraxis, mohawk homeobox, and early growth response 1, three transcription factors known to be involved in tendon development and maturation; however, we found no differences in expression between the two strains at any time point. We did not, however, measure expression of markers that could be upstream of these transcription factors during the developmental stages.
(i.e. Pou5f1). Future work should investigate the similarities between normal tendon development and regenerative models to develop the ultimate metric for assessing tissue engineering and repair strategies.

This study is not without limitations. 1) We only evaluated gene expression at early post-surgical time points. Future work should investigate gene expression at later time points, such as 5, 8, and 10 weeks to better understand the molecular events that are contributing to the mechanical results observed at these time points. 2) The PT injury that we utilize to investigate tendon healing is not clinically relevant. Tendon and ligament injuries occurring in the patient population are often accompanied by an underlying chronic condition, termed tendinopathy. Our murine model fails to incorporate aspects of tendinopathy making direct comparisons to the clinical condition difficult. While this is a limitation, investigating tendon injuries in a well-controlled environment minimizes potential confounding variables. Furthermore, there are very few models available that exhibit a consistent tendinopathic phenotype. Future work should investigate developing a suitable model system that faithfully recapitulates aspects of tendinopathy. Findings from this study could be applied to this future model. 3) We compared the mechanical healing outcomes to 20-week old C57BL/6 and MRL/MpJ native controls; however, a more appropriate control would have been age-matched to each of the post-surgical time points (i.e. 22-, 25-, and 28-week old mice) to account for any changes in native mechanical properties as a result of age. We selected to only test 20-week old animals because this is a mature animal and we expected minimal differences in mechanical properties as a result of age.

While it remains unclear if the MRL/MpJ is a model of true regeneration, findings from this study support further characterization of this model to identify potential pathways to target
with our tissue-engineered therapies. Understanding the MRL/MpJ healing process will allow the field to identify biological success criteria to benchmark tissue-engineered constructs and repairs.

MATERIALS AND METHODS

Experimental Design

Patellar tendon physical dimensions, structural and material properties, tissue morphology, and gene expression levels were assessed at three times points following a full-length, full-thickness central injury in 20-week old (20.6 ± 0.3 weeks; mean ± SD) male C57BL/6 mice and 20-week old (20.4 ± 0.5 weeks; mean ± SD) male MRL/MpJ mice (Table 5). Breeding pairs were obtained for both the C56BL/6 (stock number: 000664) and MRL/MpJ (stock number: 000486) strains from The Jackson Laboratory (Bar Harbor, ME) and then bred in-house to produce animals for this study. The age of the mice for this study was selected because twenty-week-old animals have reached skeletal maturity and the size of the PT permits for creating repeatable surgical injuries and post-injury testing. Following injury, natural tendon healing based on biomechanical outcomes at 2, 5, and 8 weeks (n=8-14 per time point), histology at 3, 7, and 14 days (n=2 per time point), and gene expression at 3, 7, and 14 days (n=3 per time point) was compared between the MRL/MpJ strain and C57BL/6 control strain. Inter-animal comparisons were also made to respective native, unoperated PTs from twenty-week-old MRL/MpJ and C57BL/6 mice.
Table 5. Experimental Design Comparing C57BL/6 and MRL/MpJ Mechanical and Biological Measures Following Central Patellar Tendon Injury

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time Post Surgery (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>DEFECT</td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td></td>
</tr>
<tr>
<td>Histology/IHC</td>
<td>9*</td>
</tr>
<tr>
<td>qPCR</td>
<td>7</td>
</tr>
<tr>
<td>Biomechanics</td>
<td>8</td>
</tr>
<tr>
<td>MRL/MpJ</td>
<td></td>
</tr>
<tr>
<td>Histology/IHC</td>
<td>9*</td>
</tr>
<tr>
<td>qPCR</td>
<td>8</td>
</tr>
<tr>
<td>Biomechanics</td>
<td>2</td>
</tr>
<tr>
<td>SHAM</td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td></td>
</tr>
<tr>
<td>Histology/IHC</td>
<td>2</td>
</tr>
<tr>
<td>Biomechanics</td>
<td>2</td>
</tr>
<tr>
<td>MRL/MpJ</td>
<td></td>
</tr>
<tr>
<td>Histology/IHC</td>
<td>2</td>
</tr>
<tr>
<td>Biomechanics</td>
<td>11</td>
</tr>
</tbody>
</table>

*Nine animals were pulled together in 3 samples of 3 tendons for each group to account for biologic variability

Ethics Statement

All protocols and procedures were reviewed and approved by the University of Cincinnati’s Institutional Animal Care and Use Committee.

Surgical Procedure

Animals were anesthetized through inhalation of 3% isoflurane and both hindlimbs were shaved and aseptically prepared using ethanol and betadine washes. Mice were placed on a sterile surgical table and incisions were made on each limb to expose the PT. Medial and lateral longitudinal incisions were created on either side of the PT and then jeweler’s forceps were slipped under the tendon to isolate it from surrounding tissue. Next, a longitudinal incision was
made along the length of the tendon approximately one-third of the distance from the lateral edge of the PT creating the lateral strut. Using a pair of modified jeweler’s forceps, one prong was placed anterior to the lateral strut and pushed through posteriorly to isolate the central portion of the PT. The central portion was removed by cutting at both the patellar and tibial insertions, and a modified jigsaw blade was used to disrupt the tibial insertion (Fig. 14b). In the contralateral limb, a sham procedure was completed in which the jeweler’s forceps were slipped under the tendon to isolate it from surrounding tissue; however, no central defect was created. Skin incisions were closed using 5-0 prolene suture (Ethicon, Somerville, NJ) and animals were allowed unrestricted cage activity following the procedure. At appropriate time points post-injury, mice were euthanized by carbon dioxide asphyxiation and cervical dislocation. Limbs were harvested for biomechanical testing, gene expression, and tissue morphology.

**Fig. 14:** Native, uninjured patellar tendon (a), tendon defect created at surgery (b), and patellar tendon repair tissue at 5 weeks (c). The tendon defect is still clearly discernable at 5 weeks.

**Biomechanical Testing**

Animals were sacrificed at 2, 5, and 8 weeks following surgery and frozen at -20°C until the day of testing. Prior to testing, animals were thawed, skin and muscle was dissected away, and the knee joint was flexed to 45°. In the defect limb, the medial and lateral struts were removed leaving the patella-PT repair tissue-tibia unit (Fig.14c). PT repair tissue length and
width was measured by taking a digital image with a ruler in plane. The contralateral, sham limb was cut down to a similar width as the PT repair tissue. The patella-PT repair tissue-tibia unit was then placed in a custom grip and fixed in place using polymethylacrylate (Dentsply International, York, PA) with the tibia cemented into the grip and secured with a staple to prevent slipping during testing. The grip with the test tissue was then loaded into a materials testing system (100R; TestResources, Shakopee, MN) and lowered to position the patella into the conical-shaped bottom grip. PT repair tissue thickness was measured by taking a digital image of the pre-loaded tissue with a ruler in plane. The tissue was tested in a 37°C PBS bath by applying a preload to 0.02N, preconditioning for 25 cycles with 0-1% strain, and then failing in uniaxial tension at 0.1% of total tendon length/second\(^{238}\). The applied load (N) and grip-to-grip displacement (mm) were recorded throughout the testing period.

Ultimate load, failure displacement, stress and strain were recorded during the testing period. A linear regression algorithm was used to calculate stiffness and modulus from the linear portion of the load-displacement and stress-strain curves, respectively. The failure location was also recorded following the completion of the mechanical test.

**EdU (5-ethynyl-2’-deoxyuridine) Injections**

Twenty-four hours prior to sacrifice, animals were administered an intraperitoneal injection of EdU (5-ethynyl-2’-deoxyuridine, Invitrogen, Grand Island, NY) at a concentration of 3µg/g body weight to assess cellular proliferation occurring at 3, 7, and 14 days following surgery.

**Histological and Immunohistochemical Sample Preparation**

After sacrifice, each limb was dislocated at the femoral joint, the skin and foot removed, and samples were fixed in 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) for 24 hours.
Samples were then decalcified with 0.5M EDTA/PBS for 7 days at 4°C. Following decalcification, samples were further trimmed and embedded in O.C.T. compound (Andwin Scientific Tissue-Tek™, Schaumburg, IL) for frozen sectioning. Serial, transverse sections were cut along the length of the tendon at 6 equally spaced levels approximately 0.5mm apart beginning distal to the patella using a Leica CM3050S cryostat (Leica, Wetzlar, Germany). Both thin (8µm) and thick (100µm) sections were taken using cryofilm (Cryofilm Type 2C, Section-lab, Hiroshima, Japan) at each level for immunohistochemical analysis. Sections were affixed to glass slides and allowed to dry for 48 hours at room temperature.

**Histology and Immunohistochemistry**

Sections were incubated in blocking solution (0.1% Triton X-100 and 1% BSA in PBS) for one hour and then incubated for either one hour at room temperature or overnight at 4°C with a combination of the following primary antibodies: rabbit anti-collagen type VI (1:50, sc-20649, Santa Cruz Biotechnology, Dallas, TX), anti-α-smooth muscle actin-Cy3 conjugated (1:500, C6198, Sigma-Aldrich, St. Louis, MO), goat anti-collagen type I (1:100, AB758, EMD Millipore, Billerica, MA), rabbit anti-collagen type III (1:200, ab7778, Abcam, Cambridge, MA), rabbit anti-tenascin-C (1:500, ab6346, Abcam), rabbit anti-p21 (1:25, sc-397, Santa Cruz Biotechnologies), or rat anti-F4/80 (1:100, 123102, BioLegend, San Diego, CA). Following incubation with primary antibody, sections were washed three times in PBS and then incubated in appropriate secondary antibody (Alexa Fluor IgG; Invitrogen) for one hour at room temperature. Goat anti-rabbit antibodies (488 nm, 594 nm, 647 nm, or 750nm) were used for all primary rabbit antibodies, goat anti-rat (647 nm) was used for the tenascin-C primary antibody, and donkey anti-goat (594 nm) was used for the collagen type I primary antibody. Following incubation, sections were washed and then stained with DAPI to visualize cell nuclei. Sections
were then photographed using the Axio Scan.Z1 microscope (Carl Zeiss Microscopy, Thornwood, NY) at a magnification of 10X. Following imaging, sections were washed again, stained for EdU following the manufacturer’s protocol (Click-iT® EdU Alexa Fluor® 647 Imaging Kit, C10340, Life Technologies), and reimaged to capture cells that were actively proliferating at the time of sacrifice. Additional sections were also stained for hematoxylin and eosin following traditional protocols for frozen sections to assess overall tissue morphology.

**Quantification of Proliferating Cells**

Grayscale images for EdU and DAPI were equally thresholded for all groups and converted to binary images. For each section, the DAPI image was overlaid on to the EdU image to identify cells that were EdU positive. Images were cropped down to include only the defect region, medial/lateral struts, paratenon, and retinaculum based on anatomical landmarks. Total cell number (all DAPI positive cells) and EdU positive nuclei (cells positive for both DAPI and EdU) were then counted by thresholding upper and lower size bounds using FIJI particle counter.

**Quantitative Real-Time PCR (qPCR)**

Following sacrifice at 3, 7, and 14 days following injury, the midsubstance portion of the defect and sham samples was isolated and 3 tendons were pooled per sample. Native, uninjured tendon midsubstance samples were isolated to serve as a control. Tissue samples were placed in RNAlater® (Invitrogen) and stored at -20°C. The RNAeasy® Mini Kit (74104; Qiagen, Venlo, Limburg) was used to isolate RNA from each sample following the manufacturer’s protocol. The tissue was removed from RNAlater® (Invitrogen), weighed, and then placed in an RNAse-free tube partially submerged in liquid nitrogen. The tissue was disrupted using a pestle, vortexed, and centrifuged. The supernatant was then passed through a spin column and an on-
column DNase digestion was performed following the RNase-Free DNase Set protocol (79254; Qiagen), followed by subsequent washes/spins. The RNA was eluted and quantified using a NanoDrop ND-1000 Spectrophotometer (NC9904842, NanoDrop Technologies, Inc, Wilmington, DE). RNA was then converted to cDNA using the High Capacity RNA-to-cDNA kit (4387406; Applied Biosystems, Grand Island, NY). Real-time qPCR reactions were run using Taqman® Gene Expression Fast Mastermix (4364103; Applied Biosystems) and Taqman® Gene Expression Assays (Applied Biosystems) for Col1a1, Col3a1, Dcn, Fmod, TnC, Tnmd, Scx, Mkx, Egr1, p21, Myc (Table 6). Delta \( C_T \) values were computed and normalized to 18S expression within each sample. Delta delta \( C_T \) values were computed by normalizing to native tendon samples for C57BL/6 and MRL/MpJ.

<table>
<thead>
<tr>
<th>Name</th>
<th>Symbol</th>
<th>Gene Expression Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eukaryotic 18S rRNA</td>
<td>18s</td>
<td>Hs99999901_s1</td>
</tr>
<tr>
<td>Collagen Type I, alpha 1 chain</td>
<td>Col1a1</td>
<td>Mm00801666_g1</td>
</tr>
<tr>
<td>Collagen Type III, alpha 1 chain</td>
<td>Col3a1</td>
<td>Mm01254476_m1</td>
</tr>
<tr>
<td>Decorin</td>
<td>Dcn</td>
<td>Mm00514535_m1</td>
</tr>
<tr>
<td>Fibromodulin</td>
<td>Fmod</td>
<td>Mm00491215_m1</td>
</tr>
<tr>
<td>Tenascin-C</td>
<td>TnC</td>
<td>Mm00495662_m1</td>
</tr>
<tr>
<td>Tenomodulin</td>
<td>Tnmd</td>
<td>Mm00491594_m1</td>
</tr>
<tr>
<td>Scleraxis</td>
<td>Scx</td>
<td>Mm01205675_m1</td>
</tr>
<tr>
<td>Mohawk Homeobox</td>
<td>Mkx</td>
<td>Mm00617017_m1</td>
</tr>
<tr>
<td>Early Growth Response-1</td>
<td>Egr1</td>
<td>Mm00656724_m1</td>
</tr>
<tr>
<td>Cyclin-Dependent Kinase Inhibitor 1A</td>
<td>p21</td>
<td>Mm04205640_g1</td>
</tr>
<tr>
<td>Myelocytomatosis Oncogene</td>
<td>Myc</td>
<td>Mm00487804_m1</td>
</tr>
</tbody>
</table>
Statistical Analysis

All data sets were verified to be homoscedastic and normally distributed prior to statistical testing. Native MRL/MpJ and C57BL/6 tendon dimensions, mechanical properties, and gene expression values were compared using independent student t-test. Native, defect, and sham mechanical properties were evaluated via 3-way ANOVA with time post-surgery, murine strain, and treatment set as fixed factors with Fisher’s least significant difference (LSD) implemented for post-hoc comparisons. Gene expression was evaluated via 2-way ANOVA with time post-surgery and murine strain set as fixed factors with Fisher’s least significant difference (LSD) implemented for post-hoc comparisons. Cellular proliferation was assessed using independent student t-test between strains at 3, 7, and 14 days following injury. The IBM SPSS Statistics 2.1.0 software (Chicago, IL) was used to perform all statistical testing.

ACKNOWLEDGEMENTS

We gratefully acknowledge the National Institutes of Health for providing research and student support (R01 AR056943). We also thank Andrew Breidenbach and Steve Gilday for surgical and technical assistance along with Dr. Lou Soslowsky for his contributions to our current biomechanical assessment protocols.
Comparing and Contrasting the Temporal Tendon Healing RNA Expression Profiles Among the C57BL/6, LG/J, and MRL/MpJ Murine Strains

Andrea L. Lalley, B.S.\textsuperscript{a}, Nathaniel A. Dyment, Ph.D.\textsuperscript{b}, Pujan Joshi, Ph.D.\textsuperscript{c}, Dong-Guk Shin, Ph.D.\textsuperscript{c}, David Rowe, M.D.\textsuperscript{b}, Jason T. Shearn, Ph.D.\textsuperscript{a}

\textsuperscript{a}Biomedical Engineering Program, College of Engineering and Applied Science, University of Cincinnati, Cincinnati, Ohio

\textsuperscript{b}Department of Reconstructive Sciences, College of Dental Medicine, University of Connecticut Health Center, Farmington, Connecticut

\textsuperscript{c}Computer Science and Engineering, University of Connecticut, Storrs, Connecticut

\textsuperscript{1}This manuscript is currently \textit{in preparation} for the PlosOne.
ABSTRACT

Current tendon tissue engineering approaches fail to restore native tissue structure and function. Identifying biological success criteria is needed to improve future therapy development. One strategy for identifying biological success criteria is to compare and contrast the RNA transcriptome profiles for successful and unsuccessful models of tendon healing. We have previously characterized the MRL/MpJ murine strain as a model that shows improved mechanical outcomes following a centralPT injury; however, we found no significant differences in the tenogenic expression profiles compared to wildtype at 3, 7, and 14 days. This study seeks to more fully characterize the RNA transcriptome at these early time points post injury using RNA-Seq, a deep-sequencing technology capable of producing millions of cDNA reads for a given sample and mapping them to a reference genome. We evaluated the transcriptome of the C57BL/6, LG/J, and MRL/MpJ strains for native tissue and following injury at 3, 7, and 14 days using a novel clustering software program and differential analysis of transcription factor expression. We found the MRL/MpJ responded to tendon injury by decreasing activation of immune response-related pathways while increasing expression of transcription factors implicated in developmental processes. Future work will seek to better characterize the cell populations responding to tendon injury and further investigate the role of the immune response during stages of tendon healing.
INTRODUCTION

Natural tendon healing fails to restore native mechanical and biological properties following injury\textsuperscript{18, 26, 27, 176}. Over the past several years, the Murphy Roths Large (MRL/MpJ) murine strain has been evaluated for its superior healing capacity following injury in a number of tissue types including the heart\textsuperscript{128}, skin\textsuperscript{126}, cornea\textsuperscript{105}, spinal cord\textsuperscript{131}, and articular cartilage\textsuperscript{129}. Our laboratory has recently evaluated the MRL/MpJ’s response to a full-length, full-thickness central patellar tendon (PT) defect and found improved biomechanical outcomes by 8 weeks post injury (Chapter 4), reaching near normal structural properties. It has long been hypothesized that evaluating genetic differences following a treatment could help to explain the resulting phenotypic differences; however, genetic analysis following tendon injury in the MRL/MpJ and C57BL/6 model system at early time points (3, 7, and 14 days following injury) revealed no differences between the two strains (Chapter 4). Unfortunately, real-time PCR and microarrays are limited because these measures can only assess expression of known targets that have been previously identified. These technologies cannot evaluate expression of novel targets or other unidentified genetic changes.

Recent advances in the field of genomics have expanded our current capabilities. RNA-Seq is a high-throughput, parallel, deep-sequencing technology that has revolutionized the field\textsuperscript{239-241}. This technology sequences and measures cDNA reads that are then mapped against a reference genome producing a quantitative transcriptional read-out of the entire genome for a given sample\textsuperscript{240}. Further, RNA-Seq is highly replicable and unique in that it provides absolute values as opposed to relative gene expression values, offering a more precise assessment of transcript level expression compared to real-time PCR and microarray experiments\textsuperscript{242}. 

84
With this technology, we have evaluated the early healing transcriptome using Illumina RNA-Seq methodology following a central PT injury in three mouse strains, the MRL/MpJ, LG/J, and C57BL/6, at 3, 7, and 14 days post injury. The MRL/MpJ mouse is a result of a series of crosses between the C57BL/6J, C3H/HeDi, AKR/J strains followed by backcrosses to the LG/J strain, with the LG/J strain accounting for 75% of the genetic background. Incorporating this strain into the genetic analysis offers an additional metric for identifying the unique expression profiles present in the MRL/MpJ mouse during healing.

Results were compared for differential gene expression using Pattern Based Clustering (PBC) to understand the global effects following injury within each strain. These frameworks were originally developed for analyzing micro-array experiments; however, more recently, we have been modifying and implementing these processes for RNA-Seq clustering. This clustering analysis was followed up by evaluating differentially expressed transcription factors among the strains, with the goal of identifying specific pathways that may be activated or deactivated in the MRL/MpJ healing process. The objective of this project was to first evaluate the global differences among the strains in terms of pathway activation/deactivation in response to injury and then to investigate the transcription factors that may be driving for the healing phenotype. By evaluating the differentially expressed transcription factors, we hope to identify potential targets for future investigation in tendon tissue engineering approaches.

RESULTS

Cluster Analysis

Comparisons Across Strains (Fig.15a-d, Table 7-14)

For each comparison, we evaluated C57BL6 to LG/J to MRL/MpJ in this order. C57BL/6 is the control strain, the LG/J strain constitutes 75% of the MRL/MpJ background, and
the MRL/MpJ displayed the desired healing outcome. We evaluated clusters of the observed
values (+2, +2) and (-2, -2) to identify the most significantly differentially activated/deactivated
pathways for each comparison. Plot clustering output is presented on pages 101-109, including
individual plot clusters for each comparison and tables reporting pathways that show significant
activation within each of the clusters.

Native Comparison (Fig. 15a, Table 7-8)

Clustering analysis revealed 255 genes comprised the (+2, +2) cluster and 530
genes comprised the (-2, -2) cluster. The (+2, +2) cluster indicates the MRL/MpJ shows
increased activation of pathways involved in cell adhesion (p=0.0002), anterior-posterior
pattern specification processes (p=0.0045), and regionalization (p=0.0056) and the (-2, -
2) cluster shows decreased activation of pathways implicated primarily in muscle
development and function including muscle contraction (p=1.41e-08), muscle
development (p=1.45e-07), myofibril assembly (p=3.23e-06), and myoblast
differentiation (p=4.44e-06).

Day 3 Post Injury Comparison (Fig. 15b, Table 9-10)

Clustering analysis showed 160 genes comprised the (+2, +2) cluster and 237
genoms comprised the (-2, -2) cluster. The (+2, +2) cluster indicates the MRL/MpJ shows
increased activation of pathways involved in exocytosis (p=0.00140) and aromatic amino
acid family metabolic processes (p=0.00750), while the (-2, -2) cluster shows the
MRL/MpJ decreases expression of genes related to epidermis development (p=9.98e-06),
ectoderm development (p=1.49e-05), the Wnt signaling pathway (p=4.41e-04), and
regulation of cell apoptotic pathways (p=3.50e-03).

Day 7 Post Injury Comparison (Fig. 15c Table 11-12)
Clustering analysis revealed 147 genes comprised the (+2, +2) cluster and 131 genes comprised the (-2, -2) cluster. The (+2, +2) cluster shows the MRL/MpJ activates pathways involved in amine metabolic/catabolic processes (p=0.000867 and p=0.0041, respectively), aromatic catabolic processes (p=0.0025), and negative regulation of the immune response (p=0.0057). The (-2, -2) cluster indicates the MRL/MpJ shows decreased collagen breakdown/production (p=0.0018 and p=0.0027, respectively). Furthermore, the MRL/MpJ shows decreased activation of the immune response (p=0.0079), specifically B cell mediated immunity (p=0.0032), leukocyte, lymphocyte, and immunoglobulin activation (p=0.0035, 0.0025, and p=0.003, respectively), and the adaptive immune response (p=0.0081).

**Day 14 Post Injury Comparison** (Fig. 15d, Table 13-14)

Clustering analysis revealed 226 genes comprised the (+2, +2) cluster and 176 genes comprised the (-2, -2) cluster. Investigating the (+2, +2) cluster indicates the MRL/MpJ activates pathways involved in nucleosome assembly (p=6.09e-05), chromatin assembly (p=1.46e-04), DNA packaging (3.93e-04), chromosome organization and biogenesis (p=0.0063), and muscle pathways, including muscle contraction (p=0.0038). The (-2,-2) cluster suggests the MRL/MpJ decreases expression of genes found in the immune response as indicated by the pathways identified in this cluster including antigen processing and presentation of peptide antigen (p=5.85e-04), regulation of lymphocyte and leukocyte mediated immunity (p=8.83e-004), and natural killer cell inhibitory signaling pathway (p=0.0061).

**Comparison Within Strains** (Fig. 16a-c, Table 15-20)
For each comparison, we evaluated Day 3 to Day 7 to Day 14 because we were interested in the genetic changes occurring with respect to time for each strain. We evaluated clusters of the observed values (+2,+2) and (-2,-2) to identify the most differentially expressed genes for each comparison. Plot clustering output is presented on pages 110-118, including individual plot clusters for each comparison and tables reporting pathways that show significant activation within each of the clusters.

*C57BL/6* (Fig. 16a, Table 15-16)

Clustering analysis showed 351 genes comprised the (+2, +2) cluster and 338 genes comprised the (-2, -2) cluster. This analysis indicates the C57BL/6 shows increased expression of genes involved in the muscle system (p=1.22e-09) and decreased activation of cell cycle regulating pathways (p=3.47e-08) over time.

*LG/J* (Fig. 16b, Table 17-18)

Clustering analysis indicated 364 genes comprised the (+2, +2) cluster and 140 genes comprised the (-2, -2) cluster. The LG/J shows activation of pathways regulating cell adhesion (p=2.31e-12), inflammatory response (p=1.58e-04), and collagen fibril organization (p=0.0021), and shows decreased activation of peptidyl –serine and –tyrosine phosphorylation (p=2.21e-04 and p=0.0053).

*MRL/MpJ* (Fig. 16c Table 19-20)

Clustering analysis revealed 384 genes comprised the (+2, +2) cluster and 284 genes comprised the (-2, -2) cluster. The MRL/MpJ showed increased activation of pathways involved in muscle cell differentiation (p=1.82e-04), glucan and glycogen metabolic processes (p=2.03e-04), developmental processes (p=3.56e-04), and collagen
fibril organization (p=0.0019). This strain showed decreased activation for pathways involving the immune response (p=2.33e-07) and inflammation (p=3.61e-04).

Identification of Differentially Regulated Transcription Factors (Tables 21 and 22)

At day 3, 70 transcription factors showed increased expression and 87 transcription factors exhibited decreased expression in the MRL/MpJ. At day 7, 319 transcription factors showed increased expression while displayed 19 decreased compared to the LG/J and C57BL/6. Of these factors, 19 were identified to show increased expression at both 3 and 7 days while 3 showed decreased expression at both time points. Many of the transcription factors that were elevated in the MRL/MpJ are involved in development (Hoxd13, Pax9, and Mapk13) and erythroid differentiation (Mafk and 2700050L05Rik) (Table 21). Several of the factors have unknown functions (Zfp879, Zfp213, Foxc1, Zfp689). The decreased transcription factors function in T-cell differentiation (Gata3), epithelial cell differentiation (Ehf), and apoptosis (Hdac3) (Table 22).
Table 21: Elevated transcription factor expression at 3 and 7 days post injury for the MRL/MpJ

<table>
<thead>
<tr>
<th>Transcription Factor</th>
<th>Function</th>
<th>MRL v BL6 Day 3</th>
<th>MRL v LGJ Day 3</th>
<th>LG/J v BL6 Day 3</th>
<th>MRL v BL6 Day 7</th>
<th>MRL v LGJ Day 7</th>
<th>LG/J v BL6 Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoxd3</td>
<td>morphogenesis, cell adhesion</td>
<td>23.08</td>
<td>1.51</td>
<td>15.30</td>
<td>2.83</td>
<td>2.11</td>
<td>1.34</td>
</tr>
<tr>
<td>Mapk13</td>
<td>cell growth, cell adhesion, differentiation</td>
<td>7.67</td>
<td>1.92</td>
<td>4.00</td>
<td>16.92</td>
<td>2.11</td>
<td>8.00</td>
</tr>
<tr>
<td>Hdac9</td>
<td>histone deacetylase, angiogenesis</td>
<td>7.18</td>
<td>2.65</td>
<td>2.71</td>
<td>7.24</td>
<td>2.40</td>
<td>3.02</td>
</tr>
<tr>
<td>Zfp879</td>
<td>function not known</td>
<td>4.00</td>
<td>2.00</td>
<td>2.00</td>
<td>3.75</td>
<td>1.50</td>
<td>2.50</td>
</tr>
<tr>
<td>Hdac11</td>
<td>prevents IL-10 expression, immune activation</td>
<td>3.48</td>
<td>2.71</td>
<td>1.28</td>
<td>2.66</td>
<td>1.18</td>
<td>2.25</td>
</tr>
<tr>
<td>Lhx2</td>
<td>hematopoietic stem cell maintenance</td>
<td>3.43</td>
<td>2.00</td>
<td>1.71</td>
<td>3.00</td>
<td>1.80</td>
<td>1.67</td>
</tr>
<tr>
<td>Lass4</td>
<td>ceramide synthesis</td>
<td>2.95</td>
<td>1.63</td>
<td>1.81</td>
<td>2.38</td>
<td>1.49</td>
<td>1.60</td>
</tr>
<tr>
<td>Pax9</td>
<td>tooth development</td>
<td>2.67</td>
<td>1.60</td>
<td>1.67</td>
<td>3.30</td>
<td>1.32</td>
<td>2.51</td>
</tr>
<tr>
<td>Mafk</td>
<td>erythroid differentiation</td>
<td>2.27</td>
<td>1.22</td>
<td>1.87</td>
<td>3.14</td>
<td>1.89</td>
<td>1.66</td>
</tr>
<tr>
<td>Zfp213</td>
<td>function not known</td>
<td>2.18</td>
<td>1.32</td>
<td>1.65</td>
<td>1.70</td>
<td>1.31</td>
<td>1.30</td>
</tr>
<tr>
<td>Foxc1</td>
<td>function not known - embryonic and ocular development?</td>
<td>1.92</td>
<td>1.21</td>
<td>1.58</td>
<td>1.97</td>
<td>1.18</td>
<td>1.67</td>
</tr>
<tr>
<td>Notch3</td>
<td>cell differentiation, proliferation, apoptosis, T cell generation</td>
<td>1.74</td>
<td>1.18</td>
<td>1.47</td>
<td>2.68</td>
<td>1.21</td>
<td>2.22</td>
</tr>
<tr>
<td>Zfp689</td>
<td>function not known - lupus?</td>
<td>1.73</td>
<td>1.29</td>
<td>1.34</td>
<td>2.15</td>
<td>1.14</td>
<td>1.89</td>
</tr>
<tr>
<td>Hes6</td>
<td>neuronal differentiation</td>
<td>1.62</td>
<td>1.11</td>
<td>1.46</td>
<td>2.40</td>
<td>1.11</td>
<td>2.17</td>
</tr>
<tr>
<td>Mad2l2</td>
<td>function not known - neuronal development?</td>
<td>1.47</td>
<td>1.14</td>
<td>1.29</td>
<td>4.64</td>
<td>1.18</td>
<td>3.94</td>
</tr>
<tr>
<td>Nr2f2</td>
<td>antioxidant stress response in macrophages</td>
<td>1.31</td>
<td>1.17</td>
<td>1.12</td>
<td>1.59</td>
<td>1.22</td>
<td>1.30</td>
</tr>
<tr>
<td>2700050D05Rik</td>
<td>erythroid differentiation</td>
<td>1.31</td>
<td>1.15</td>
<td>1.14</td>
<td>1.74</td>
<td>1.41</td>
<td>1.23</td>
</tr>
<tr>
<td>Cdk7</td>
<td>cyclin dependent kinase</td>
<td>1.27</td>
<td>1.12</td>
<td>1.13</td>
<td>1.38</td>
<td>1.10</td>
<td>1.25</td>
</tr>
<tr>
<td>Setd1b</td>
<td>pluripotency</td>
<td>1.23</td>
<td>1.11</td>
<td>1.11</td>
<td>1.83</td>
<td>1.18</td>
<td>1.55</td>
</tr>
</tbody>
</table>

Table 22: Decreased transcription factor expression at 3 and 7 days post injury for the MRL/MpJ

<table>
<thead>
<tr>
<th>Transcription Factor</th>
<th>Function</th>
<th>MRL v BL6 Day 3</th>
<th>MRL v LGJ Day 3</th>
<th>LG/J v BL6 Day 3</th>
<th>MRL v BL6 Day 7</th>
<th>MRL v LGJ Day 7</th>
<th>LG/J v BL6 Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gata3</td>
<td>T-cell differentiation</td>
<td>-14.33</td>
<td>-2.67</td>
<td>-5.38</td>
<td>-3.00</td>
<td>-1.33</td>
<td>-2.25</td>
</tr>
<tr>
<td>Ehf</td>
<td>epithelial cell differentiation, carcinogenesis</td>
<td>-4.57</td>
<td>-2.08</td>
<td>-2.19</td>
<td>-4.08</td>
<td>-3.61</td>
<td>-1.13</td>
</tr>
<tr>
<td>Hdac3</td>
<td>histone deacetylase, apoptosis</td>
<td>-2.05</td>
<td>-1.46</td>
<td>-1.40</td>
<td>-1.24</td>
<td>-1.12</td>
<td>-1.10</td>
</tr>
</tbody>
</table>
Validation of RNA-Seq Results

To validate the RNA-Seq expression profiles, quantitative real-time PCR was performed on 14 genes of interest (Table 23) to evaluate overall temporal expression trends. For 12 of the 14 genes, qRT-PCR temporal expression patterns were generally in agreement with the measured RNA-Seq data (Fig. 17a, b, c), while 2 of the 14 genes (Hoxa13 and Pou5f1) did not match RNA-Seq expression profile patterns (Fig. 17d). Those genes that did not match RNA-Seq results were transcription factors expressed at the upper detection limits of the Taqman expression assays. Based on these results, RNA-Seq is an adequate tool for assessing gene expression and may be better suited for assessing expression of weakly expressed genes.

DISCUSSION

Identifying and characterizing a mammalian model of regeneration has the potential to greatly impact the field of regenerative medicine. Over the past decade, researchers have investigated the MRL/MpJ mouse, a strain that exhibits superior healing outcomes in a number of injury models\(^ {105, 126, 128} \). Despite this work, it still remains unknown what molecular mechanisms are driving the improved healing response, although many hypotheses have been proposed including mutations producing a p21 deficiency, increased activation of stem-related markers, and abnormalities in the immune and inflammatory response following injury\(^ {105, 138, 141} \). Researchers have also determined the MRL/MpJ healing response may depend on the type and location of the injury\(^ {134} \). This study was designed to determine the differentially activated pathways that are involved in the MRL/MpJ healing response following a central PT injury. We have previously shown the MRL/MpJ displays an improved healing outcome compared to wildtype at 8 weeks post injury. Evaluating the early response to injury, measured at 3, 7, and 14
days, could provide information in determining how early signaling events might contribute to long-term repair outcome.

We first conducted a series of clustering experiments to understand the global differences in pathway activation existing among the C57BL/6, LG/J, and MRL/MpJ strains following tendon injury. Differential analysis of the native tissue indicated the MRL/MpJ naturally expresses higher levels of genes that are involved in cell adhesion, pattern specification, and anterior-posterior patterning, all of which could indicate the MRL/MpJ native environment is more similar to the environment of a developing animal. Interestingly, the MRL/MpJ showed decreased pathway activation of muscle development and differentiation pathways compared to the other two strains in the native tissue. While the MRL/MpJ showed increased activation of exocytosis pathways at day 3, it showed depressed levels of developmental pathways of the epidermis, ectoderm, and organ compared to the C57BL/6 and LG/J. The primary finding of this analysis; however, is the substantially decreased immunological response following injury compared to the C57BL/6 and LG/J, primarily at post-surgical days 7 and 14. The vast majority of significantly regulated pathways indicates the MRL/MpJ decreases activation of a number of immune/inflammatory-related pathways including leukocyte and lymphocyte activation, adaptive immunity, immunoglobulin-mediated immune response, and antigen processing and presentation. The pathways that were activated in the MRL/MpJ indicate it is likely responding to the injury by increased levels of cellular proliferation as indicated by activation of pathways involved in nucleosome assembly, chromatin assembly, DNA packaging, and amine and aromatic metabolism. These findings support findings from Chapter 4, which showed the MRL/MpJ displayed increased levels of cellular proliferation as measured by EdU uptake by the
cells at 7 and 14 days post injury. Furthermore, staining for F4/80 for macrophage infiltration to the wound site indicated a dampened immune response in the MRL/MpJ.

Pathway activation assessed over time within each strain showed both the LG/J and MRL/MpJ activated pathways for collagen fibril organization, while the C57BL/6 did not. The early activation of genes in this pathway could help to explain the increased mechanical properties at late post-surgical time points.

Taking the transcriptome analysis a step further, we used the Avadis NGS software to identify transcription factors that displayed unique expression profiles following injury. Based on the mechanical results reported in Chapter 4 that showed the MRL/MpJ has improved healing outcomes at 2 weeks, we chose to limit our analysis to transcription factors that showed differential expression at 3 and 7 days only. We identified 19 transcription factors that were upregulated and 3 transcription factors that were downregulated in the MRL/MpJ compared to both the LG/J and C57BL/6. Of the factors that showed increased expression, several are found to be involved during early developmental stages (Hoxd3, Mapk13, Notch3, and Pax9). Interestingly, Hoxd3 has been identified to act in concert with Hoxa13 to regulate expression of Six2, a transcription factor expressed in developing tendons. Yamamoto-Shiraishi et al. demonstrated that without Hoxa13 and Hoxd13 expression, Six2 failed to activate and tendons did not form in embryonic mice\(^ {244}\). Pax9 has been implicated in tooth development; however, a 2005 study demonstrated it to be a downstream effector of Gli3 during vertebrate limb development as well\(^ {245}\). Our results, coupled with these previous studies, suggest the MRL/MpJ may ‘reactive’ developmental transcription factors more readily than the other strains, allowing it to be better suited for injury response.
Evaluation of the transcription factors that showed decreased expression compared to C57BL/6 and LG/J produced only three factors. Interestingly, GATA3, known as the trans-acting T-cell-specific transcription factor GATA-3, is highly involved in T cell regulation, particularly T helper 2 cell differentiation\textsuperscript{246,247}. This finding supports our findings from the clustering analysis, which indicated decreased immune pathway activation following injury.

Taken together, we hypothesize the MRL/MpJ phenotype may be a result of a decreased immunological response coupled with increased activation of developmental transcription factors at early post-surgical time points. Of course, much more work needs to be completed to verify this claim. First, we need to identify and characterize the cell populations that 1) express the transcription factors that show increased expression in the MRL/MpJ strain and 2) express the transcription factors that show decreased expression in the C57BL/6. This will provide us with a better understanding of the cell populations that respond to the tendon injury and allow us to characterize their complete phenotype. Secondly, we need to evaluate the effects of ablating aspects of the immune response on tendon healing to determine how it impacts the ultimate repair outcome. Obviously, the immune system is extremely complex and inter-connected. Disrupting portions of the system can produce unanticipated, catastrophic results to the animal. We need to identify targeted approaches for investigating this concept, perhaps through the use of CRISPR technology\textsuperscript{248}. Finally, the most significant mechanical improvements between the MRL/MpJ and C57BL/6 strains were observed at 8 weeks post injury. At the beginning of these experiments, we hypothesized that early molecular events would drive the accumulation of long-term mechanical outcomes; however, this assumption may not be true. We should also include analysis of repair tissue at late post-surgical time points, such as 5, 8, and 10 weeks, to ascertain how the molecular profile is altered during the healing process.
This study is not without limitations. 1) We limited our clustering analysis to (+2, +2) and (-2, -2) expression patterns. The Plot Cluster analysis software allows comparisons to be constructed among any of the data sets and provides clustering outputs in the form of (-2, -1, 0, 1, 2). We chose to evaluate the (+2, +2) and (-2, -2) patterns for each comparison because we were most interested in identifying pathways that showed the greatest fold changes between groups. Of course, other patterns could be evaluated including (+1, +2), (-1, -2), (+1, +1), and (-1, -1) and future work should investigate these possible outputs. For the purposes of understanding the global responses to tendon injury across strains, our approach for investigating the (+2, +2) and (-2, -2) patterns is sufficient. 2) We have not previously characterized the LG/J’s mechanical response to aPT defect. Previous reports suggest the LG/J displays an improved healing phenotype following injury in articular cartilage; however, we have not tested its response to a centralPT defect\textsuperscript{249}. We have shown that different mouse strains show variability in healing responses based on the type of injury or injury location, so fully characterizing the LG/J model remains a priority for future studies to validate its inclusion in this clustering analysis.

While we have employed extensive sequencing technologies and novel analysis tools, the molecular mechanisms driving the MRL/MpJ phenotype remain unknown. However, results from this study provide a direction for future work, namely investigating the role of the immune response and activation of development-related transcription factors. Fully characterizing this model system has tremendous potential for improving clinical approaches for treatment of musculoskeletal conditions.
MATERIALS AND METHODS

Surgical Procedure

Animals were anesthetized through inhalation of 3% isoflurane and both hindlimbs were shaved and aseptically prepared using ethanol and betadine washes. Mice were placed on a sterile surgical table and incisions were made on each limb to expose the PT. Medial and lateral longitudinal incisions were created on either side of the PT and then jeweler’s forceps were slipped under the tendon to isolate it from surrounding tissue. Next, a longitudinal incision was made along the length of the tendon approximately one-third of the distance from the lateral edge of the PT creating the lateral strut. Using a pair of modified jeweler’s forceps, one prong was placed anterior to the lateral strut and pushed through posteriorly to isolate the central portion of the PT. The central portion was removed by cutting at both the patellar and tibial insertions, and a modified jigsaw blade was used to disrupt the tibial insertion. Skin incisions were closed using 5-0 prolene suture (Ethicon, Somerville, NJ) and animals were allowed unrestricted cage activity following the procedure. At appropriate time points post-injury, mice were euthanized by carbon dioxide asphyxiation and cervical dislocation. Limbs were harvested for RNA-Seq experiments with qRT-PCR validation.

Total RNA Isolation, Quantification, cDNA Library Creation and Sequencing

Following sacrifice at 3, 7, and 14 days following injury, the midsubstance portions of the defect samples were isolated and 3 tendons were pulled per sample. Native, uninjured tendon midsubstances were isolated to serve as a control. Tissue samples were placed in RNALater® (Invitrogen) and stored at -20°C. The RNAspray® Mini Kit (74104; Qiagen, Venlo, Limburg) was used to isolate RNA from each sample following the manufacturer’s protocol. The tissue was removed from RNALater® (Invitrogen), weighed, and then placed in an RNase-
free tube partially submerged in liquid nitrogen. The tissue was disrupted using a pestle, vortexed, and centrifuged. The supernatant was then passed through a spin column and an on-column DNase digestion was performed following the RNase-Free DNase Set protocol (79254; Qiagen), followed by subsequent washes/spins. The RNA was eluted and quantified using a NanoDrop ND-1000 Spectrophotometer (NC9904842, NanoDrop Technologies, Inc, Wilmington, DE).

Total RNA for each sample was then submitted to the Cincinnati Children’s Hospital Medical Center Gene Expression Core Facility for amplification and cDNA library creation. First, RNA quality and quantity were evaluated using the Agilent Bioanalyzer (Agilent Technologies, Inc). The Ovation® RNA-Seq System V2 (7102; NuGEN Technologies, Inc., San Carlos, CA) was used to amplify the RNA samples, to create 2-4 micrograms of double stranded cDNA. The Nextera DNA Sample Preparation Kit (FC-121-1031; Illumina, San Diego, CA) was then used to create DNA library templates from the double stranded cDNA. Samples were pooled and then submitted to the Cincinnati Children’s Hospital Medical Center Genetic Variation and Gene Discovery Core for sequencing using the Illumina HiSeq2500 (Illumina). The parameters for the sequencing were set to single read, 50 base pair read length, with the number of reads requested per sample set to 25-30 million.

Alignment and Analysis of Illumina Reads

The sequence reads were aligned to the Mouse NCBI37/mm9 (NCBI and the Mouse Genome Sequencing Consortium) reference genome using Avadis NGS software (Strand Genomics, San Francisco, CA). The Cufflinks package was used for transcript identification and assembly.
Pattern Based Clustering

Gene expression was measured in fragments per exon kilobase per million mapped sequence reads (FPKM). All genes on the mm9 reference genome were assigned to a functional category using the gene ontology (GO) classification and functional categories (Gene Ontology Consortium, 2001) and Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

The identified transcripts and expression values were processed through subsequent analysis following Pattern Based Clustering (SPBC) and visualized using a plot-clustering program. This program combines gene expression profiles, transcription factor binding site analysis, and information derived from the GO and KEGG databases to produce a statistically valid, comprehensive method of evaluating and clustering time-series RNA-Seq data sets. GOStat is a software package (Bioconductor) incorporated into the clustering program used to measure GO term enrichments within each of the individual clusters, producing a p-value. Small p-values indicate that the GO terms falling in a particular cluster are unlikely to be grouped into the cluster by randomly. First, significantly regulated genes were identified and grouped on fold change patterns by implementing previously characterized statistical methods. Observed fold change patterns were assigned to a set of values of 0, 1, or 2. A 0 assignment indicates little to no fold change, 1 indicates a 1.5- to 2-fold change, and 2 indicates >2-fold change. Genes showing little to no fold changes were removed from the analysis to facilitate accelerated processing times of the data sets. Four different comparisons were completed among the strains at each time point as follows (Fig. 18a): 1) C57BL/6, LG/J, and MRL/MpJ Native; 2) C57BL/6, LG/J, and MRL/MpJ at D3; 3) C57BL/6, LG/J, and MRL/MpJ at D7; 4) C57BL/6, LG/J, and MRL/MpJ at D14. Three comparisons were completed within each strain across time points as
follows (Fig. 18b): 1) C57BL/6 D3, D7, and D14; 2) LG/J D3, D7, and D14; 3) MRL/MpJ D3, D7, and D14.

- **Comparisons Across Strains**

  1. C57BL/6 N → LG/J N → MRL/MpJ

  2. C57BL/6 D3 → LG/J D3 → MRL/MpJ

  3. C57BL/6 D7 → LG/J D7 → MRL/MpJ

  4. C57BL/6 D14 → LG/J D14 → MRL/MpJ

- **Comparisons Within Strain**

  1. C57BL/6 D3 → C57BL/6 D7 → C57BL/6 D14

  2. LG/J D3 → LG/J D7 → LG/J D14


**Fig. 18:** Summary of clustering comparisons. Clustering analysis was completed for seven different comparisons including across strains at each time point (Native and 3, 7, and 14 days post injury) (a) and within each strain across time points (3, 7, and 14 days) (b).

**Differential Transcription Factor Expression Among Strains**

Based on the significant increase in linear stiffness at 2 weeks for the MRL/MpJ and the slight decline in biomechanical properties for both mice strains between 2 and 5 weeks (as reported in Chapter 4), we focused our analysis on (1) transcripts that showed a 1.1-fold increase in expression in the MRL/MpJ at 3 and 7 days compared to the LG/J and C57BL/6 strains, but were no different at 14 days and, (2) transcripts that showed a 1.1-fold decrease in expression in the MRL/MpJ at 3 and 7 days compared to the LG/J and C57BL/6 strains, but were no different at 14 days. The analysis was further limited to transcription factor expression only.

**Real-Time qPCR Validation**

First-strand cDNA synthesis was performed with the High Capacity RNA-to-cDNA kit (4387406; Applied Biosystems, Grand Island, NY). Real-time qPCR reactions were run using Taqman® Gene Expression Fast Mastermix (4364103; Applied Biosystems) and Taqman® Gene Expression Assays (Applied Biosystems) for Col1a1, Col3a1, Dcn, Fmod, TnC, Tnmd, Scx, Mkx, Egr1, p21, Myc, Ifng, Pou5f1, and Hoxa13 (Table 23). All reactions were run in triplicate.
to account for technical variability and averaged. Delta $C_T$ values were computed and
normalized to 18S expression within each sample.

<table>
<thead>
<tr>
<th>Name</th>
<th>Symbol</th>
<th>Gene Expression Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eukaryotic 18S rRNA</td>
<td>18s</td>
<td>Hs99999901_s1</td>
</tr>
<tr>
<td>Collagen Type I, alpha 1 chain</td>
<td>Col1a1</td>
<td>Mm00801666_g1</td>
</tr>
<tr>
<td>Collagen Type III, alpha 1 chain</td>
<td>Col3a1</td>
<td>Mm01254476_m1</td>
</tr>
<tr>
<td>Decorin</td>
<td>Dcn</td>
<td>Mm00514535_m1</td>
</tr>
<tr>
<td>Fibromodulin</td>
<td>Fmod</td>
<td>Mm00491215_m1</td>
</tr>
<tr>
<td>Tenascin-C</td>
<td>TnC</td>
<td>Mm00495662_m1</td>
</tr>
<tr>
<td>Tenomodulin</td>
<td>Tnmd</td>
<td>Mm00491594_m1</td>
</tr>
<tr>
<td>Scleraxis</td>
<td>Scx</td>
<td>Mm01205675_m1</td>
</tr>
<tr>
<td>Mohawk Homeobox</td>
<td>Mkx</td>
<td>Mm00617017_m1</td>
</tr>
<tr>
<td>Early Growth Response-1</td>
<td>Egr1</td>
<td>Mm00656724_m1</td>
</tr>
<tr>
<td>Cyclin-Dependent Kinase Inhibitor 1A</td>
<td>p21</td>
<td>Mm04205640_g1</td>
</tr>
<tr>
<td>Myelocytomatosis Oncogene</td>
<td>Myc</td>
<td>Mm00487804_m1</td>
</tr>
<tr>
<td>Interferon Gamma</td>
<td>Ifng</td>
<td>Mm01168134_m1</td>
</tr>
<tr>
<td>POU Domain, Class 5, Transcription Factor 1</td>
<td>Pou5f1</td>
<td>Mm03053917_g1</td>
</tr>
<tr>
<td>Homeobox A13</td>
<td>Hoxa13</td>
<td>Mm00433967_m1</td>
</tr>
</tbody>
</table>

ACKNOWLEDGEMENTS

The authors would like to thank the Cincinnati Children’s Hospital Medical Center’s
Gene Expression Microarray Core and the Genetic Variation and Gene Discovery Core facilities
for RNA quality analysis, cDNA amplification, library creation, and next-generation sequencing
services.
**Fig 15: Comparisons Across Strains**

**Fig. 15a Comparison 1- Native**

C57BL/6 N ➔ LG/J N ➔ MRL/MpJ N

(+2, +2) ➔ (-2, -2)

---

**Table 7: (+2, +2) GO terms and p-values (Native Strain Comparison)**

<table>
<thead>
<tr>
<th>GO Id</th>
<th>P Value</th>
<th>Exp Count</th>
<th>Count</th>
<th>Size</th>
<th>GO Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0007155</td>
<td>2.20E-04</td>
<td>4.67</td>
<td>14</td>
<td>556</td>
<td>cell adhesion</td>
</tr>
<tr>
<td>GO:0022610</td>
<td>2.20E-04</td>
<td>4.67</td>
<td>14</td>
<td>556</td>
<td>biological adhesion</td>
</tr>
<tr>
<td>GO:0030431</td>
<td>6.87E-04</td>
<td>0.04</td>
<td>2</td>
<td>5</td>
<td>sleep</td>
</tr>
<tr>
<td>GO:0007389</td>
<td>4.50E-03</td>
<td>2.06</td>
<td>7</td>
<td>245</td>
<td>pattern specification process</td>
</tr>
<tr>
<td>GO:0003002</td>
<td>5.60E-03</td>
<td>1.62</td>
<td>6</td>
<td>193</td>
<td>regionalization</td>
</tr>
<tr>
<td>GO:0009952</td>
<td>6.00E-03</td>
<td>1.16</td>
<td>5</td>
<td>138</td>
<td>anterior/posterior pattern formation</td>
</tr>
<tr>
<td>GO:0001964</td>
<td>6.80E-03</td>
<td>0.13</td>
<td>2</td>
<td>15</td>
<td>startle response</td>
</tr>
<tr>
<td>GO:0035115</td>
<td>7.70E-03</td>
<td>0.13</td>
<td>2</td>
<td>16</td>
<td>embryonic forelimb morphogenesis</td>
</tr>
<tr>
<td>GO:0050890</td>
<td>8.30E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>cognition</td>
</tr>
<tr>
<td>GO:0046462</td>
<td>8.30E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>monoacylglycerol metabolic process</td>
</tr>
<tr>
<td>GO:0032314</td>
<td>8.30E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>regulation of Rac GTPase activity</td>
</tr>
<tr>
<td>GO:0051823</td>
<td>8.30E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>regulation of synapse structural plasticity</td>
</tr>
</tbody>
</table>
Table 8: (-2, -2) GO terms and p-values (Native Strain Comparison)

<table>
<thead>
<tr>
<th>GO Id</th>
<th>P Value</th>
<th>Exp Count</th>
<th>Count</th>
<th>Size</th>
<th>GO Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0006936</td>
<td>1.41E-08</td>
<td>1.42</td>
<td>12</td>
<td>72</td>
<td>muscle contraction</td>
</tr>
<tr>
<td>GO:0003012</td>
<td>1.95E-08</td>
<td>1.46</td>
<td>12</td>
<td>74</td>
<td>muscle system process</td>
</tr>
<tr>
<td>GO:0055001</td>
<td>1.45E-07</td>
<td>0.28</td>
<td>6</td>
<td>14</td>
<td>muscle cell development</td>
</tr>
<tr>
<td>GO:0068128</td>
<td>2.40E-07</td>
<td>8.85</td>
<td>27</td>
<td>449</td>
<td>cation transport</td>
</tr>
<tr>
<td>GO:0045214</td>
<td>6.63E-07</td>
<td>0.2</td>
<td>5</td>
<td>10</td>
<td>sarcomere organization</td>
</tr>
<tr>
<td>GO:0030001</td>
<td>8.87E-07</td>
<td>7.74</td>
<td>24</td>
<td>393</td>
<td>metal ion transport</td>
</tr>
<tr>
<td>GO:007519</td>
<td>1.24E-06</td>
<td>2.11</td>
<td>12</td>
<td>107</td>
<td>skeletal muscle development</td>
</tr>
<tr>
<td>GO:0048628</td>
<td>1.69E-06</td>
<td>0.39</td>
<td>6</td>
<td>20</td>
<td>myoblast maturation</td>
</tr>
<tr>
<td>GO:0006811</td>
<td>1.78E-06</td>
<td>13.54</td>
<td>33</td>
<td>687</td>
<td>ion transport</td>
</tr>
<tr>
<td>GO:0048627</td>
<td>2.33E-06</td>
<td>0.41</td>
<td>6</td>
<td>21</td>
<td>myoblast development</td>
</tr>
<tr>
<td>GO:0055002</td>
<td>3.23E-06</td>
<td>0.26</td>
<td>5</td>
<td>13</td>
<td>striated muscle cell development</td>
</tr>
<tr>
<td>GO:0030239</td>
<td>3.23E-06</td>
<td>0.26</td>
<td>5</td>
<td>13</td>
<td>myofibril assembly</td>
</tr>
<tr>
<td>GO:0045445</td>
<td>4.44E-06</td>
<td>0.69</td>
<td>7</td>
<td>35</td>
<td>myoblast differentiation</td>
</tr>
<tr>
<td>GO:0048747</td>
<td>6.92E-06</td>
<td>1.34</td>
<td>9</td>
<td>68</td>
<td>muscle fiber development</td>
</tr>
<tr>
<td>GO:0048741</td>
<td>6.92E-06</td>
<td>1.34</td>
<td>9</td>
<td>68</td>
<td>skeletal muscle fiber development</td>
</tr>
<tr>
<td>GO:0075171</td>
<td>1.06E-05</td>
<td>3.49</td>
<td>14</td>
<td>177</td>
<td>muscle development</td>
</tr>
<tr>
<td>GO:006941</td>
<td>1.44E-05</td>
<td>0.55</td>
<td>6</td>
<td>28</td>
<td>striated muscle contraction</td>
</tr>
<tr>
<td>GO:0014706</td>
<td>1.65E-05</td>
<td>2.7</td>
<td>12</td>
<td>137</td>
<td>striated muscle development</td>
</tr>
<tr>
<td>GO:0031032</td>
<td>1.98E-05</td>
<td>0.35</td>
<td>5</td>
<td>18</td>
<td>actomyosin structure organization and biogenesis</td>
</tr>
<tr>
<td>GO:0048469</td>
<td>1.45E-04</td>
<td>1.54</td>
<td>8</td>
<td>78</td>
<td>cell maturation</td>
</tr>
<tr>
<td>GO:0007127</td>
<td>1.93E-04</td>
<td>0.55</td>
<td>5</td>
<td>28</td>
<td>meiosis I</td>
</tr>
<tr>
<td>GO:0051146</td>
<td>3.19E-04</td>
<td>0.61</td>
<td>5</td>
<td>31</td>
<td>striated muscle cell differentiation</td>
</tr>
<tr>
<td>GO:007010</td>
<td>3.31E-04</td>
<td>7.19</td>
<td>18</td>
<td>365</td>
<td>cytoskeleton organization and biogenesis</td>
</tr>
<tr>
<td>GO:0007128</td>
<td>3.93E-04</td>
<td>0.16</td>
<td>3</td>
<td>8</td>
<td>meiotic prophase I</td>
</tr>
<tr>
<td>GO:0051324</td>
<td>3.93E-04</td>
<td>0.16</td>
<td>3</td>
<td>8</td>
<td>prophase</td>
</tr>
<tr>
<td>GO:0042692</td>
<td>4.04E-04</td>
<td>1.36</td>
<td>7</td>
<td>69</td>
<td>muscle cell differentiation</td>
</tr>
<tr>
<td>GO:0015672</td>
<td>4.40E-04</td>
<td>5.52</td>
<td>15</td>
<td>280</td>
<td>monovalent inorganic cation transport</td>
</tr>
<tr>
<td>GO:0043269</td>
<td>6.76E-04</td>
<td>0.41</td>
<td>4</td>
<td>21</td>
<td>regulation of ion transport</td>
</tr>
<tr>
<td>GO:0021700</td>
<td>8.46E-04</td>
<td>1.99</td>
<td>8</td>
<td>101</td>
<td>developmental maturation</td>
</tr>
<tr>
<td>GO:006096</td>
<td>9.50E-04</td>
<td>0.77</td>
<td>5</td>
<td>39</td>
<td>glycolysis</td>
</tr>
<tr>
<td>GO:0044275</td>
<td>1.10E-03</td>
<td>1.18</td>
<td>6</td>
<td>60</td>
<td>cellular carbohydrate catabolic process</td>
</tr>
<tr>
<td>GO:006816</td>
<td>1.60E-03</td>
<td>2.21</td>
<td>8</td>
<td>112</td>
<td>calcium ion transport</td>
</tr>
<tr>
<td>GO:0016052</td>
<td>1.70E-03</td>
<td>1.28</td>
<td>6</td>
<td>65</td>
<td>carbohydrate catabolic process</td>
</tr>
<tr>
<td>GO:0006091</td>
<td>1.80E-03</td>
<td>2.76</td>
<td>9</td>
<td>140</td>
<td>generation of precursor metabolites and energy</td>
</tr>
<tr>
<td>GO:0051606</td>
<td>2.10E-03</td>
<td>1.34</td>
<td>6</td>
<td>68</td>
<td>detection of stimulus</td>
</tr>
<tr>
<td>GO:0019320</td>
<td>2.20E-03</td>
<td>0.93</td>
<td>5</td>
<td>47</td>
<td>hexose catabolic process</td>
</tr>
<tr>
<td>GO:006007</td>
<td>2.20E-03</td>
<td>0.93</td>
<td>5</td>
<td>47</td>
<td>glucose catabolic process</td>
</tr>
<tr>
<td>GO:0046365</td>
<td>2.20E-03</td>
<td>0.93</td>
<td>5</td>
<td>47</td>
<td>monosaccharide catabolic process</td>
</tr>
<tr>
<td>GO:006813</td>
<td>2.20E-03</td>
<td>2.86</td>
<td>9</td>
<td>145</td>
<td>potassium ion transport</td>
</tr>
<tr>
<td>GO:006810</td>
<td>2.50E-03</td>
<td>42.44</td>
<td>60</td>
<td>2154</td>
<td>transport</td>
</tr>
<tr>
<td>GO:0044262</td>
<td>2.60E-03</td>
<td>4.08</td>
<td>11</td>
<td>207</td>
<td>cellular carbohydrate metabolic process</td>
</tr>
<tr>
<td>GO:0051234</td>
<td>3.40E-03</td>
<td>42.98</td>
<td>60</td>
<td>2181</td>
<td>establishment of localization</td>
</tr>
<tr>
<td>GO:0046164</td>
<td>3.40E-03</td>
<td>1.02</td>
<td>5</td>
<td>52</td>
<td>alcohol catabolic process</td>
</tr>
<tr>
<td>GO:0048878</td>
<td>5.40E-03</td>
<td>5.12</td>
<td>12</td>
<td>260</td>
<td>chemical homeostasis</td>
</tr>
<tr>
<td>GO:0015674</td>
<td>5.40E-03</td>
<td>2.68</td>
<td>8</td>
<td>136</td>
<td>di-, tri-valent inorganic cation transport</td>
</tr>
<tr>
<td>GO:0055013</td>
<td>7.60E-03</td>
<td>0.14</td>
<td>2</td>
<td>7</td>
<td>cardiac muscle cell development</td>
</tr>
<tr>
<td>GO:006006</td>
<td>9.10E-03</td>
<td>1.79</td>
<td>6</td>
<td>91</td>
<td>glucose metabolic process</td>
</tr>
</tbody>
</table>
Table 9: (+2, +2) GO terms and p-values (Day 3 Strain Comparison)

<table>
<thead>
<tr>
<th>GO Id</th>
<th>P Value</th>
<th>Exp Count</th>
<th>Count</th>
<th>Size</th>
<th>GO Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0006887</td>
<td>1.40E-03</td>
<td>0.49</td>
<td>4</td>
<td>90</td>
<td>exocytosis</td>
</tr>
<tr>
<td>GO:0017157</td>
<td>4.20E-03</td>
<td>0.1</td>
<td>2</td>
<td>18</td>
<td>regulation of exocytosis</td>
</tr>
<tr>
<td>GO:0046903</td>
<td>6.30E-03</td>
<td>1.18</td>
<td>5</td>
<td>214</td>
<td>secretion</td>
</tr>
<tr>
<td>GO:0009072</td>
<td>6.30E-03</td>
<td>0.12</td>
<td>2</td>
<td>22</td>
<td>aromatic amino acid family metabolic process</td>
</tr>
<tr>
<td>GO:0048489</td>
<td>7.50E-03</td>
<td>0.13</td>
<td>2</td>
<td>24</td>
<td>synaptic vesicle transport</td>
</tr>
<tr>
<td>GO Id</td>
<td>P Value</td>
<td>Exp Count</td>
<td>Count</td>
<td>Size</td>
<td>GO Term</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
<td>-------</td>
<td>------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>GO:0009888</td>
<td>2.32E-07</td>
<td>2.96</td>
<td>15</td>
<td>349</td>
<td>tissue development</td>
</tr>
<tr>
<td>GO:0008544</td>
<td>9.98E-06</td>
<td>1.05</td>
<td>8</td>
<td>124</td>
<td>epidermis development</td>
</tr>
<tr>
<td>GO:0007398</td>
<td>1.49E-05</td>
<td>1.11</td>
<td>8</td>
<td>131</td>
<td>ectoderm development</td>
</tr>
<tr>
<td>GO:0031424</td>
<td>9.85E-05</td>
<td>0.25</td>
<td>4</td>
<td>29</td>
<td>keratinization</td>
</tr>
<tr>
<td>GO:0048729</td>
<td>3.55E-04</td>
<td>0.94</td>
<td>6</td>
<td>111</td>
<td>tissue morphogenesis</td>
</tr>
<tr>
<td>GO:007223</td>
<td>4.41E-04</td>
<td>0.15</td>
<td>3</td>
<td>18</td>
<td>Wnt receptor signaling pathway, calcium modulating pathway</td>
</tr>
<tr>
<td>GO:0048513</td>
<td>8.75E-04</td>
<td>11.53</td>
<td>23</td>
<td>1360</td>
<td>organ development</td>
</tr>
<tr>
<td>GO:0045840</td>
<td>1.40E-03</td>
<td>0.06</td>
<td>2</td>
<td>7</td>
<td>positive regulation of mitosis</td>
</tr>
<tr>
<td>GO:0009913</td>
<td>1.60E-03</td>
<td>0.51</td>
<td>4</td>
<td>60</td>
<td>epidermal cell differentiation</td>
</tr>
<tr>
<td>GO:0045109</td>
<td>1.90E-03</td>
<td>0.07</td>
<td>2</td>
<td>8</td>
<td>intermediate filament organization</td>
</tr>
<tr>
<td>GO:0001503</td>
<td>2.30E-03</td>
<td>0.92</td>
<td>5</td>
<td>109</td>
<td>ossification</td>
</tr>
<tr>
<td>GO:0031214</td>
<td>2.40E-03</td>
<td>0.93</td>
<td>5</td>
<td>110</td>
<td>biomineral formation</td>
</tr>
<tr>
<td>GO:0043065</td>
<td>3.50E-03</td>
<td>1.47</td>
<td>6</td>
<td>173</td>
<td>positive regulation of apoptosis</td>
</tr>
<tr>
<td>GO:0043068</td>
<td>3.70E-03</td>
<td>1.48</td>
<td>6</td>
<td>175</td>
<td>positive regulation of programmed cell death</td>
</tr>
<tr>
<td>GO:0048730</td>
<td>4.10E-03</td>
<td>0.65</td>
<td>4</td>
<td>77</td>
<td>epidermis morphogenesis</td>
</tr>
<tr>
<td>GO:0045104</td>
<td>6.90E-03</td>
<td>0.13</td>
<td>2</td>
<td>15</td>
<td>intermediate filament cytoskeleton organization and biogenesis</td>
</tr>
<tr>
<td>GO:0042060</td>
<td>7.40E-03</td>
<td>0.77</td>
<td>4</td>
<td>91</td>
<td>wound healing</td>
</tr>
<tr>
<td>GO:0050793</td>
<td>8.10E-03</td>
<td>6.15</td>
<td>13</td>
<td>725</td>
<td>regulation of developmental process</td>
</tr>
<tr>
<td>GO:0010042</td>
<td>8.40E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>response to manganese ion</td>
</tr>
<tr>
<td>GO:0007035</td>
<td>8.40E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>vacular acidification</td>
</tr>
<tr>
<td>GO:0032799</td>
<td>8.40E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>low-density lipoprotein receptor metabolic process</td>
</tr>
<tr>
<td>GO:0060064</td>
<td>8.40E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>Spemann organizer formation at the anterior end of the primitive streak</td>
</tr>
<tr>
<td>GO:0060061</td>
<td>8.40E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>Spemann organizer formation</td>
</tr>
<tr>
<td>GO:0032026</td>
<td>8.40E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>response to magnesium ion</td>
</tr>
<tr>
<td>GO:0032025</td>
<td>8.40E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>response to cobalt ion</td>
</tr>
<tr>
<td>GO:0032803</td>
<td>8.40E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>regulation of low-density lipoprotein receptor catabolic process</td>
</tr>
<tr>
<td>GO:0032802</td>
<td>8.40E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>low-density lipoprotein receptor catabolic process</td>
</tr>
<tr>
<td>GO:0034213</td>
<td>8.40E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>quinolinate catabolic process</td>
</tr>
<tr>
<td>GO:0045787</td>
<td>8.90E-03</td>
<td>0.14</td>
<td>2</td>
<td>17</td>
<td>positive regulation of cell cycle</td>
</tr>
<tr>
<td>GO:0007498</td>
<td>9.10E-03</td>
<td>0.43</td>
<td>3</td>
<td>51</td>
<td>mesoderm development</td>
</tr>
<tr>
<td>GO:0048731</td>
<td>9.20E-03</td>
<td>13.87</td>
<td>23</td>
<td>1636</td>
<td>system development</td>
</tr>
<tr>
<td>GO:0007088</td>
<td>9.90E-03</td>
<td>0.15</td>
<td>2</td>
<td>18</td>
<td>regulation of mitosis</td>
</tr>
</tbody>
</table>
Fig. 15c Comparison 3 – Day 7

C57BL/6 D7 → LG/J D7 → MRL/MpJ D7

(+2, +2) (−2, −2)
<table>
<thead>
<tr>
<th>GO Id</th>
<th>P Value</th>
<th>Exp Count</th>
<th>Count</th>
<th>Size</th>
<th>GO Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0002920</td>
<td>8.27E-04</td>
<td>0.04</td>
<td>2</td>
<td>7</td>
<td>regulation of humoral immune response</td>
</tr>
<tr>
<td>GO:0006576</td>
<td>8.67E-04</td>
<td>0.43</td>
<td>4</td>
<td>67</td>
<td>biogenic amine metabolic process</td>
</tr>
<tr>
<td>GO:0048585</td>
<td>1.50E-03</td>
<td>0.23</td>
<td>3</td>
<td>36</td>
<td>negative regulation of response to stimulus</td>
</tr>
<tr>
<td>GO:0006575</td>
<td>1.80E-03</td>
<td>0.52</td>
<td>4</td>
<td>82</td>
<td>amino acid derivative metabolic process</td>
</tr>
<tr>
<td>GO:0019439</td>
<td>2.50E-03</td>
<td>0.08</td>
<td>2</td>
<td>12</td>
<td>aromatic compound catabolic process</td>
</tr>
<tr>
<td>GO:0006519</td>
<td>2.90E-03</td>
<td>0.08</td>
<td>4</td>
<td>82</td>
<td>amino acid derivative metabolic process</td>
</tr>
<tr>
<td>GO:0042402</td>
<td>2.90E-03</td>
<td>0.08</td>
<td>2</td>
<td>13</td>
<td>biogenic amine catabolic process</td>
</tr>
<tr>
<td>GO:0006519</td>
<td>2.90E-03</td>
<td>0.08</td>
<td>4</td>
<td>82</td>
<td>amino acid and derivative metabolic process</td>
</tr>
<tr>
<td>GO:0042402</td>
<td>2.90E-03</td>
<td>0.08</td>
<td>2</td>
<td>13</td>
<td>biogenic amine catabolic process</td>
</tr>
<tr>
<td>GO:0006519</td>
<td>2.90E-03</td>
<td>0.08</td>
<td>4</td>
<td>82</td>
<td>amino acid and derivative metabolic process</td>
</tr>
<tr>
<td>GO:0042402</td>
<td>2.90E-03</td>
<td>0.08</td>
<td>2</td>
<td>13</td>
<td>biogenic amine catabolic process</td>
</tr>
<tr>
<td>GO:0006519</td>
<td>2.90E-03</td>
<td>0.08</td>
<td>4</td>
<td>82</td>
<td>amino acid and derivative metabolic process</td>
</tr>
<tr>
<td>GO:0042402</td>
<td>2.90E-03</td>
<td>0.08</td>
<td>2</td>
<td>13</td>
<td>biogenic amine catabolic process</td>
</tr>
<tr>
<td>GO:0006519</td>
<td>2.90E-03</td>
<td>0.08</td>
<td>4</td>
<td>82</td>
<td>amino acid and derivative metabolic process</td>
</tr>
</tbody>
</table>
Table 12: (-2, -2) GO terms and p-values (Day 7 Strain Comparison)

<table>
<thead>
<tr>
<th>GO Id</th>
<th>P Value</th>
<th>Exp Count</th>
<th>Count</th>
<th>Size</th>
<th>GO Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0017156</td>
<td>1.49E-04</td>
<td>0.1</td>
<td>3</td>
<td>24</td>
<td>calcium ion-dependent exocytosis</td>
</tr>
<tr>
<td>GO:0007601</td>
<td>7.08E-04</td>
<td>0.41</td>
<td>4</td>
<td>93</td>
<td>visual perception</td>
</tr>
<tr>
<td>GO:0050953</td>
<td>7.37E-04</td>
<td>0.41</td>
<td>4</td>
<td>94</td>
<td>sensory perception of light stimulus</td>
</tr>
<tr>
<td>GO:0021879</td>
<td>1.10E-03</td>
<td>0.05</td>
<td>2</td>
<td>12</td>
<td>forebrain neuron differentiation</td>
</tr>
<tr>
<td>GO:0021872</td>
<td>1.60E-03</td>
<td>0.06</td>
<td>2</td>
<td>14</td>
<td>generation of neurons in the forebrain</td>
</tr>
<tr>
<td>GO:0035074</td>
<td>1.80E-03</td>
<td>0.07</td>
<td>2</td>
<td>15</td>
<td>collagen catabolic process</td>
</tr>
<tr>
<td>GO:0050870</td>
<td>1.90E-03</td>
<td>0.25</td>
<td>3</td>
<td>57</td>
<td>positive regulation of T cell activation</td>
</tr>
<tr>
<td>GO:0044259</td>
<td>2.10E-03</td>
<td>0.07</td>
<td>2</td>
<td>16</td>
<td>multicyclophosphorylated macromolecular metabolic process</td>
</tr>
<tr>
<td>GO:0044256</td>
<td>2.10E-03</td>
<td>0.07</td>
<td>2</td>
<td>16</td>
<td>protein digestion</td>
</tr>
<tr>
<td>GO:0044626</td>
<td>2.10E-03</td>
<td>0.07</td>
<td>2</td>
<td>16</td>
<td>multicellular organism macromolecular catabolic process</td>
</tr>
<tr>
<td>GO:0044268</td>
<td>2.10E-03</td>
<td>0.07</td>
<td>2</td>
<td>16</td>
<td>multicellular organism protein metabolic process</td>
</tr>
<tr>
<td>GO:0044243</td>
<td>2.10E-03</td>
<td>0.07</td>
<td>2</td>
<td>16</td>
<td>multicellular organism catabolic process</td>
</tr>
<tr>
<td>GO:0044254</td>
<td>2.10E-03</td>
<td>0.07</td>
<td>2</td>
<td>16</td>
<td>multicellular organism protein catabolic process</td>
</tr>
<tr>
<td>GO:0046649</td>
<td>2.50E-03</td>
<td>0.95</td>
<td>5</td>
<td>218</td>
<td>lymphocyte activation</td>
</tr>
<tr>
<td>GO:0032963</td>
<td>2.70E-03</td>
<td>0.08</td>
<td>2</td>
<td>18</td>
<td>collagen metabolic process</td>
</tr>
<tr>
<td>GO:0016064</td>
<td>3.00E-03</td>
<td>0.29</td>
<td>3</td>
<td>67</td>
<td>immunoglobulin mediated immune response</td>
</tr>
<tr>
<td>GO:0019724</td>
<td>3.20E-03</td>
<td>0.3</td>
<td>3</td>
<td>68</td>
<td>B cell mediated immunity</td>
</tr>
<tr>
<td>GO:0044236</td>
<td>3.30E-03</td>
<td>0.09</td>
<td>2</td>
<td>20</td>
<td>multicellular organism metabolic process</td>
</tr>
<tr>
<td>GO:0045321</td>
<td>3.50E-03</td>
<td>1.02</td>
<td>5</td>
<td>235</td>
<td>leukocyte activation</td>
</tr>
<tr>
<td>GO:0002684</td>
<td>3.70E-03</td>
<td>0.64</td>
<td>4</td>
<td>146</td>
<td>positive regulation of immune system process</td>
</tr>
<tr>
<td>GO:0050852</td>
<td>3.70E-03</td>
<td>0.09</td>
<td>2</td>
<td>21</td>
<td>T cell receptor signaling pathway</td>
</tr>
<tr>
<td>GO:0051251</td>
<td>3.90E-03</td>
<td>0.32</td>
<td>3</td>
<td>73</td>
<td>positive regulation of lymphocyte activation</td>
</tr>
<tr>
<td>GO:0019835</td>
<td>4.00E-03</td>
<td>0.1</td>
<td>2</td>
<td>22</td>
<td>cytolysis</td>
</tr>
<tr>
<td>GO:0002696</td>
<td>4.00E-03</td>
<td>0.32</td>
<td>3</td>
<td>74</td>
<td>positive regulation of leukocyte activation</td>
</tr>
<tr>
<td>GO:0050867</td>
<td>4.00E-03</td>
<td>0.32</td>
<td>3</td>
<td>74</td>
<td>positive regulation of cell activation</td>
</tr>
<tr>
<td>GO:0000072</td>
<td>4.30E-03</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>M phase specific microtubule process</td>
</tr>
<tr>
<td>GO:0021905</td>
<td>4.30E-03</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>forebrain-midbrain boundary formation</td>
</tr>
<tr>
<td>GO:0021902</td>
<td>4.30E-03</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>commitment of a neuronal cell to a specific type of neuron in the forebrain</td>
</tr>
<tr>
<td>GO:0019896</td>
<td>4.30E-03</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>axon transport of mitochondrion</td>
</tr>
<tr>
<td>GO:0051639</td>
<td>4.30E-03</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>actin filament network formation</td>
</tr>
<tr>
<td>GO:0001775</td>
<td>4.50E-03</td>
<td>1.09</td>
<td>5</td>
<td>250</td>
<td>cell activation</td>
</tr>
<tr>
<td>GO:0002208</td>
<td>4.80E-03</td>
<td>0.1</td>
<td>2</td>
<td>24</td>
<td>somatic diversification of immunoglobulins during immune response</td>
</tr>
<tr>
<td>GO:0002204</td>
<td>4.80E-03</td>
<td>0.1</td>
<td>2</td>
<td>24</td>
<td>somatic recombination of immunoglobulin genes during immune response</td>
</tr>
<tr>
<td>GO:0045190</td>
<td>4.80E-03</td>
<td>0.1</td>
<td>2</td>
<td>24</td>
<td>isotype switching</td>
</tr>
<tr>
<td>GO:0050863</td>
<td>5.60E-03</td>
<td>0.36</td>
<td>3</td>
<td>83</td>
<td>regulation of T cell activation</td>
</tr>
<tr>
<td>GO:0002381</td>
<td>5.60E-03</td>
<td>0.11</td>
<td>2</td>
<td>26</td>
<td>immunoglobulin production during immune response</td>
</tr>
<tr>
<td>GO:0045562</td>
<td>6.10E-03</td>
<td>0.12</td>
<td>2</td>
<td>27</td>
<td>positive regulation of T cell differentiation</td>
</tr>
<tr>
<td>GO:0050896</td>
<td>6.40E-03</td>
<td>7.63</td>
<td>15</td>
<td>1749</td>
<td>response to stimulus</td>
</tr>
<tr>
<td>GO:0006955</td>
<td>6.50E-03</td>
<td>1.69</td>
<td>6</td>
<td>387</td>
<td>immune response</td>
</tr>
<tr>
<td>GO:0016447</td>
<td>7.00E-03</td>
<td>0.13</td>
<td>2</td>
<td>29</td>
<td>somatic recombination of immunoglobulin gene segments</td>
</tr>
<tr>
<td>GO:0006887</td>
<td>7.00E-03</td>
<td>0.39</td>
<td>3</td>
<td>90</td>
<td>exocytosis</td>
</tr>
<tr>
<td>GO:0045621</td>
<td>7.50E-03</td>
<td>0.13</td>
<td>2</td>
<td>30</td>
<td>positive regulation of lymphocyte differentiation</td>
</tr>
<tr>
<td>GO:0016445</td>
<td>7.50E-03</td>
<td>0.13</td>
<td>2</td>
<td>30</td>
<td>somatic diversification of immunoglobulins</td>
</tr>
<tr>
<td>GO:0042102</td>
<td>7.50E-03</td>
<td>0.13</td>
<td>2</td>
<td>30</td>
<td>positive regulation of T cell proliferation</td>
</tr>
<tr>
<td>GO:0050851</td>
<td>7.50E-03</td>
<td>0.13</td>
<td>2</td>
<td>30</td>
<td>antigen receptor-mediated signaling pathway</td>
</tr>
<tr>
<td>GO:0002682</td>
<td>7.90E-03</td>
<td>0.79</td>
<td>4</td>
<td>181</td>
<td>regulation of immune system process</td>
</tr>
<tr>
<td>GO:0050778</td>
<td>7.90E-03</td>
<td>0.41</td>
<td>3</td>
<td>94</td>
<td>positive regulation of immune response</td>
</tr>
<tr>
<td>GO:0045597</td>
<td>8.10E-03</td>
<td>0.41</td>
<td>3</td>
<td>95</td>
<td>positive regulation of cell differentiation</td>
</tr>
<tr>
<td>GO:0002250</td>
<td>8.10E-03</td>
<td>0.41</td>
<td>3</td>
<td>95</td>
<td>adaptive immune response</td>
</tr>
<tr>
<td>GO:0002460</td>
<td>8.10E-03</td>
<td>0.41</td>
<td>3</td>
<td>95</td>
<td>adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains</td>
</tr>
<tr>
<td>GO:0002449</td>
<td>8.40E-03</td>
<td>0.42</td>
<td>3</td>
<td>96</td>
<td>lymphocyte mediated immunity</td>
</tr>
<tr>
<td>GO:0001756</td>
<td>8.50E-03</td>
<td>0.14</td>
<td>2</td>
<td>32</td>
<td>somitogenesis</td>
</tr>
<tr>
<td>GO:0014832</td>
<td>8.70E-03</td>
<td>0.01</td>
<td>1</td>
<td>2</td>
<td>urinary bladder smooth muscle contraction</td>
</tr>
<tr>
<td>GO:0014848</td>
<td>8.70E-03</td>
<td>0.01</td>
<td>1</td>
<td>2</td>
<td>urinary tract smooth muscle contraction</td>
</tr>
<tr>
<td>GO:0043366</td>
<td>8.70E-03</td>
<td>0.01</td>
<td>1</td>
<td>2</td>
<td>beta selection</td>
</tr>
<tr>
<td>GO:0030046</td>
<td>8.70E-03</td>
<td>0.01</td>
<td>1</td>
<td>2</td>
<td>parallel actin filament bundle formation</td>
</tr>
<tr>
<td>GO:0002562</td>
<td>9.00E-03</td>
<td>0.14</td>
<td>2</td>
<td>33</td>
<td>recombination within a single locus</td>
</tr>
<tr>
<td>GO:0016444</td>
<td>9.00E-03</td>
<td>0.14</td>
<td>2</td>
<td>33</td>
<td>somatic cell DNA recombination</td>
</tr>
<tr>
<td>GO:0002455</td>
<td>9.00E-03</td>
<td>0.14</td>
<td>2</td>
<td>33</td>
<td>humoral immune response mediated by circulating immunoglobulin</td>
</tr>
<tr>
<td>GO:0002200</td>
<td>9.50E-03</td>
<td>0.15</td>
<td>2</td>
<td>34</td>
<td>somatic diversification of immune receptors</td>
</tr>
</tbody>
</table>
Table 13: (+2, +2) GO terms and p-values (Day 14 Strain Comparison)

<table>
<thead>
<tr>
<th>GO Id</th>
<th>P Value</th>
<th>Exp Count</th>
<th>Count</th>
<th>Size</th>
<th>GO Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0006334</td>
<td>6.09E-05</td>
<td>0.68</td>
<td>6</td>
<td>77</td>
<td>nucleosome assembly</td>
</tr>
<tr>
<td>GO:0031497</td>
<td>1.46E-04</td>
<td>0.8</td>
<td>6</td>
<td>90</td>
<td>chromatin assembly</td>
</tr>
<tr>
<td>GO:0022607</td>
<td>2.15E-04</td>
<td>1.22</td>
<td>7</td>
<td>137</td>
<td>cellular component assembly</td>
</tr>
<tr>
<td>GO:0085004</td>
<td>3.05E-04</td>
<td>0.91</td>
<td>6</td>
<td>103</td>
<td>protein-DNA complex assembly</td>
</tr>
<tr>
<td>GO:0006323</td>
<td>3.93E-04</td>
<td>0.96</td>
<td>6</td>
<td>108</td>
<td>DNA packaging</td>
</tr>
<tr>
<td>GO:0006333</td>
<td>5.25E-04</td>
<td>1.01</td>
<td>6</td>
<td>114</td>
<td>chromatin assembly or disassembly</td>
</tr>
<tr>
<td>GO:0043269</td>
<td>8.07E-04</td>
<td>0.19</td>
<td>3</td>
<td>21</td>
<td>regulation of ion transport</td>
</tr>
<tr>
<td>GO:006941</td>
<td>1.90E-03</td>
<td>0.25</td>
<td>3</td>
<td>28</td>
<td>striated muscle contraction</td>
</tr>
<tr>
<td>GO:0042434</td>
<td>2.70E-03</td>
<td>0.08</td>
<td>2</td>
<td>9</td>
<td>indole derivative metabolic process</td>
</tr>
<tr>
<td>GO:0042430</td>
<td>2.70E-03</td>
<td>0.08</td>
<td>2</td>
<td>9</td>
<td>indole and derivative metabolic process</td>
</tr>
<tr>
<td>GO:0006586</td>
<td>2.70E-03</td>
<td>0.08</td>
<td>2</td>
<td>9</td>
<td>indolalkylamine metabolic process</td>
</tr>
<tr>
<td>GO:007009</td>
<td>3.30E-03</td>
<td>0.09</td>
<td>2</td>
<td>10</td>
<td>plasma membrane organization and biogenesis</td>
</tr>
<tr>
<td>GO:0006936</td>
<td>3.80E-03</td>
<td>0.64</td>
<td>4</td>
<td>72</td>
<td>muscle contraction</td>
</tr>
<tr>
<td>GO:0003012</td>
<td>4.20E-03</td>
<td>0.66</td>
<td>4</td>
<td>74</td>
<td>muscle system process</td>
</tr>
<tr>
<td>GO:0051924</td>
<td>5.70E-03</td>
<td>0.12</td>
<td>2</td>
<td>13</td>
<td>regulation of calcium ion transport</td>
</tr>
<tr>
<td>GO:0009063</td>
<td>6.00E-03</td>
<td>0.37</td>
<td>3</td>
<td>42</td>
<td>amino acid catabolic process</td>
</tr>
<tr>
<td>GO:0051276</td>
<td>6.30E-03</td>
<td>2.75</td>
<td>8</td>
<td>310</td>
<td>chromosome organization and biogenesis</td>
</tr>
<tr>
<td>GO:0009308</td>
<td>6.50E-03</td>
<td>2.2</td>
<td>7</td>
<td>248</td>
<td>amine metabolic process</td>
</tr>
<tr>
<td>GO:0030514</td>
<td>6.60E-03</td>
<td>0.12</td>
<td>2</td>
<td>14</td>
<td>negative regulation of BMP signaling pathway</td>
</tr>
<tr>
<td>GO:006325</td>
<td>6.70E-03</td>
<td>2.21</td>
<td>7</td>
<td>249</td>
<td>establishment and/or maintenance of chromatin architecture</td>
</tr>
<tr>
<td>GO:0021703</td>
<td>8.80E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>locus ceruleus development</td>
</tr>
<tr>
<td>GO:0032769</td>
<td>8.80E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>negative regulation of monoxygenase activity</td>
</tr>
<tr>
<td>GO:007208</td>
<td>8.80E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>serotonin receptor, phospholipase C activating pathway</td>
</tr>
<tr>
<td>GO:0051354</td>
<td>8.80E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>negative regulation of oxidoreductase activity</td>
</tr>
<tr>
<td>GO:0051001</td>
<td>8.80E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>negative regulation of nitric-oxide synthase activity</td>
</tr>
<tr>
<td>GO:0015817</td>
<td>8.80E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>histidine transport</td>
</tr>
</tbody>
</table>
Table 14: (-2, -2) GO terms and p-values (Day 14 Strain Comparison)

<table>
<thead>
<tr>
<th>GO Id</th>
<th>P Value</th>
<th>Exp Count</th>
<th>Count</th>
<th>Size</th>
<th>GO Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0006955</td>
<td>2.23E-05</td>
<td>2.38</td>
<td>11</td>
<td>387</td>
<td>immune response</td>
</tr>
<tr>
<td>GO:0002376</td>
<td>5.81E-04</td>
<td>4.01</td>
<td>12</td>
<td>653</td>
<td>immune system process</td>
</tr>
<tr>
<td>GO:0048002</td>
<td>5.85E-04</td>
<td>0.17</td>
<td>3</td>
<td>27</td>
<td>antigen processing and presentation of peptide antigen</td>
</tr>
<tr>
<td>GO:0002706</td>
<td>8.83E-04</td>
<td>0.19</td>
<td>3</td>
<td>31</td>
<td>regulation of lymphocyte mediated immunity</td>
</tr>
<tr>
<td>GO:0002703</td>
<td>8.83E-04</td>
<td>0.19</td>
<td>3</td>
<td>31</td>
<td>regulation of leukocyte mediated immunity</td>
</tr>
<tr>
<td>GO:0002697</td>
<td>1.60E-03</td>
<td>0.23</td>
<td>3</td>
<td>38</td>
<td>regulation of immune effector process</td>
</tr>
<tr>
<td>GO:0042755</td>
<td>1.90E-03</td>
<td>0.07</td>
<td>2</td>
<td>11</td>
<td>eating behavior</td>
</tr>
<tr>
<td>GO:0001818</td>
<td>2.30E-03</td>
<td>0.07</td>
<td>2</td>
<td>12</td>
<td>negative regulation of cytokine production</td>
</tr>
<tr>
<td>GO:0002769</td>
<td>2.30E-03</td>
<td>0.56</td>
<td>4</td>
<td>91</td>
<td>chemotaxis</td>
</tr>
<tr>
<td>GO:0042330</td>
<td>2.30E-03</td>
<td>0.56</td>
<td>4</td>
<td>91</td>
<td>taxis</td>
</tr>
<tr>
<td>GO:0019882</td>
<td>3.10E-03</td>
<td>0.29</td>
<td>3</td>
<td>48</td>
<td>antigen processing and presentation</td>
</tr>
<tr>
<td>GO:0019886</td>
<td>3.20E-03</td>
<td>0.09</td>
<td>2</td>
<td>14</td>
<td>antigen processing and presentation of exogenous peptide antigen via MHC class II</td>
</tr>
<tr>
<td>GO:0002495</td>
<td>3.20E-03</td>
<td>0.09</td>
<td>2</td>
<td>14</td>
<td>antigen processing and presentation of peptide antigen via MHC class II</td>
</tr>
<tr>
<td>GO:002504</td>
<td>4.20E-03</td>
<td>0.1</td>
<td>2</td>
<td>16</td>
<td>antigen processing and presentation of peptide or polysaccharide antigen via MHC class II</td>
</tr>
<tr>
<td>GO:002478</td>
<td>4.20E-03</td>
<td>0.1</td>
<td>2</td>
<td>16</td>
<td>antigen processing and presentation of exogenous peptide antigen</td>
</tr>
<tr>
<td>GO:0007339</td>
<td>4.70E-03</td>
<td>0.11</td>
<td>2</td>
<td>18</td>
<td>binding of sperm to zona pellucida</td>
</tr>
<tr>
<td>GO:0035036</td>
<td>5.30E-03</td>
<td>0.11</td>
<td>2</td>
<td>18</td>
<td>sperm-egg recognition</td>
</tr>
<tr>
<td>GO:002824</td>
<td>5.30E-03</td>
<td>0.11</td>
<td>2</td>
<td>18</td>
<td>positive regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains</td>
</tr>
<tr>
<td>GO:0002821</td>
<td>5.30E-03</td>
<td>0.11</td>
<td>2</td>
<td>18</td>
<td>positive regulation of adaptive immune response</td>
</tr>
<tr>
<td>GO:0031341</td>
<td>5.90E-03</td>
<td>0.12</td>
<td>2</td>
<td>19</td>
<td>regulation of cell killing</td>
</tr>
<tr>
<td>GO:0001910</td>
<td>5.90E-03</td>
<td>0.12</td>
<td>2</td>
<td>19</td>
<td>regulation of leukocyte mediated cytotoxicity</td>
</tr>
<tr>
<td>GO:002428</td>
<td>6.10E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>antigen processing and presentation of peptide antigen</td>
</tr>
<tr>
<td>GO:0002769</td>
<td>6.10E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>natural killer cell inhibitory signaling pathway</td>
</tr>
<tr>
<td>GO:002767</td>
<td>6.10E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>immune response-inhibiting cell surface receptor</td>
</tr>
<tr>
<td>GO:002765</td>
<td>6.10E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>immune response-inhibiting signal transduction</td>
</tr>
<tr>
<td>GO:0030886</td>
<td>6.10E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>regulation of myeloid dendritic cell activation</td>
</tr>
<tr>
<td>GO:0030885</td>
<td>6.10E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>regulation of myeloid dendritic cell activation</td>
</tr>
<tr>
<td>GO:0019896</td>
<td>6.10E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>axon transport of mitochondrion</td>
</tr>
<tr>
<td>GO:002476</td>
<td>6.10E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>antigen processing and presentation of endogenous</td>
</tr>
<tr>
<td>GO:0051151</td>
<td>6.10E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>negative regulation of smooth muscle cell differentiation</td>
</tr>
<tr>
<td>GO:0004988</td>
<td>6.50E-03</td>
<td>0.12</td>
<td>2</td>
<td>20</td>
<td>cell-cell recognition</td>
</tr>
<tr>
<td>GO:0019884</td>
<td>7.20E-03</td>
<td>0.13</td>
<td>2</td>
<td>21</td>
<td>antigen processing and presentation of exogenous</td>
</tr>
<tr>
<td>GO:0002705</td>
<td>7.90E-03</td>
<td>0.14</td>
<td>2</td>
<td>22</td>
<td>positive regulation of leukocyte mediated immunity</td>
</tr>
<tr>
<td>GO:0002708</td>
<td>7.90E-03</td>
<td>0.14</td>
<td>2</td>
<td>22</td>
<td>positive regulation of lymphocyte mediated immunity</td>
</tr>
<tr>
<td>GO:0007626</td>
<td>8.40E-03</td>
<td>1.26</td>
<td>5</td>
<td>205</td>
<td>locomotory behavior</td>
</tr>
<tr>
<td>GO:001909</td>
<td>9.40E-03</td>
<td>0.15</td>
<td>2</td>
<td>24</td>
<td>leukocyte mediated cytotoxicity</td>
</tr>
<tr>
<td>GO:001906</td>
<td>9.40E-03</td>
<td>0.15</td>
<td>2</td>
<td>24</td>
<td>cell killing</td>
</tr>
<tr>
<td>GO:002699</td>
<td>9.40E-03</td>
<td>0.15</td>
<td>2</td>
<td>24</td>
<td>positive regulation of immune effector process</td>
</tr>
</tbody>
</table>
Fig 16: Comparisons Within Strains Over Time

Fig. 16a Comparison 1- C57BL/6

C57BL/6 D3 → C57BL/6 D7 → C57BL/6 D14

(+2, +2)  (-2, -2)
Table 15: (+2, +2) GO terms and p-values (C57BL/6 Time Comparison)

<table>
<thead>
<tr>
<th>GO Id</th>
<th>P Value</th>
<th>Exp Count</th>
<th>Count</th>
<th>Size</th>
<th>GO Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0006936</td>
<td>8.79E-10</td>
<td>1.11</td>
<td>12</td>
<td>72</td>
<td>muscle contraction</td>
</tr>
<tr>
<td>GO:0003012</td>
<td>1.22E-09</td>
<td>1.14</td>
<td>12</td>
<td>74</td>
<td>muscle system process</td>
</tr>
<tr>
<td>GO:0055001</td>
<td>3.37E-08</td>
<td>0.22</td>
<td>6</td>
<td>14</td>
<td>muscle cell development</td>
</tr>
<tr>
<td>GO:0045445</td>
<td>4.56E-08</td>
<td>0.54</td>
<td>8</td>
<td>35</td>
<td>myoblast differentiation</td>
</tr>
<tr>
<td>GO:0048747</td>
<td>8.06E-08</td>
<td>1.05</td>
<td>10</td>
<td>68</td>
<td>muscle fiber development</td>
</tr>
<tr>
<td>GO:0048741</td>
<td>8.06E-08</td>
<td>1.05</td>
<td>10</td>
<td>68</td>
<td>skeletal muscle fiber development</td>
</tr>
<tr>
<td>GO:0048628</td>
<td>4.03E-07</td>
<td>0.31</td>
<td>6</td>
<td>20</td>
<td>myoblast maturation</td>
</tr>
<tr>
<td>GO:0048627</td>
<td>5.57E-07</td>
<td>0.32</td>
<td>6</td>
<td>21</td>
<td>myoblast development</td>
</tr>
<tr>
<td>GO:0007519</td>
<td>7.53E-07</td>
<td>1.65</td>
<td>11</td>
<td>107</td>
<td>skeletal muscle development</td>
</tr>
<tr>
<td>GO:0055002</td>
<td>9.65E-07</td>
<td>0.2</td>
<td>5</td>
<td>13</td>
<td>striated muscle cell development</td>
</tr>
<tr>
<td>GO:0030239</td>
<td>9.65E-07</td>
<td>0.2</td>
<td>5</td>
<td>13</td>
<td>myofibril assembly</td>
</tr>
<tr>
<td>GO:006941</td>
<td>3.54E-06</td>
<td>0.43</td>
<td>6</td>
<td>28</td>
<td>striated muscle contraction</td>
</tr>
<tr>
<td>GO:0031032</td>
<td>6.03E-06</td>
<td>0.28</td>
<td>5</td>
<td>18</td>
<td>actomyosin structure organization and biogenesis</td>
</tr>
<tr>
<td>GO:0014706</td>
<td>8.61E-06</td>
<td>2.11</td>
<td>11</td>
<td>137</td>
<td>striated muscle development</td>
</tr>
<tr>
<td>GO:0042692</td>
<td>1.04E-05</td>
<td>1.06</td>
<td>8</td>
<td>69</td>
<td>muscle cell differentiation</td>
</tr>
<tr>
<td>GO:0045214</td>
<td>1.07E-05</td>
<td>0.15</td>
<td>4</td>
<td>10</td>
<td>sarcomere organization</td>
</tr>
<tr>
<td>GO:0007517</td>
<td>9.30E-05</td>
<td>2.73</td>
<td>11</td>
<td>177</td>
<td>muscle development</td>
</tr>
<tr>
<td>GO:0051146</td>
<td>1.02E-04</td>
<td>0.48</td>
<td>5</td>
<td>31</td>
<td>striated muscle cell differentiation</td>
</tr>
<tr>
<td>GO:0006073</td>
<td>1.19E-04</td>
<td>0.49</td>
<td>5</td>
<td>32</td>
<td>glucan metabolic process</td>
</tr>
<tr>
<td>GO:0005977</td>
<td>1.19E-04</td>
<td>0.49</td>
<td>5</td>
<td>32</td>
<td>glycogen metabolic process</td>
</tr>
<tr>
<td>GO:0030001</td>
<td>1.24E-04</td>
<td>6.06</td>
<td>17</td>
<td>393</td>
<td>metal ion transport</td>
</tr>
<tr>
<td>GO:0006811</td>
<td>1.47E-04</td>
<td>10.6</td>
<td>24</td>
<td>687</td>
<td>ion transport</td>
</tr>
<tr>
<td>GO:0006112</td>
<td>1.85E-04</td>
<td>0.54</td>
<td>5</td>
<td>35</td>
<td>energy reserve metabolic process</td>
</tr>
<tr>
<td>GO:0006812</td>
<td>2.02E-04</td>
<td>6.93</td>
<td>18</td>
<td>449</td>
<td>cation transport</td>
</tr>
<tr>
<td>GO:0005978</td>
<td>4.00E-04</td>
<td>0.15</td>
<td>3</td>
<td>10</td>
<td>glycogen biosynthetic process</td>
</tr>
<tr>
<td>GO:0009250</td>
<td>4.00E-04</td>
<td>0.15</td>
<td>3</td>
<td>10</td>
<td>glucan biosynthetic process</td>
</tr>
<tr>
<td>GO:0015672</td>
<td>4.03E-04</td>
<td>4.32</td>
<td>13</td>
<td>280</td>
<td>monovalent inorganic cation transport</td>
</tr>
<tr>
<td>GO:0006937</td>
<td>5.38E-04</td>
<td>0.39</td>
<td>4</td>
<td>25</td>
<td>regulation of muscle contraction</td>
</tr>
<tr>
<td>GO:0005976</td>
<td>8.28E-04</td>
<td>0.74</td>
<td>5</td>
<td>48</td>
<td>polysaccharide metabolic process</td>
</tr>
<tr>
<td>GO:0044264</td>
<td>8.28E-04</td>
<td>0.74</td>
<td>5</td>
<td>48</td>
<td>cellular polysaccharide metabolic process</td>
</tr>
<tr>
<td>GO:0048469</td>
<td>1.20E-03</td>
<td>1.2</td>
<td>6</td>
<td>78</td>
<td>cell maturation</td>
</tr>
<tr>
<td>GO:0045662</td>
<td>1.30E-03</td>
<td>0.06</td>
<td>2</td>
<td>4</td>
<td>negative regulation of myoblast differentiation</td>
</tr>
<tr>
<td>GO:0033692</td>
<td>2.00E-03</td>
<td>0.26</td>
<td>3</td>
<td>17</td>
<td>cellular polysaccharide biosynthetic process</td>
</tr>
<tr>
<td>GO:0007339</td>
<td>2.00E-03</td>
<td>0.26</td>
<td>3</td>
<td>17</td>
<td>binding of sperm to zona pellucida</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>positive regulation of G-protein coupled receptor protein</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>signaling pathway</td>
</tr>
<tr>
<td>GO:0000271</td>
<td>2.40E-03</td>
<td>0.28</td>
<td>3</td>
<td>18</td>
<td>polysaccharide biosynthetic process</td>
</tr>
<tr>
<td>GO:0035036</td>
<td>2.40E-03</td>
<td>0.28</td>
<td>3</td>
<td>18</td>
<td>sperm-egg recognition</td>
</tr>
<tr>
<td>GO:0015980</td>
<td>3.00E-03</td>
<td>0.99</td>
<td>5</td>
<td>64</td>
<td>energy derivation by oxidation of organic compounds</td>
</tr>
<tr>
<td>GO:0009988</td>
<td>3.30E-03</td>
<td>0.31</td>
<td>3</td>
<td>20</td>
<td>cell-cell recognition</td>
</tr>
<tr>
<td>GO:0051148</td>
<td>3.40E-03</td>
<td>0.09</td>
<td>2</td>
<td>6</td>
<td>negative regulation of muscle cell differentiation</td>
</tr>
<tr>
<td>GO:0055065</td>
<td>4.10E-03</td>
<td>1.06</td>
<td>5</td>
<td>69</td>
<td>metal ion homeostasis</td>
</tr>
<tr>
<td>GO:0007338</td>
<td>4.20E-03</td>
<td>0.66</td>
<td>4</td>
<td>43</td>
<td>single fertilization</td>
</tr>
<tr>
<td>GO:0001600</td>
<td>4.40E-03</td>
<td>0.34</td>
<td>3</td>
<td>22</td>
<td>two-component signal transduction system (phosphorelay)</td>
</tr>
<tr>
<td>GO:0021700</td>
<td>4.60E-03</td>
<td>1.56</td>
<td>6</td>
<td>101</td>
<td>developmental maturation</td>
</tr>
<tr>
<td>GO:0001666</td>
<td>5.00E-03</td>
<td>0.35</td>
<td>3</td>
<td>23</td>
<td>response to hypoxia</td>
</tr>
<tr>
<td>GO:0022607</td>
<td>5.30E-03</td>
<td>2.11</td>
<td>7</td>
<td>137</td>
<td>cellular component assembly</td>
</tr>
<tr>
<td>GO:0045661</td>
<td>6.20E-03</td>
<td>0.12</td>
<td>2</td>
<td>8</td>
<td>regulation of myoblast differentiation</td>
</tr>
<tr>
<td>GO:0007623</td>
<td>8.00E-03</td>
<td>0.42</td>
<td>3</td>
<td>27</td>
<td>circadian rhythm</td>
</tr>
<tr>
<td>GO:0069444</td>
<td>8.00E-03</td>
<td>0.42</td>
<td>3</td>
<td>27</td>
<td>membrane fusion</td>
</tr>
<tr>
<td>GO:0030036</td>
<td>9.80E-03</td>
<td>2.38</td>
<td>7</td>
<td>154</td>
<td>actin cytoskeleton organization and biogenesis</td>
</tr>
</tbody>
</table>
## Table 16: (-2, -2) GO terms and p-values (C57BL/6 Time Comparison)

<table>
<thead>
<tr>
<th>GO Id</th>
<th>P Value</th>
<th>Exp Count</th>
<th>Count</th>
<th>Size</th>
<th>GO Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0022402</td>
<td>4.96E-09</td>
<td>4.98</td>
<td>22</td>
<td>321</td>
<td>cell cycle process</td>
</tr>
<tr>
<td>GO:0007049</td>
<td>3.47E-08</td>
<td>9.19</td>
<td>29</td>
<td>593</td>
<td>cell cycle</td>
</tr>
<tr>
<td>GO:0006323</td>
<td>1.04E-07</td>
<td>1.67</td>
<td>12</td>
<td>108</td>
<td>DNA packaging</td>
</tr>
<tr>
<td>GO:0031497</td>
<td>1.34E-07</td>
<td>1.1</td>
<td>1</td>
<td>90</td>
<td>chromatin assembly</td>
</tr>
<tr>
<td>GO:0000279</td>
<td>1.69E-07</td>
<td>3.69</td>
<td>17</td>
<td>238</td>
<td>M phase</td>
</tr>
<tr>
<td>GO:0022607</td>
<td>1.99E-07</td>
<td>2.12</td>
<td>13</td>
<td>137</td>
<td>cellular component assembly</td>
</tr>
<tr>
<td>GO:0022403</td>
<td>2.53E-07</td>
<td>4.25</td>
<td>18</td>
<td>274</td>
<td>cell cycle phase</td>
</tr>
<tr>
<td>GO:0006334</td>
<td>2.83E-07</td>
<td>1.19</td>
<td>10</td>
<td>77</td>
<td>nucleosome assembly</td>
</tr>
<tr>
<td>GO:0065004</td>
<td>5.39E-07</td>
<td>1.6</td>
<td>11</td>
<td>103</td>
<td>protein-DNA complex assembly</td>
</tr>
<tr>
<td>GO:0009611</td>
<td>5.56E-07</td>
<td>4.48</td>
<td>18</td>
<td>289</td>
<td>response to wounding</td>
</tr>
<tr>
<td>GO:0051301</td>
<td>7.11E-07</td>
<td>3.63</td>
<td>16</td>
<td>234</td>
<td>cell division</td>
</tr>
<tr>
<td>GO:0006959</td>
<td>2.40E-06</td>
<td>6</td>
<td>20</td>
<td>387</td>
<td>immune response</td>
</tr>
<tr>
<td>GO:0000278</td>
<td>4.39E-07</td>
<td>1.19</td>
<td>10</td>
<td>77</td>
<td>M phase of mitotic cell cycle</td>
</tr>
<tr>
<td>GO:0000277</td>
<td>7.46E-06</td>
<td>3.38</td>
<td>14</td>
<td>218</td>
<td>mitotic cell cycle</td>
</tr>
<tr>
<td>GO:0000087</td>
<td>8.10E-06</td>
<td>2.51</td>
<td>12</td>
<td>162</td>
<td>response to stress</td>
</tr>
<tr>
<td>GO:0007067</td>
<td>8.10E-06</td>
<td>2.51</td>
<td>12</td>
<td>162</td>
<td>mitosis</td>
</tr>
<tr>
<td>GO:0006954</td>
<td>1.40E-05</td>
<td>3.1</td>
<td>13</td>
<td>200</td>
<td>inflammatory response</td>
</tr>
<tr>
<td>GO:0006950</td>
<td>9.71E-06</td>
<td>4.81</td>
<td>15</td>
<td>310</td>
<td>chromosome organization and biogenesis</td>
</tr>
<tr>
<td>GO:0006952</td>
<td>1.44E-04</td>
<td>5.55</td>
<td>16</td>
<td>358</td>
<td>defense response</td>
</tr>
<tr>
<td>GO:0006259</td>
<td>2.13E-04</td>
<td>5.16</td>
<td>15</td>
<td>333</td>
<td>DNA metabolic process</td>
</tr>
<tr>
<td>GO:0006260</td>
<td>2.37E-04</td>
<td>2.08</td>
<td>9</td>
<td>134</td>
<td>DNA replication</td>
</tr>
<tr>
<td>GO:0034622</td>
<td>3.00E-04</td>
<td>3.64</td>
<td>12</td>
<td>235</td>
<td>cellular macromolecular complex assembly</td>
</tr>
<tr>
<td>GO:0050896</td>
<td>3.36E-04</td>
<td>27.12</td>
<td>45</td>
<td>1749</td>
<td>response to stimulus</td>
</tr>
<tr>
<td>GO:0065003</td>
<td>4.69E-04</td>
<td>4.39</td>
<td>13</td>
<td>281</td>
<td>macromolecular complex assembly</td>
</tr>
<tr>
<td>GO:0034621</td>
<td>1.40E-03</td>
<td>4.34</td>
<td>12</td>
<td>280</td>
<td>cellular macromolecular complex subunit orgniz</td>
</tr>
<tr>
<td>GO:0006325</td>
<td>1.70E-03</td>
<td>3.86</td>
<td>11</td>
<td>249</td>
<td>establishment and/or maintenance of chromatin architecture</td>
</tr>
<tr>
<td>GO:0043933</td>
<td>1.90E-03</td>
<td>5.12</td>
<td>13</td>
<td>330</td>
<td>macromolecular complex subunit organization</td>
</tr>
<tr>
<td>GO:0006909</td>
<td>2.70E-03</td>
<td>0.59</td>
<td>4</td>
<td>38</td>
<td>phagocytosis</td>
</tr>
<tr>
<td>GO:0008219</td>
<td>3.30E-03</td>
<td>10.28</td>
<td>20</td>
<td>663</td>
<td>cell death</td>
</tr>
<tr>
<td>GO:0012502</td>
<td>3.60E-03</td>
<td>1.97</td>
<td>7</td>
<td>127</td>
<td>induction of programmed cell death</td>
</tr>
<tr>
<td>GO:006917</td>
<td>3.60E-03</td>
<td>1.97</td>
<td>7</td>
<td>127</td>
<td>induction of apoptosis</td>
</tr>
<tr>
<td>GO:0016265</td>
<td>3.70E-03</td>
<td>10.39</td>
<td>20</td>
<td>670</td>
<td>death</td>
</tr>
<tr>
<td>GO:0006915</td>
<td>4.00E-03</td>
<td>9.74</td>
<td>19</td>
<td>628</td>
<td>apoptosis</td>
</tr>
<tr>
<td>GO:0007050</td>
<td>4.60E-03</td>
<td>0.68</td>
<td>4</td>
<td>44</td>
<td>cell cycle arrest</td>
</tr>
<tr>
<td>GO:0012501</td>
<td>4.70E-03</td>
<td>9.88</td>
<td>19</td>
<td>637</td>
<td>programmed cell death</td>
</tr>
<tr>
<td>GO:0006525</td>
<td>6.20E-03</td>
<td>0.12</td>
<td>2</td>
<td>8</td>
<td>arginine metabolic process</td>
</tr>
<tr>
<td>GO:0051130</td>
<td>7.30E-03</td>
<td>0.78</td>
<td>4</td>
<td>50</td>
<td>positive regulation of cellular component organization and biogenesis</td>
</tr>
<tr>
<td>GO:0000050</td>
<td>8.00E-03</td>
<td>0.14</td>
<td>2</td>
<td>9</td>
<td>urea cycle</td>
</tr>
<tr>
<td>GO:0043603</td>
<td>8.00E-03</td>
<td>0.14</td>
<td>2</td>
<td>9</td>
<td>amide metabolic process</td>
</tr>
<tr>
<td>GO:0019627</td>
<td>8.00E-03</td>
<td>0.14</td>
<td>2</td>
<td>9</td>
<td>urea metabolic process</td>
</tr>
<tr>
<td>GO:0006857</td>
<td>9.90E-03</td>
<td>0.16</td>
<td>2</td>
<td>10</td>
<td>oligopeptide transport</td>
</tr>
<tr>
<td>GO:0051607</td>
<td>9.90E-03</td>
<td>0.16</td>
<td>2</td>
<td>10</td>
<td>defense response to virus</td>
</tr>
</tbody>
</table>
Fig. 16b Comparison 2- LG/J

LG/J D3 → LG/J D7 → LG/J D14

(+2, +2) → (-2, -2)
Table 17: (+2, +2) GO terms and p-values (LG/J Time Comparison)

<table>
<thead>
<tr>
<th>GO Id</th>
<th>P Value</th>
<th>Exp Count</th>
<th>Count</th>
<th>Size</th>
<th>GO Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0007155</td>
<td>2.31E-12</td>
<td>9.83</td>
<td>37</td>
<td>556</td>
<td>cell adhesion</td>
</tr>
<tr>
<td>GO:0022610</td>
<td>2.31E-12</td>
<td>9.83</td>
<td>37</td>
<td>556</td>
<td>biological adhesion</td>
</tr>
<tr>
<td>GO:0006817</td>
<td>6.56E-06</td>
<td>1.33</td>
<td>9</td>
<td>75</td>
<td>phosphate transport</td>
</tr>
<tr>
<td>GO:0006811</td>
<td>3.08E-05</td>
<td>12.15</td>
<td>28</td>
<td>687</td>
<td>ion transport</td>
</tr>
<tr>
<td>GO:0015698</td>
<td>3.32E-05</td>
<td>2.44</td>
<td>11</td>
<td>138</td>
<td>inorganic anion transport</td>
</tr>
<tr>
<td>GO:0001501</td>
<td>3.85E-05</td>
<td>2.94</td>
<td>12</td>
<td>166</td>
<td>skeletal development</td>
</tr>
<tr>
<td>GO:0006820</td>
<td>4.34E-05</td>
<td>2.97</td>
<td>12</td>
<td>168</td>
<td>anion transport</td>
</tr>
<tr>
<td>GO:0006958</td>
<td>6.58E-05</td>
<td>0.44</td>
<td>5</td>
<td>25</td>
<td>complement activation, classical pathway</td>
</tr>
<tr>
<td>GO:0006959</td>
<td>1.48E-04</td>
<td>0.81</td>
<td>6</td>
<td>46</td>
<td>humoral immune response</td>
</tr>
<tr>
<td>GO:0002526</td>
<td>1.58E-04</td>
<td>1.17</td>
<td>7</td>
<td>66</td>
<td>acute inflammatory response</td>
</tr>
<tr>
<td>GO:0002541</td>
<td>1.64E-04</td>
<td>0.53</td>
<td>5</td>
<td>30</td>
<td>activation of plasma proteins during acute inflammatory response</td>
</tr>
<tr>
<td>GO:0006956</td>
<td>1.64E-04</td>
<td>0.53</td>
<td>5</td>
<td>30</td>
<td>complement activation</td>
</tr>
<tr>
<td>GO:0051216</td>
<td>1.74E-04</td>
<td>1.18</td>
<td>7</td>
<td>67</td>
<td>cartilage development</td>
</tr>
<tr>
<td>GO:0002455</td>
<td>2.62E-04</td>
<td>0.58</td>
<td>5</td>
<td>33</td>
<td>humoral immune response mediated by circulating immunoglobulin</td>
</tr>
<tr>
<td>GO:0006954</td>
<td>8.61E-04</td>
<td>3.54</td>
<td>11</td>
<td>200</td>
<td>inflammatory response</td>
</tr>
<tr>
<td>GO:0006508</td>
<td>8.98E-04</td>
<td>9.18</td>
<td>20</td>
<td>519</td>
<td>proteolysis</td>
</tr>
<tr>
<td>GO:0045663</td>
<td>9.23E-04</td>
<td>0.05</td>
<td>2</td>
<td>3</td>
<td>positive regulation of myoblast differentiation</td>
</tr>
<tr>
<td>GO:0016064</td>
<td>1.10E-03</td>
<td>1.18</td>
<td>6</td>
<td>67</td>
<td>immunoglobulin mediated immune response</td>
</tr>
<tr>
<td>GO:0021954</td>
<td>1.20E-03</td>
<td>0.48</td>
<td>4</td>
<td>27</td>
<td>central nervous system neuron development</td>
</tr>
<tr>
<td>GO:0019724</td>
<td>1.20E-03</td>
<td>1.2</td>
<td>6</td>
<td>68</td>
<td>B cell mediated immunity</td>
</tr>
<tr>
<td>GO:0040007</td>
<td>1.30E-03</td>
<td>4.92</td>
<td>13</td>
<td>278</td>
<td>growth</td>
</tr>
<tr>
<td>GO:0016337</td>
<td>1.40E-03</td>
<td>3.22</td>
<td>10</td>
<td>182</td>
<td>cell-cell adhesion</td>
</tr>
<tr>
<td>GO:0030199</td>
<td>2.10E-03</td>
<td>0.27</td>
<td>3</td>
<td>15</td>
<td>collagen fibril organization</td>
</tr>
<tr>
<td>GO:0021953</td>
<td>2.30E-03</td>
<td>0.57</td>
<td>4</td>
<td>32</td>
<td>central nervous system neuron differentiation</td>
</tr>
<tr>
<td>GO:0002443</td>
<td>2.50E-03</td>
<td>1.86</td>
<td>7</td>
<td>105</td>
<td>leukocyte mediated immunity</td>
</tr>
<tr>
<td>GO:0019226</td>
<td>2.70E-03</td>
<td>4.09</td>
<td>11</td>
<td>231</td>
<td>transmission of nerve impulse</td>
</tr>
<tr>
<td>GO:0009791</td>
<td>3.20E-03</td>
<td>1.01</td>
<td>5</td>
<td>57</td>
<td>post-embryonic development</td>
</tr>
<tr>
<td>GO:0051149</td>
<td>4.40E-03</td>
<td>0.11</td>
<td>2</td>
<td>6</td>
<td>positive regulation of muscle cell differentiation</td>
</tr>
<tr>
<td>GO:0009611</td>
<td>5.30E-03</td>
<td>5.11</td>
<td>12</td>
<td>289</td>
<td>response to wounding</td>
</tr>
<tr>
<td>GO:0045834</td>
<td>6.10E-03</td>
<td>0.12</td>
<td>2</td>
<td>7</td>
<td>positive regulation of lipid metabolic process</td>
</tr>
<tr>
<td>GO:0015813</td>
<td>6.10E-03</td>
<td>0.12</td>
<td>2</td>
<td>7</td>
<td>L-glutamate transport</td>
</tr>
<tr>
<td>GO:0048856</td>
<td>6.10E-03</td>
<td>31.59</td>
<td>46</td>
<td>1809</td>
<td>anatomical structure development</td>
</tr>
<tr>
<td>GO:0048518</td>
<td>6.30E-03</td>
<td>17.07</td>
<td>28</td>
<td>965</td>
<td>positive regulation of biological process</td>
</tr>
<tr>
<td>GO:0002250</td>
<td>6.70E-03</td>
<td>1.68</td>
<td>6</td>
<td>95</td>
<td>adaptive immune response based on somatic recombination of</td>
</tr>
<tr>
<td>GO:0002460</td>
<td>6.70E-03</td>
<td>1.68</td>
<td>6</td>
<td>95</td>
<td>immune receptors built from immunoglobulin superfamily domains</td>
</tr>
<tr>
<td>GO:0045087</td>
<td>7.00E-03</td>
<td>1.7</td>
<td>6</td>
<td>96</td>
<td>innate immune response</td>
</tr>
<tr>
<td>GO:0002449</td>
<td>7.00E-03</td>
<td>1.7</td>
<td>6</td>
<td>96</td>
<td>lymphocyte mediated immunity</td>
</tr>
<tr>
<td>GO:0007288</td>
<td>7.30E-03</td>
<td>3.41</td>
<td>9</td>
<td>193</td>
<td>synaptic transmission</td>
</tr>
<tr>
<td>GO:0002253</td>
<td>7.40E-03</td>
<td>1.22</td>
<td>5</td>
<td>69</td>
<td>activation of immune response</td>
</tr>
<tr>
<td>GO:0048731</td>
<td>7.60E-03</td>
<td>28.93</td>
<td>42</td>
<td>1636</td>
<td>system development</td>
</tr>
<tr>
<td>GO:0007611</td>
<td>7.80E-03</td>
<td>1.24</td>
<td>5</td>
<td>70</td>
<td>learning and/or memory</td>
</tr>
<tr>
<td>GO:0009605</td>
<td>8.00E-03</td>
<td>8.17</td>
<td>16</td>
<td>462</td>
<td>response to external stimulus</td>
</tr>
<tr>
<td>GO:0008217</td>
<td>8.00E-03</td>
<td>0.8</td>
<td>4</td>
<td>45</td>
<td>regulation of blood pressure</td>
</tr>
<tr>
<td>GO:0045661</td>
<td>8.10E-03</td>
<td>0.14</td>
<td>2</td>
<td>8</td>
<td>regulation of myoblast differentiation</td>
</tr>
<tr>
<td>GO:0007271</td>
<td>8.10E-03</td>
<td>0.14</td>
<td>2</td>
<td>8</td>
<td>synaptic transmission, cholinergic</td>
</tr>
<tr>
<td>GO:0021952</td>
<td>8.10E-03</td>
<td>0.14</td>
<td>2</td>
<td>8</td>
<td>central nervous system projection neuron axonogenesis</td>
</tr>
<tr>
<td>GO:0015800</td>
<td>8.10E-03</td>
<td>0.14</td>
<td>2</td>
<td>8</td>
<td>acidic amino acid transport</td>
</tr>
<tr>
<td>GO:0051179</td>
<td>8.70E-03</td>
<td>44.11</td>
<td>59</td>
<td>2494</td>
<td>localization</td>
</tr>
<tr>
<td>GO:0002252</td>
<td>8.90E-03</td>
<td>2.33</td>
<td>7</td>
<td>132</td>
<td>immune effector process</td>
</tr>
</tbody>
</table>

114
Table 18: (-2, -2) GO terms and p-values (LG/J Time Comparison)

<table>
<thead>
<tr>
<th>GO Id</th>
<th>P Value</th>
<th>Exp Count</th>
<th>Count</th>
<th>Size</th>
<th>GO Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0033138</td>
<td>2.21E-04</td>
<td>0.02</td>
<td>2</td>
<td>4</td>
<td>positive regulation of peptidyl-serine phosphorylation</td>
</tr>
<tr>
<td>GO:0033135</td>
<td>2.21E-04</td>
<td>0.02</td>
<td>2</td>
<td>4</td>
<td>regulation of peptidyl-serine phosphorylation</td>
</tr>
<tr>
<td>GO:0006955</td>
<td>5.80E-04</td>
<td>2.38</td>
<td>9</td>
<td>387</td>
<td>immune response</td>
</tr>
<tr>
<td>GO:0050730</td>
<td>1.20E-03</td>
<td>0.21</td>
<td>3</td>
<td>35</td>
<td>regulation of peptidyl-tyrosine phosphorylation</td>
</tr>
<tr>
<td>GO:0046888</td>
<td>1.90E-03</td>
<td>0.07</td>
<td>2</td>
<td>11</td>
<td>negative regulation of hormone secretion</td>
</tr>
<tr>
<td>GO:0000279</td>
<td>3.30E-03</td>
<td>1.46</td>
<td>6</td>
<td>238</td>
<td>M phase</td>
</tr>
<tr>
<td>GO:0051048</td>
<td>3.70E-03</td>
<td>0.09</td>
<td>2</td>
<td>15</td>
<td>negative regulation of secretion</td>
</tr>
<tr>
<td>GO:0051046</td>
<td>5.10E-03</td>
<td>0.35</td>
<td>3</td>
<td>57</td>
<td>regulation of secretion</td>
</tr>
<tr>
<td>GO:0018105</td>
<td>5.30E-03</td>
<td>0.11</td>
<td>2</td>
<td>18</td>
<td>peptidyl-serine phosphorylation</td>
</tr>
<tr>
<td>GO:0018209</td>
<td>5.30E-03</td>
<td>0.11</td>
<td>2</td>
<td>18</td>
<td>peptidyl-serine modification</td>
</tr>
<tr>
<td>GO:0018108</td>
<td>5.30E-03</td>
<td>0.36</td>
<td>3</td>
<td>58</td>
<td>peptidyl-tyrosine phosphorylation</td>
</tr>
<tr>
<td>GO:0018212</td>
<td>5.30E-03</td>
<td>0.36</td>
<td>3</td>
<td>58</td>
<td>peptidyl-tyrosine modification</td>
</tr>
<tr>
<td>GO:0050731</td>
<td>5.90E-03</td>
<td>0.12</td>
<td>2</td>
<td>19</td>
<td>positive regulation of peptidyl-tyrosine phosphorylation</td>
</tr>
<tr>
<td>GO:0042508</td>
<td>6.10E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>tyrosine phosphorylation of Stat1 protein</td>
</tr>
<tr>
<td>GO:0009853</td>
<td>6.10E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>photorespiration</td>
</tr>
<tr>
<td>GO:009854</td>
<td>6.10E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>oxidative photosynthetic carbon pathway</td>
</tr>
<tr>
<td>GO:0043313</td>
<td>6.10E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>regulation of neutrophil degranulation</td>
</tr>
<tr>
<td>GO:0043124</td>
<td>6.10E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>negative regulation of I-kappaB kinase/NF-kappaB cascade</td>
</tr>
<tr>
<td>GO:0051639</td>
<td>6.10E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>actin filament network formation</td>
</tr>
<tr>
<td>GO:0055091</td>
<td>6.10E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>phospholipid homeostasis</td>
</tr>
<tr>
<td>GO:0055090</td>
<td>6.10E-03</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
<td>acylglycerol homeostasis</td>
</tr>
<tr>
<td>GO:002376</td>
<td>6.30E-03</td>
<td>4.01</td>
<td>10</td>
<td>653</td>
<td>immune system process</td>
</tr>
<tr>
<td>GO:0022403</td>
<td>6.60E-03</td>
<td>1.68</td>
<td>6</td>
<td>274</td>
<td>cell cycle phase</td>
</tr>
<tr>
<td>GO:0000165</td>
<td>7.00E-03</td>
<td>0.76</td>
<td>4</td>
<td>124</td>
<td>MAPKKK cascade</td>
</tr>
<tr>
<td>GO:001932</td>
<td>7.30E-03</td>
<td>0.4</td>
<td>3</td>
<td>65</td>
<td>regulation of protein amino acid phosphorylation</td>
</tr>
<tr>
<td>GO:0007262</td>
<td>8.40E-03</td>
<td>1.26</td>
<td>5</td>
<td>205</td>
<td>locomotory behavior</td>
</tr>
<tr>
<td>GO:0006936</td>
<td>9.70E-03</td>
<td>0.44</td>
<td>3</td>
<td>72</td>
<td>muscle contraction</td>
</tr>
<tr>
<td>GO:0022607</td>
<td>9.90E-03</td>
<td>0.84</td>
<td>4</td>
<td>137</td>
<td>cellular component assembly</td>
</tr>
</tbody>
</table>
Fig. 16c Comparison 3- MRL/MpJ


(+2, +2)  (-2, -2)
<table>
<thead>
<tr>
<th>GO Id</th>
<th>P Value</th>
<th>Exp Count</th>
<th>Count</th>
<th>Size</th>
<th>GO Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0051146</td>
<td>1.73E-04</td>
<td>0.54</td>
<td>5</td>
<td>31</td>
<td>striated muscle cell differentiation</td>
</tr>
<tr>
<td>GO:0042692</td>
<td>1.82E-04</td>
<td>1.19</td>
<td>7</td>
<td>69</td>
<td>muscle cell differentiation</td>
</tr>
<tr>
<td>GO:006073</td>
<td>2.03E-04</td>
<td>0.55</td>
<td>5</td>
<td>32</td>
<td>glucan metabolic process</td>
</tr>
<tr>
<td>GO:005977</td>
<td>2.03E-04</td>
<td>0.55</td>
<td>5</td>
<td>32</td>
<td>glycogen metabolic process</td>
</tr>
<tr>
<td>GO:006813</td>
<td>2.09E-04</td>
<td>2.51</td>
<td>10</td>
<td>145</td>
<td>potassium ion transport</td>
</tr>
<tr>
<td>GO:006603</td>
<td>2.97E-04</td>
<td>0.03</td>
<td>2</td>
<td>2</td>
<td>phosphocreatine metabolic process</td>
</tr>
<tr>
<td>GO:006112</td>
<td>3.13E-04</td>
<td>0.6</td>
<td>5</td>
<td>35</td>
<td>energy reserve metabolic process</td>
</tr>
<tr>
<td>GO:0021700</td>
<td>3.56E-04</td>
<td>1.75</td>
<td>8</td>
<td>101</td>
<td>developmental maturation</td>
</tr>
<tr>
<td>GO:0048469</td>
<td>3.90E-04</td>
<td>1.35</td>
<td>7</td>
<td>78</td>
<td>cell maturation</td>
</tr>
<tr>
<td>GO:0060078</td>
<td>4.12E-04</td>
<td>0.36</td>
<td>4</td>
<td>21</td>
<td>regulation of postsynaptic membrane potential</td>
</tr>
<tr>
<td>GO:006873</td>
<td>4.24E-04</td>
<td>3.25</td>
<td>11</td>
<td>188</td>
<td>cellular ion homeostasis</td>
</tr>
<tr>
<td>GO:008869</td>
<td>4.51E-04</td>
<td>24.14</td>
<td>41</td>
<td>1397</td>
<td>cellular developmental process</td>
</tr>
<tr>
<td>GO:008513</td>
<td>5.13E-04</td>
<td>23.5</td>
<td>40</td>
<td>1360</td>
<td>organ development</td>
</tr>
<tr>
<td>GO:0055082</td>
<td>5.52E-04</td>
<td>3.35</td>
<td>11</td>
<td>194</td>
<td>cellular chemical homeostasis</td>
</tr>
<tr>
<td>GO:005978</td>
<td>5.58E-04</td>
<td>0.17</td>
<td>3</td>
<td>10</td>
<td>glycogen biosynthetic process</td>
</tr>
<tr>
<td>GO:009250</td>
<td>5.58E-04</td>
<td>0.17</td>
<td>3</td>
<td>10</td>
<td>glycogen biosynthetic process</td>
</tr>
<tr>
<td>GO:008878</td>
<td>5.90E-04</td>
<td>2.51</td>
<td>10</td>
<td>145</td>
<td>potassium ion transport</td>
</tr>
<tr>
<td>GO:0048731</td>
<td>9.22E-04</td>
<td>28.27</td>
<td>45</td>
<td>1636</td>
<td>system development</td>
</tr>
<tr>
<td>GO:006814</td>
<td>1.10E-03</td>
<td>2.07</td>
<td>8</td>
<td>120</td>
<td>sodium ion transport</td>
</tr>
<tr>
<td>GO:0042391</td>
<td>1.20E-03</td>
<td>1.64</td>
<td>7</td>
<td>95</td>
<td>regulation of membrane potential</td>
</tr>
<tr>
<td>GO:0048704</td>
<td>1.30E-03</td>
<td>0.83</td>
<td>5</td>
<td>48</td>
<td>embryonic skeletal morphogenesis</td>
</tr>
<tr>
<td>GO:005976</td>
<td>1.30E-03</td>
<td>0.83</td>
<td>5</td>
<td>48</td>
<td>polysaccharide metabolic process</td>
</tr>
<tr>
<td>GO:0044264</td>
<td>1.30E-03</td>
<td>0.83</td>
<td>5</td>
<td>48</td>
<td>cellular polysaccharide metabolic process</td>
</tr>
<tr>
<td>GO:0043062</td>
<td>1.60E-03</td>
<td>1.71</td>
<td>7</td>
<td>99</td>
<td>extracellular structure organization and biogenesis</td>
</tr>
<tr>
<td>GO:0043576</td>
<td>1.70E-03</td>
<td>0.07</td>
<td>2</td>
<td>4</td>
<td>regulation of respiratory gaseous exchange</td>
</tr>
<tr>
<td>GO:0042447</td>
<td>1.70E-03</td>
<td>0.07</td>
<td>2</td>
<td>4</td>
<td>hormone catabolic process</td>
</tr>
<tr>
<td>GO:006599</td>
<td>1.70E-03</td>
<td>0.07</td>
<td>2</td>
<td>4</td>
<td>phosphagen metabolic process</td>
</tr>
<tr>
<td>GO:008935</td>
<td>1.70E-03</td>
<td>0.07</td>
<td>2</td>
<td>4</td>
<td>peripheral nervous system neuron development</td>
</tr>
<tr>
<td>GO:0031999</td>
<td>1.90E-03</td>
<td>0.26</td>
<td>3</td>
<td>15</td>
<td>collagen fibril organization</td>
</tr>
<tr>
<td>GO:006810</td>
<td>2.20E-03</td>
<td>37.22</td>
<td>54</td>
<td>2154</td>
<td>transport</td>
</tr>
<tr>
<td>GO:0048856</td>
<td>2.30E-03</td>
<td>31.26</td>
<td>47</td>
<td>1809</td>
<td>anatomical structure development</td>
</tr>
<tr>
<td>GO:008544</td>
<td>2.30E-03</td>
<td>0.93</td>
<td>5</td>
<td>54</td>
<td>adult locomotory behavior</td>
</tr>
<tr>
<td>GO:001501</td>
<td>2.30E-03</td>
<td>2.87</td>
<td>9</td>
<td>166</td>
<td>skeletal development</td>
</tr>
<tr>
<td>GO:0015674</td>
<td>2.40E-03</td>
<td>2.35</td>
<td>8</td>
<td>136</td>
<td>di-, tri-valent inorganic cation transport</td>
</tr>
<tr>
<td>GO:006820</td>
<td>2.50E-03</td>
<td>2.9</td>
<td>9</td>
<td>168</td>
<td>anion transport</td>
</tr>
<tr>
<td>GO:0042592</td>
<td>2.60E-03</td>
<td>7.95</td>
<td>17</td>
<td>460</td>
<td>homeostatic process</td>
</tr>
<tr>
<td>GO:0015688</td>
<td>2.70E-03</td>
<td>2.38</td>
<td>8</td>
<td>138</td>
<td>inorganic anion transport</td>
</tr>
<tr>
<td>GO:0050966</td>
<td>2.80E-03</td>
<td>0.09</td>
<td>2</td>
<td>5</td>
<td>detection of mechanical stimulus involved in sensory perception of pain</td>
</tr>
<tr>
<td>GO:0048934</td>
<td>2.80E-03</td>
<td>0.09</td>
<td>2</td>
<td>5</td>
<td>peripheral nervous system neuron differentiation</td>
</tr>
<tr>
<td>GO:0033692</td>
<td>2.80E-03</td>
<td>0.29</td>
<td>3</td>
<td>17</td>
<td>cellular polysaccharide biosynthetic process</td>
</tr>
<tr>
<td>GO:0060079</td>
<td>2.80E-03</td>
<td>0.29</td>
<td>3</td>
<td>17</td>
<td>regulation of excitatory postsynaptic membrane potential</td>
</tr>
<tr>
<td>GO:006091</td>
<td>2.90E-03</td>
<td>2.42</td>
<td>8</td>
<td>140</td>
<td>generation of precursor metabolites and energy</td>
</tr>
<tr>
<td>GO:0051234</td>
<td>2.90E-03</td>
<td>37.69</td>
<td>54</td>
<td>2181</td>
<td>establishment of localization</td>
</tr>
<tr>
<td>GO:004262</td>
<td>3.20E-03</td>
<td>3.58</td>
<td>10</td>
<td>207</td>
<td>cellular carbohydrate metabolic process</td>
</tr>
<tr>
<td>GO:006816</td>
<td>3.20E-03</td>
<td>1.94</td>
<td>7</td>
<td>112</td>
<td>calcium ion transport</td>
</tr>
<tr>
<td>GO:000271</td>
<td>3.40E-03</td>
<td>0.31</td>
<td>3</td>
<td>18</td>
<td>polysaccharide biosynthetic process</td>
</tr>
<tr>
<td>GO:004705</td>
<td>3.70E-03</td>
<td>1.04</td>
<td>5</td>
<td>60</td>
<td>skeletal morphogenesis</td>
</tr>
<tr>
<td>GO:0044275</td>
<td>3.70E-03</td>
<td>1.04</td>
<td>5</td>
<td>60</td>
<td>cellular carbohydrate catabolic process</td>
</tr>
<tr>
<td>GO:0019725</td>
<td>3.70E-03</td>
<td>4.27</td>
<td>11</td>
<td>247</td>
<td>cellular homeostasis</td>
</tr>
<tr>
<td>GO:0055074</td>
<td>4.20E-03</td>
<td>1.07</td>
<td>5</td>
<td>62</td>
<td>calcium ion homeostasis</td>
</tr>
<tr>
<td>GO:003007</td>
<td>4.40E-03</td>
<td>0.67</td>
<td>4</td>
<td>39</td>
<td>heart morphogenesis</td>
</tr>
<tr>
<td>GO:0015980</td>
<td>4.90E-03</td>
<td>1.11</td>
<td>5</td>
<td>64</td>
<td>energy derivation by oxidation of organic compounds</td>
</tr>
<tr>
<td>GO:0048706</td>
<td>5.20E-03</td>
<td>1.12</td>
<td>5</td>
<td>65</td>
<td>embryonic skeletal development</td>
</tr>
<tr>
<td>GO:0016502</td>
<td>5.20E-03</td>
<td>1.12</td>
<td>5</td>
<td>65</td>
<td>carbohydrate catabolic process</td>
</tr>
<tr>
<td>GO:0042691</td>
<td>1.82E-04</td>
<td>1.19</td>
<td>7</td>
<td>69</td>
<td>negative regulation of response to external stimulus</td>
</tr>
<tr>
<td>GO:0032102</td>
<td>5.30E-03</td>
<td>0.36</td>
<td>3</td>
<td>21</td>
<td>negative regulation of response to external stimulus</td>
</tr>
<tr>
<td>GO:0032502</td>
<td>5.50E-03</td>
<td>45.93</td>
<td>62</td>
<td>2658</td>
<td>developmental process</td>
</tr>
<tr>
<td>GO:0032501</td>
<td>5.60E-03</td>
<td>60.62</td>
<td>78</td>
<td>3508</td>
<td>multicellular organismal process</td>
</tr>
<tr>
<td>GO:0055080</td>
<td>6.20E-03</td>
<td>2.18</td>
<td>7</td>
<td>126</td>
<td>cation homeostasis</td>
</tr>
<tr>
<td>GO:0055065</td>
<td>6.70E-03</td>
<td>1.19</td>
<td>5</td>
<td>69</td>
<td>metal ion homeostasis</td>
</tr>
<tr>
<td>GO:0055066</td>
<td>7.70E-03</td>
<td>1.73</td>
<td>6</td>
<td>100</td>
<td>di-, tri-valent inorganic cation homeostasis</td>
</tr>
<tr>
<td>GO:0051963</td>
<td>7.70E-03</td>
<td>0.14</td>
<td>2</td>
<td>8</td>
<td>regulation of synaptogenesis</td>
</tr>
<tr>
<td>GO:006817</td>
<td>9.50E-03</td>
<td>1.3</td>
<td>5</td>
<td>75</td>
<td>phosphate transport</td>
</tr>
<tr>
<td>GO Id</td>
<td>P Value</td>
<td>Exp Count</td>
<td>Count</td>
<td>Size</td>
<td>GO Term</td>
</tr>
<tr>
<td>------------</td>
<td>----------</td>
<td>-----------</td>
<td>-------</td>
<td>-------</td>
<td>------------------------------------------------------</td>
</tr>
<tr>
<td>GO:0022403</td>
<td>2.70E-08</td>
<td>3.67</td>
<td>18</td>
<td>274</td>
<td>cell cycle phase</td>
</tr>
<tr>
<td>GO:000279</td>
<td>1.22E-07</td>
<td>3.19</td>
<td>16</td>
<td>238</td>
<td>M phase</td>
</tr>
<tr>
<td>GO:000278</td>
<td>2.28E-07</td>
<td>2.92</td>
<td>15</td>
<td>218</td>
<td>mitotic cell cycle</td>
</tr>
<tr>
<td>GO:006955</td>
<td>2.33E-07</td>
<td>5.19</td>
<td>20</td>
<td>387</td>
<td>immune response</td>
</tr>
<tr>
<td>GO:000087</td>
<td>2.60E-07</td>
<td>2.17</td>
<td>13</td>
<td>162</td>
<td>M phase of mitotic cell cycle</td>
</tr>
<tr>
<td>GO:007067</td>
<td>2.60E-07</td>
<td>2.17</td>
<td>13</td>
<td>162</td>
<td>mitosis</td>
</tr>
<tr>
<td>GO:0022402</td>
<td>2.96E-07</td>
<td>4.3</td>
<td>18</td>
<td>321</td>
<td>cell cycle process</td>
</tr>
<tr>
<td>GO:002376</td>
<td>5.52E-07</td>
<td>8.75</td>
<td>26</td>
<td>653</td>
<td>immune system process</td>
</tr>
<tr>
<td>GO:0051301</td>
<td>5.68E-07</td>
<td>3.14</td>
<td>15</td>
<td>234</td>
<td>cell division</td>
</tr>
<tr>
<td>GO:006950</td>
<td>1.58E-06</td>
<td>11.73</td>
<td>30</td>
<td>875</td>
<td>response to stress</td>
</tr>
<tr>
<td>GO:007049</td>
<td>4.16E-06</td>
<td>7.95</td>
<td>23</td>
<td>593</td>
<td>cell cycle</td>
</tr>
<tr>
<td>GO:0045087</td>
<td>5.38E-06</td>
<td>1.29</td>
<td>9</td>
<td>96</td>
<td>innate immune response</td>
</tr>
<tr>
<td>GO:000611</td>
<td>7.80E-06</td>
<td>3.87</td>
<td>15</td>
<td>289</td>
<td>response to wounding</td>
</tr>
<tr>
<td>GO:0050896</td>
<td>1.79E-05</td>
<td>23.45</td>
<td>44</td>
<td>1749</td>
<td>response to stimulus</td>
</tr>
<tr>
<td>GO:006952</td>
<td>9.36E-05</td>
<td>4.8</td>
<td>15</td>
<td>358</td>
<td>defense response</td>
</tr>
<tr>
<td>GO:007017</td>
<td>2.74E-04</td>
<td>2.12</td>
<td>9</td>
<td>158</td>
<td>microtubule-based process</td>
</tr>
<tr>
<td>GO:006954</td>
<td>3.61E-04</td>
<td>2.68</td>
<td>10</td>
<td>200</td>
<td>inflammatory response</td>
</tr>
<tr>
<td>GO:009605</td>
<td>4.78E-04</td>
<td>6.19</td>
<td>16</td>
<td>462</td>
<td>response to external stimulus</td>
</tr>
<tr>
<td>GO:0031341</td>
<td>1.90E-03</td>
<td>0.25</td>
<td>3</td>
<td>19</td>
<td>regulation of cell killing</td>
</tr>
<tr>
<td>GO:001910</td>
<td>1.90E-03</td>
<td>0.25</td>
<td>3</td>
<td>19</td>
<td>regulation of leukocyte mediated cytotoxicity</td>
</tr>
<tr>
<td>GO:003142</td>
<td>2.50E-03</td>
<td>0.08</td>
<td>2</td>
<td>6</td>
<td>negative regulation of cell killing</td>
</tr>
<tr>
<td>GO:006882</td>
<td>2.50E-03</td>
<td>0.08</td>
<td>2</td>
<td>6</td>
<td>cellular zinc ion homeostasis</td>
</tr>
<tr>
<td>GO:001911</td>
<td>2.50E-03</td>
<td>0.08</td>
<td>2</td>
<td>6</td>
<td>negative regulation of leukocyte mediated cytotoxicity</td>
</tr>
<tr>
<td>GO:004649</td>
<td>2.60E-03</td>
<td>2.92</td>
<td>9</td>
<td>218</td>
<td>lymphocyte activation</td>
</tr>
<tr>
<td>GO:007059</td>
<td>3.00E-03</td>
<td>0.6</td>
<td>4</td>
<td>45</td>
<td>chromosome segregation</td>
</tr>
<tr>
<td>GO:007018</td>
<td>3.30E-03</td>
<td>1.01</td>
<td>5</td>
<td>75</td>
<td>microtubule-based movement</td>
</tr>
<tr>
<td>GO:0055069</td>
<td>3.50E-03</td>
<td>0.09</td>
<td>2</td>
<td>7</td>
<td>zinc ion homeostasis</td>
</tr>
<tr>
<td>GO:001909</td>
<td>3.80E-03</td>
<td>0.32</td>
<td>3</td>
<td>24</td>
<td>leukocyte mediated cytotoxicity</td>
</tr>
<tr>
<td>GO:001906</td>
<td>3.80E-03</td>
<td>0.32</td>
<td>3</td>
<td>24</td>
<td>cell killing</td>
</tr>
<tr>
<td>GO:0045321</td>
<td>4.40E-03</td>
<td>3.15</td>
<td>9</td>
<td>235</td>
<td>leukocyte activation</td>
</tr>
<tr>
<td>GO:001775</td>
<td>6.50E-03</td>
<td>3.35</td>
<td>9</td>
<td>250</td>
<td>cell activation</td>
</tr>
<tr>
<td>GO:0030705</td>
<td>7.10E-03</td>
<td>1.21</td>
<td>5</td>
<td>90</td>
<td>cytoskeleton-dependent intracellular transport</td>
</tr>
<tr>
<td>GO:002707</td>
<td>7.40E-03</td>
<td>0.13</td>
<td>2</td>
<td>10</td>
<td>negative regulation of lymphocyte mediated immunity</td>
</tr>
<tr>
<td>GO:002704</td>
<td>7.40E-03</td>
<td>0.13</td>
<td>2</td>
<td>10</td>
<td>negative regulation of leukocyte mediated immunity</td>
</tr>
<tr>
<td>GO:0042060</td>
<td>7.50E-03</td>
<td>1.22</td>
<td>5</td>
<td>91</td>
<td>wound healing</td>
</tr>
<tr>
<td>GO:002706</td>
<td>8.00E-03</td>
<td>0.42</td>
<td>3</td>
<td>31</td>
<td>regulation of lymphocyte mediated immunity</td>
</tr>
<tr>
<td>GO:002703</td>
<td>8.00E-03</td>
<td>0.42</td>
<td>3</td>
<td>31</td>
<td>regulation of leukocyte mediated immunity</td>
</tr>
<tr>
<td>GO:002715</td>
<td>9.00E-03</td>
<td>0.15</td>
<td>2</td>
<td>11</td>
<td>regulation of natural killer cell mediated immunity</td>
</tr>
<tr>
<td>GO:002846</td>
<td>9.00E-03</td>
<td>0.15</td>
<td>2</td>
<td>11</td>
<td>cell proliferation in forebrain</td>
</tr>
<tr>
<td>GO:0042269</td>
<td>9.00E-03</td>
<td>0.15</td>
<td>2</td>
<td>11</td>
<td>regulation of natural killer cell mediated cytotoxicity</td>
</tr>
</tbody>
</table>
Fig. 17a: Comparison of temporal gene expression patterns obtained from RNA-Seq and qPCR technologies for C57BL/6, LG/J, and MRL/MpJ. Expression of collagen type I (Col1a1), collagen type III (Col3a1), scleraxis (Scx), and mohawk homeobox (Mkx) are represented. Overall, the temporal expression patterns measured using qPCR and RNASeq within each strain for each gene were similar.
Fig. 17b: Comparison of temporal gene expression patterns obtained from RNA-Seq and qPCR technologies for C57BL/6, LG/J, and MRL/MpJ. Expression of early growth response 1 (Egr1), tenascin-C (TnC), tenomodulin (Tnmd), and fibromodulin (Fmod) are represented. Overall, the temporal expression patterns measured using qPCR and RNASeq within each strain for each gene were similar.
Fig. 17c: Comparison of temporal gene expression patterns obtained from RNA-Seq and qPCR technologies for C57BL/6, LG/J, and MRL/MpJ. Expression of cyclin-dependent kinase inhibitor 1 (p21), v-myc avian myelocytomatosis viral oncogene homolog (Myc), decorin (Dcn), and interferon gamma (IFNG) are represented. Overall, the temporal expression patterns measured using qPCR and RNASeq within each strain for each gene were similar, with the exception of MRL/MpJ’s recorded IFNG expression. This can be attributed to low expression values for this gene with Ct values measured at the upper limits of the Taqman gene expression assay.
Fig. 17d: Comparison of temporal gene expression patterns obtained from RNA-Seq and qPCR technologies for C57BL/6, LG/J, and MRL/MpJ. Expression of POU class 5 homeobox 1 (Pou5f1) and homeobox A13 (Hoxa13) are represented. The temporal expression patterns for both Pou5f1 and Hoxa13 were different between the qPCR and RNASeq measures. This can be attributed to low expression values for these two genes with Ct values measured at the upper limits of the Taqman gene expression assays.
Chapter 6

The MRL/MpJ Does Not Recover Native Mechanical Properties Following a Critical-Size Femoral Osteotomy

Andrea L. Lalley, B.S.\textsuperscript{a}, Chris Casstevens, M.D.\textsuperscript{b}, Steven Dailey, M.D.\textsuperscript{b}, Nathaniel A. Dyment, Ph.D.\textsuperscript{c}, Jayne Gavrity\textsuperscript{d}, Xi Jiang, Ph.D.\textsuperscript{c}, Jason Inzana\textsuperscript{d}, Steven A. Goldstein\textsuperscript{c}, Hani Awad, Ph.D.\textsuperscript{d}, David Rowe, M.D.\textsuperscript{c}, Keith Kenter, M.D.\textsuperscript{b}, David L. Butler, Ph.D.\textsuperscript{a}, Jason T. Shearn, Ph.D.\textsuperscript{a}

\textsuperscript{a}Biomedical Engineering Program, College of Engineering and Applied Science, University of Cincinnati, Cincinnati, Ohio

\textsuperscript{b}Department of Orthopaedic Surgery, College of Medicine, University of Cincinnati, Cincinnati, Ohio

\textsuperscript{c}Department of Reconstructive Sciences, College of Dental Medicine, University of Connecticut Health Center, Farmington, Connecticut

\textsuperscript{d}Department of Biomedical Engineering, School of Medicine and Dentistry, University of Rochester, Rochester, New York

\textsuperscript{e}Orthopaedic Research Laboratories, Department of Orthopaedic Surgery, School of Medicine, University of Michigan, Ann Arbor, Michigan

\textsuperscript{1}This manuscript is currently in preparation for the Journal of Orthopaedic Traumatology
Nonunion can occur due to poor stabilization following fracture or significant bone loss resulting from tumor resection. Ten percent of fractures result in nonunion and current clinical approaches are inconsistent in restoring native tissue structure and function. We have established a murine model of nonunion by creating a critical-size femoral osteotomy fixed using a custom-designed titanium plate to study the formation of nonunions and develop potential therapies. The MRL/MpJ murine strain has been identified as a superior healer following injury in a number of tissue systems. This study aims to evaluate the MRL/MpJ’s response to a critical-size femoral osteotomy to determine differences that may exist in the healing processes between the MRL/MpJ and C57BL/6 strains. Histological and biomechanical outcomes were assessed at 2, 5, and 8 weeks post injury. We found that the mechanical results between strains were not different at any time point, with a nonunion resulting at a rate of 100% and 75% at 5 weeks and 80% and 88% at 8 weeks for the C57BL/6 and MRL/MpJ, respectively. Histological assessment of the healing tissue showed capping of the fracture ends in both strains by 5 weeks; however, the MRL/MpJ showed increased expression of alkaline phosphatase and tartrate resistant acid phosphatase in the callus regions, suggesting a prolonged healing response compared to wildtype. Future work will investigate alternative fracture models using the MRL/MpJ to determine if this strain is capable of more successfully repairing a bony defect compared to wildtype.
INTRODUCTION

Approximately 7.9 million patients are affected by fractures in the United States annually. The majority of individuals heal successfully following fracture through casting and immobilization; however, 10% of affected patients exhibit impaired bone healing leading to delayed union or nonunion. A nonunion fracture can be defined as an arrest of the periosteal and endosteal responses prohibiting bridge formation between the fracture ends. More specifically, the Food and Drug Administration defines a nonunion as a fracture that has not healed within 9 months of sustaining the injury and fails to show healing progression over 3 months of check-ups. Nonunions can occur due to excessive bone loss due to injury or tumor resection, infection, vascular deficiencies, and/or improper stabilization following injury. Co-morbidities such as smoking, alcohol use, diabetes, and malnutrition also contribute to nonunion.

There are several strategies for treating a nonunion; however, a variety of risk factors, the type of injury, and patient age can make selecting a specific therapy difficult. Bone grafting, intramedullary-screw fixation, and plating are common practices for treating a nonunion; however, each approach presents challenges, often requiring multiple surgeries to adequately repair the damage. It has been reported that approximately 50% and 47% of femoral nonunions fail to heal adequately when treated with nail dynamization (removal of nails 10-12 weeks postoperatively to stimulate remodeling) and nail exchange, respectively. Plate fixation offers a more satisfactory outcome; however, infection and decreased mechanical loading to the healing tissue can complicate the success outcome.

We have established a novel murine nonunion model by creating a critical-size femoral osteotomy. The osteotomy is fixed with a titanium plate allowing us to characterize the
formation of a nonunion and test potential therapies to improve the healing outcomes. Findings from a previous study showed an inability of a double transgenic mouse line (pOBCol3.6GFPtpz (Col1) and pCol2-ECFP (Col2)) to successfully bridge the osteotomy up to 5 weeks following injury; however, questions remain as to why this type of fracture fails to heal normally.

Identifying a model that is capable of repairing a critical-size osteotomy successfully offers an opportunity to identify unique properties of this process and apply them to current clinical practices. To this end, have been investigating a murine strain, the MRL/MpJ, that exhibits improved healing outcomes following injury in a number of tissues including skin, heart, articular cartilage, cornea, and more recently, tendon (Chapter 4). It is unknown how this strain responds to an osseous injury. Thus, the objective of this study was to determine the MRL/MpJ response to a critical-size femoral osteotomy and compare with the C57BL/6 strain. We hypothesized the MRL/MpJ would exhibit an improved healing response based on mechanical and histological outcome measures.

RESULTS

Native Mechanical and Morphological Properties of the MRL/MpJ and C57BL/6 Femur

The overall femur shape was found to be similar between the two strains. The MRL/MpJ femora were larger on average, measuring longer (18.25 ± 1.34 mm vs. 17.02 ± 1.01 mm, mean±SD) and wider (2.52 ± 0.21 mm vs. 2.10 ± 0.12 mm, mean±SD) compared to C57BL/6 controls (Fig. 19).
We evaluated the native mechanical properties of the MRL/MpJ and C57BL/6 femora. Torsional rigidity, yield torque, and rotation at yield were not different between the two strains (p>0.05), while maximum torque, rotation at failure, and energy to failure were all significantly greater in the MRL/MpJ (p<0.05) (Table 24). Immunostaining of the native C57BL/6 and MRL/MpJ femora showed minimal differences in structure or cellular activity. Alkaline phosphatase (AP) is a hydrolase enzyme expressed by osteoblasts, functioning in bone mineralization. Tartrate-resistant acid phosphatase (TRAP) is an enzyme expressed by osteoclasts, functioning in bone resorption. Both enzymes function during normal bone turnover and following injury to form new tissue. Both strains displayed similar cellular proliferation and expression levels of AP and TRAP at the distal growth plate, with minimal AP expression, TRAP expression, or cellular proliferation observed along the length of the diaphysis (Fig. 21a,h).
Table 24: Mechanical properties for C57BL/6 and MRL/MpJ native femur (mean±SD)

<table>
<thead>
<tr>
<th></th>
<th>Torsional Rigidity [N-mm^2/rad]</th>
<th>Yield Torque (N.mm)</th>
<th>Maximum Torque (N.mm)</th>
<th>Rotation at Yield (rad/mm)</th>
<th>Rotation at Failure (rad/mm)</th>
<th>Energy to Failure (N-rad/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C57BL/6</strong></td>
<td>Normal (N=8/8 recorded)</td>
<td>1406 ± 434.72</td>
<td>31.23 ± 7.33</td>
<td>40.64 ± 5.18</td>
<td>0.023 ± 0.007</td>
<td>0.034 ± 0.008</td>
</tr>
<tr>
<td><strong>MRL/MpJ</strong></td>
<td>Normal (N=7/7 recorded)</td>
<td>1577.43 ± 224.73</td>
<td>46.16 ± 11.49</td>
<td>57.23 ± 6.59^a</td>
<td>0.029 ± 0.006</td>
<td>0.044 ± 0.007^a</td>
</tr>
</tbody>
</table>

^a significantly different than C57BL/6 native femur

The MRL/MpJ Fails to Regain Native Mechanical Properties Up to 8 Weeks Following Osteotomy

The majority of the C57BL/6 and MRL/MpJ defects failed to successfully bridge the critical-sized osteotomy, resulting in a nonunion at both 5 and 8 weeks. A nonunion was defined as a failure of the test tissue to withstand an applied load during torsion testing and verification by x-ray (Fig. 20c-f). At 5 weeks, the C57BL/6 and MRL/MpJ resulted in nonunion formation at a rate of 100% (8/8) and 75% (6/8), respectively and at 8 weeks, the C57BL/6 and MRL/MpJ resulted in nonunion formation at a rate of 80% (8/10) and 88% (7/8), respectively. As such, these mechanical results were excluded from any statistical analysis.

At 5 weeks, two of the MRL/MpJ repairs showed measurable torsional rigidity; however it was far inferior to the native control (90.50±19.09 vs. 1577.43±224.73, mean±SD). At 8 weeks, two of the C56BL/6 and one of the MRL/MpJ repairs exhibited structural integrity; however, again, it was inferior to the respective native controls (103.5±70.64 vs. 1406.00±434.72 and 207.00 vs. 1577.43±224.73, mean±SD). For these samples, results for yield torque, maximum torque, rotation at yield, rotation at failure, and energy to failure showed in similar trends, with the defect tissue failing far below native properties (Table 25).
Fig. 20: X-ray comparisons between C57BL/6 and MRL/MpJ for native tissue (a-b), 2 weeks post osteotomy (c-d), and 5 weeks post osteotomy (e-f). Neither strain was capable of fully repairing the critical size defect at 2 or 5 weeks.

The MRL/MpJ Responded to Sham Procedure Showing Significantly Increased Mechanical Properties

While the defect tissue mechanical results were omitted from statistical testing, testing for the sham tissue was conducted via a two-way ANOVA with time and strain set as fixed factors. Time post-surgery and mouse strain each significantly affected the sham tissue properties (p<0.05).

At 5 weeks, the C57BL/6 showed increased torsional rigidity compared to normal (2341.00 ± 1006.57N-mm²/rad vs. 1406.00 ± 434.72 N-mm²/rad, mean±SD); however, all other recorded parameters were significantly inferior to normal (Table 25, p<0.05). By 8 weeks, torsional rigidity was still greater than normal (2092.90 ± 435.79N-mm²/rad, p<0.05) and all
other parameters had returned to normal levels, with the exception of rotation at failure still below normal (Table 25, 0.024 ± 0.010 rad/mm vs. 0.034 ± 0.008 rad/mm, mean±SD).

In contrast, the MRL/MpJ exhibited increased torsional rigidity (3255.29 ± 1248.68 N-mm²/rad vs. 1577.43 ± 224.73 N-mm²/rad, mean±SD) and maximum torque (73.73 ± 18.40 N.mm vs. 57.23 ± 6.59 N.mm, mean±SD) compared to normal at 5 weeks, and by 8 weeks, torsional rigidity, yield torque, and maximum torque were all significantly greater than normal (p<0.05). Further comparison of the MRL/MpJ and C57BL/6 sham properties at corresponding time points shows the MRL/MpJ has significantly greater values with respect to yield torque, maximum torque, rotation at yield, and energy to failure (Table 25, p<0.05).
Table 25: Mechanical parameters for defect, sham, and age-matched native controls following critical size femoral osteotomy (mean±SD). Defect samples were not included in statistical analysis because of poor healing outcomes resulting in nonunion.

<table>
<thead>
<tr>
<th></th>
<th>Torsional Rigidity [N-mm^2/rad]</th>
<th>Yield Torque (N.mm)</th>
<th>Maximum Torque (N.mm)</th>
<th>Rotation at Yield (rad/mm)</th>
<th>Rotation at Failure (rad/mm)</th>
<th>Energy to Failure (N-rad/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (N=8/8 recorded)</td>
<td>1406.00 ± 434.72</td>
<td>31.23 ± 7.33</td>
<td>40.64 ± 5.18</td>
<td>0.023 ± 0.007</td>
<td>0.034 ± 0.008</td>
<td>0.780 ± 0.177</td>
</tr>
<tr>
<td>5-wk Defect (N=0/8 recorded)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>5-wk Sham (N=4/8 recorded)</td>
<td>2341.00 ± 1006.57^a</td>
<td>10.60 ± 5.39^a</td>
<td>12.00 ± 5.74^a</td>
<td>0.005 ± 0.002^a</td>
<td>0.009 ± 0.004^a</td>
<td>0.073 ± 0.056^a</td>
</tr>
<tr>
<td>8-wk Defect (N=2/10 recorded)</td>
<td>103.5 ± 70.64</td>
<td>1.05 ± 0.07</td>
<td>1.30 ± 0.14</td>
<td>0.012 ± 0.008</td>
<td>0.013 ± 0.007</td>
<td>0.020 ± 0.000</td>
</tr>
<tr>
<td>8-wk Sham (N=10/10 recorded)</td>
<td>2092.90 ± 435.79^a</td>
<td>38.82 ± 15.32</td>
<td>43.73 ± 16.54</td>
<td>0.020 ± 0.007</td>
<td>0.024 ± 0.010^a</td>
<td>0.743 ± 0.352</td>
</tr>
<tr>
<td>MRL/MpJ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (N=7/7 recorded)</td>
<td>1577.43 ± 224.73</td>
<td>46.16 ± 11.49</td>
<td>57.23 ± 6.59</td>
<td>0.029 ± 0.006</td>
<td>0.044 ± 0.007</td>
<td>1.494 ± 0.325</td>
</tr>
<tr>
<td>5-wk Defect (N=2/8 recorded)</td>
<td>90.5 ± 19.09</td>
<td>1.7 ± 0.28</td>
<td>1.75 ± 0.35</td>
<td>0.019 ± 0.001</td>
<td>0.021 ± 0.001</td>
<td>0.020 ± 0.003</td>
</tr>
<tr>
<td>5-wk Sham (N=8/8 recorded)</td>
<td>3255.29 ± 1248.68^b,c</td>
<td>58.74 ± 17.05^c</td>
<td>73.73 ± 18.40^b,c</td>
<td>0.021 ± 0.012^b,c</td>
<td>0.011 ± 0.001^b</td>
<td>1.308 ± 0.612^c</td>
</tr>
<tr>
<td>8-wk Defect (N=1/8 recorded)</td>
<td>207.00</td>
<td>3.24</td>
<td>6.52</td>
<td>0.015</td>
<td>0.061</td>
<td>0.256</td>
</tr>
<tr>
<td>8-wk Sham (N=8/8 recorded)</td>
<td>2513.25 ± 334.63^b</td>
<td>68.83 ± 22.19^b,c</td>
<td>79.55 ± 16.63^b,c</td>
<td>0.028 ± 0.011^c</td>
<td>0.033 ± 0.014^b,c</td>
<td>1.676 ± 0.669^c</td>
</tr>
</tbody>
</table>

^a significantly different than C57BL/6 native femur, ^b significantly different than MRL/MpJ native femur, ^c significantly different than time-matched C57BL/6 sham femur
MRL/MpJ Shows Enhanced Osteoblastic and Osteoclastic Activity at 2 and 5 Weeks Post-Osteotomy

Both strains responded to the critical-size osteotomy by forming a callus and attempting to bridge the defect, although each attempt was unsuccessful. At both 2 and 5 weeks, granulation tissue is observed in the defect region; however, there is no mineralization within this region as seen via x-ray (Fig. 20c-f) and calcein blue staining (Fig. 21f,m). The MRL/MpJ appears to produce a more robust periosteal response compared to the C57BL/6 at both time points (Fig. 21b-n), with larger callus formation, increased expression of both AP and TRAP at both 2 and 5 weeks (Fig. 21b-n), and increased bone formation. By 5 weeks, both strains show capping at the callus ends; however the MRL/MpJ continues to express increased levels of AP and TRAP within the callus region. There does not appear to be differences in cellular proliferation at either time point between the strains.
Fig. 21: Cryohistology for C57BL/6 and MRL/MpJ native tissue (a,h) and 2- and 5-weeks post osteotomy (b-g, i-n). Critical defects fail to successfully bridge the defect in C57BL/6 and MRL/MpJ strains at either time point (b,i); however, the MRL/MpJ shows enhanced AP and TRAP activity at both 2 (d, k) and 5 (g, n) weeks following osteotomy in the callus region. Both strains show callus capping by 5 weeks (f, m).
DISCUSSION

The goal of this study was to characterize the MRL/MpJ’s response to a critical-size femoral osteotomy based on histological and biomechanical response measures. The rate of nonunion formation and the inconsistencies associated with current treatment strategies necessitates that we identify alternative approaches to improving the long-term outcomes. Previous work by our group and others have demonstrated the MRL/MpJ mouse strain exhibits improved healing outcomes following injury in a number of musculoskeletal tissues, including tendon (Chapter 4) and articular cartilage. We chose to evaluate this strain’s response to a critical-size femoral osteotomy, as previous work by our group indicated an inability of the wildtype strain to successfully repair this type of injury.

Unfortunately, most likely due to the size of the osteotomy, neither strain was capable of bridging the defect space and showed no differences in mechanical outcomes. Histological investigation showed capping of the fracture ends at 5 weeks, further confirming the mechanical results. Despite the poor mechanical results, the MRL/MpJ does qualitatively show enhanced expression of enzymes involved in bone formation and turnover, AP and TRAP, compared to the wildtype at both 2 and 5 weeks. This suggests that, despite the capping of the fracture ends, the MRL/MpJ displays a prolonged response to injury, similar to what was observed in the MRL/MpJ’s response to a tendon injury (Chapter 4). It could be that the shear volume of removed bone was simply too large for cells to bridge the defect.

To our knowledge, there are no published studies evaluating the MRL/MpJ’s response to an osseous injury; however, there are numerous previous studies that also show minimal differences between the MRL/MpJ and C57BL/6 healing responses in other tissue and injury types. Cimini et al. created a cryogenic injury to the right ventricle of both C57BL/6
and MRL/MpJ mice and found no difference in infarct area, infarct thickness, or left ventricle thickness up to 96 days following injury between the two strains\textsuperscript{260}. These results were coupled with similar blood vessel densities and rates of cellular proliferation\textsuperscript{260}. Beare et al. created two 4mm dorsal excisional skin wounds in both C57BL/6 and MRL/MpJ mice and found the MRL/MpJ repaired the injury in a similar manner as wildtype, via granulation tissue formation and collagen deposition, resulting in a scar\textsuperscript{134}. No differences in macrophage infiltration or smooth muscle actin alpha expression were observed \textsuperscript{134}. Interestingly, ear hole punches in the same set of animals healed without the formation of scar, exhibiting completely ‘regenerated’ tissue. The authors of this study, along with other investigators who reported poor healing outcomes, suggest that differences in the tissue architecture and microenvironment govern the healing outcomes in the MRL/MpJ strain\textsuperscript{132, 134}.

In this study, it appears that the MRL/MpJ does employ an alternative response to injury despite its inability to bridge the defect based on the mechanical results of the sham procedure (Table 25). The MRL/MpJ strain exhibited a more robust response to this treatment compared to the C57BL/6. Compared to the respective native controls at five weeks, the MRL/MpJ sham showed significantly greater torsional rigidity and maximum torque while the C57BL/6 sham only showed greater torsional rigidity. By 8 weeks, the MRL/MpJ sham displayed increased torsional rigidity, yield torque, and maximum torque while the C57BL/6 only showed increased torsional rigidity compared to native. While the sham procedure did not include an excisional injury, it did induce a healing response, attributable to the presence of the bone screws and plate as evident by the observed changes in the mechanical measures. This suggests the MRL/MpJ may have the capacity for improved osseous healing compared to wildtype; however, future work investigating a smaller bony defect will need to be completed to verify this claim.
This study is not without limitations. 1) We only evaluated the creation of a critical-size osteotomy. Previous work by our group investigated the healing response to both sub-critical and critical-size femoral osteotomies and found the wildtype was capable of bridging a sub-critical defect 2 weeks post injury. Based on this result, we did not include this treatment group as a part of this study; however, comparing the MRL/MpJ and C57BL/6 responses to a sub-critical defect could help to identify differences that exist in the two processes, even if they both would successfully bridge the defect region. 2) The method of placing the fixation plate for fracture stabilization is invasive. Placing and fixing the fixation plate onto the femur involves stripping the muscle away from the bone and drilling 4 screw holes through both cortices. Further, using a dremel to create the screw holes and osteotomy generates heat that can negatively affect the femur and surrounding tissue, potentially inducing thermal injury. Future model development work should investigate alternative methods of creating femoral osteotomies to minimize the severity of the procedure.

While the MRL/MpJ did not repair a critical-size femoral osteotomy to a greater degree than the C57BL/6, findings from this study suggest the MRL/MpJ may be employing an alternative healing response. Future work should investigate additional models of bone injury to identify and better understand the mechanisms that are activated in response to injury in both the MRL/MpJ and C57BL/6. Findings from these studies have the potential to improve our approaches to tissue engineering for musculoskeletal applications.

MATERIALS AND METHODS

Biomechanics and histology were assessed following the creation of a 1.6mm long critical-size femoral osteotomy in 10-week old C57BL/6 and MRL/MpJ male mice. Breeding pairs for both the C57BL/6 (stock number: 000664) and MRL/MpJ (stock number: 000486)
were obtained from The Jackson Laboratory (Bar Harbor, ME) and then bred in-house to produce animals for this study. Following osteotomy, natural healing based on mechanical outcomes at 5 and 8 weeks (n=7-10 per time point) and histology at 2 and 5 weeks (n=2 per time point) was compared between the MRL/MpJ and C57BL/6 strains (Table 26). Comparisons were also made to the respective sham and native femora from age-matched controls.

Table 26. Experimental design comparing C57BL/6 and MRL/MpJ mechanical and biological measures following critical-size femoral osteotomy

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time Post Surgery (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>NATIVE</td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td></td>
</tr>
<tr>
<td>Histology/IHC</td>
<td>2</td>
</tr>
<tr>
<td>Biomechanics</td>
<td>8</td>
</tr>
<tr>
<td>MRL/MpJ</td>
<td></td>
</tr>
<tr>
<td>Histology/IHC</td>
<td>2</td>
</tr>
<tr>
<td>Biomechanics</td>
<td>7</td>
</tr>
<tr>
<td>DEFECT</td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td></td>
</tr>
<tr>
<td>Histology/IHC</td>
<td>2</td>
</tr>
<tr>
<td>Biomechanics</td>
<td>8</td>
</tr>
<tr>
<td>MRL/MpJ</td>
<td></td>
</tr>
<tr>
<td>Histology/IHC</td>
<td>2</td>
</tr>
<tr>
<td>Biomechanics</td>
<td>8</td>
</tr>
<tr>
<td>SHAM</td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td></td>
</tr>
<tr>
<td>Histology/IHC</td>
<td>2</td>
</tr>
<tr>
<td>Biomechanics</td>
<td>8</td>
</tr>
<tr>
<td>MRL/MpJ</td>
<td></td>
</tr>
<tr>
<td>Histology/IHC</td>
<td>2</td>
</tr>
<tr>
<td>Biomechanics</td>
<td>8</td>
</tr>
</tbody>
</table>

Ethics Statement

All protocols and procedures were approved by the University of Cincinnati’s Institutional Animal Care and Use Committee.

Surgical Procedure

Animals were anesthetized through inhalation of 3% isoflurane and both hindlimbs were shaved and aseptically prepared using ethanol and betadine washes. Mice were placed on the
sterile surgical table in the prone position and a longitudinal incision was made exposing the quadriceps and hamstring muscles. A Jeweler’s forceps was used to isolate the mid-shaft of the femur from surrounding musculature. The surgeon then placed a custom fixation plate along the length of the femur using a specially designed clamp. This titanium plate is equipped with a central taper to allow easy placement with the clamp, four screw holes positioned linearly along the length of the plate, and tabs on the bottom allowing the plate to ‘snap’ into place on the femur and minimize fixation plate-bone contact (Fig. 22a-c). Once the fixation plate was placed and clamped using a specially designed clamp (Fig. 22d), four screw holes were drilled through both cortical shells using a hand-held Dremel (Dremel, Racine, WI) and four 000 X 5/32 Self/Tap screws (FF000CE156; Morris, Sourthbridge, MA, Fig. 22e) were screwed into place affixing the plate to the femur. The clamp was removed, and a critical-size (1.6 mm long) osteotomy was created using a Dremel equipped with a #54 black oxide drill bit (20154; Irwin Tools, Huntersville, NC) (Fig. 23).
Fig. 22: Surgical equipment for creating the femoral osteotomy.  a-c Top, side, and bottom view of the titanium fixation plate designed and manufactured at the University of Michigan (Ann Arbor, MI).  d Specially designed clamp used to hold the titanium plate in place during the plating procedure.  e Screws used to fix the plate on the femur.

The defect site was irrigated with saline to remove excess bone and debris and then the muscles were repositioned over the fixation plate. A sham procedure was done in the contralateral limb by fixing the titanium plate to the femur, but no osteotomy was performed. No surgery was performed on the uninjured, control groups. Following surgery, surgical animals were administered a 2 mg/kg intraperitoneal injection of buprenorphine and permitted unrestricted cage activity until the time of sacrifice.
Fig. 23: A critical-size femoral osteotomy created at surgery.

Biomechanical Analysis

Following sacrifice at 5 and 8 weeks post-osteotomy, the femora were disarticulated from the acetobulofemoral joint and skin and muscle were removed. Test specimens were then wrapped in saline-soaked gauze, frozen at -80°C, and shipped overnight to the Awad Laboratory at the University of Rochester for biomechanical testing.

Following X-ray, samples were hydrated in phosphate buffered saline for one hour and then potted using poly(methyl methacrylate) bone cement (DePuy Orthopaedics, Inc., Warsaw, IN) in a specially designed jig equipped with 6.35 mm² aluminum tube holders ensuring consistent gage length and axial alignment throughout testing. Specimens were allowed to cure for two hours. Femora were rehydrated and then mounted on an EnduraTec TestBench™ system (200 N-mm torque cell; Bose Corporation, Minnetonka, MN). Prior to test initiation, the fixation plates were cut using a hand Dremel equipped with a stainless steel, diamond coated disc perpendicularly through the tapered portion. Specimens were tested in torsion at 1°/s until failure. X-rays were taken pre- and post-testing to assess failure mode. Ultimate torque, yield torque, and torsional rigidity were obtained by plotting the torque data against the rotational deformation (normalized to gage length).
Histological Analysis

Following sacrifice at 2 and 5 weeks, femora were excised and all muscle and skin were removed. Specimens were fixed in 4% paraformaldehyde (AA433689M, Fisher Scientific, Pittsburgh, PA) for 3 days at 4°C and then placed in phosphate-buffered saline and sent overnight to the Rowe Laboratory at the University of Connecticut for histological and immunohistochemical analysis. X-rays were taken (24kV for 8s, LX 60, Faxitron, USA) and then the fixation plates were removed from the specimens. Femora were soaked overnight in a 30% sucrose/phosphate-buffered saline solution. The following day, samples were embedded in O.C.T. compound (Andwin Scientific Tissue-Tek™, Schaumburg, IL) for frozen sectioning. Coronal sections were cut along the length of the femur at 2 levels using a Leica CM3050S cryostat (Leica, Wetzlar, Germany). Sections were taken using cryofilm (Cryofilm Type 2C, Section-lab, Hiroshima, Japan) at each level, affixed to a glass slide, and allowed to dry for 48 hours at room temperature.

All fluorescent evaluation was completed using a Zeiss Axio Scan.Z1 scanning fluorescent microscope (Carl Zeiss, Thornwood, NY) and imaged with a monochrome digital camera (Zeiss AxioCamHRm) at a magnification of 10X. Sections were soaked in PBS for 10 minutes, stained with 30mg/mL calcein blue solution (#M1255-1G; Sigma-Aldrich, St. Louis, MO) for 30 minutes to visualize mineralized tissue, and then cover-slipped in 50% glycerin for imaging. Following imaging, coverslips were removed, sections were washed with PBS, and then stained with fluorescent ELF-97 phosphatase substrate (E6589; Life Technologies, Grand Island, NY) to identify osteoclasts expressing tartrate-resistant acid phosphatase (TRAP). A yellow filter for tetracycline was used to capture the fluorescent signal (Custom HQ409sp, 425dcxr, HQ555/30, C-104285; Chroma Technology; Bellows Falls, VT). Coverslips were
removed, slides were washed with PBS, stained with liquid fast-red substrate (ab64254; Abcam, Cambridge, England) to assess alkaline phosphatase (AP) activity and EdU following the manufacturer’s protocol (Click-iT® EdU Alexa Fluor® 647 Imaging Kit, C10340, Life Technologies) to assess cellular proliferation, and reimaged. AP and EdU signals were captured with a TRITC (Chroma 49005 ET) and Cy 5 (Chroma 49006 ET) filter, respectively. Finally, slides were stained with toluidine blue (89640, Sigma-Aldrich) to evaluate overall tissue morphology and cartilage proteoglycans in the callus.

**Statistical Analysis**

Because the majority of the critical-size defects resulted in nonunion, and thus showed no measurable torsional rigidity, defect samples were not included in the statistical analysis. Biomechanical properties for contralateral shams and natives were compared using two-way ANOVA with time post-injury and strain as fixed factors with Fisher’s least significant difference used for post-hoc comparisons (p<0.05). All statistical testing was carried out using IBM SPSS Statistics 2.1.0 (Chicago, Illinois) software.

**ACKNOWLEDGEMENTS**

We gratefully acknowledge the National Institutes of Health for providing research and student support (R01 AR056943). The authors would like to thank Dr. Brian Grawe (University of Cincinnati, Cincinnati, Ohio) for surgical assistance and Jeffrey Meganck (University of Michigan, Ann Arbor, Michigan) for machining fracture fixation plates.
Loss of Mohawk Homeobox Function Produces Morphological Abnormalities and Biomechanical Deficiencies in the Adult Murine Patellar Tendon

Andrea L. Lalley, B.S., Han Liu, Ph.D., Rulang Jiang, Ph.D., Jason T. Shearn, Ph.D.

Biomedical Engineering Program, College of Engineering and Applied Science, University of Cincinnati, Cincinnati, Ohio

Division of Developmental Biology, Cincinnati Children’s Hospital Research Foundation, Cincinnati, Ohio

1This manuscript is currently in preparation for the Journal of Orthopaedic Research
ABSTRACT

To improve tissue engineering strategies, engineers and biologists are now collaborating to identify mechanisms that regulate successful tissue formation during normal development and then use these findings to inform future repair approaches. The mohawk homeobox transcription factor (Mkx) was recently found to function in tenocyte differentiation and tendon maturation during late pre-natal and early post-natal developmental stages; however, it is unknown what the long-term effects of ablating gene function would be on post-natal tendon development and maturation. We evaluated the morphological and mechanical outcomes for the patellar tendon in 20-week old Mkx-/- and Mkx+/- littermates. We found that the Mkx-/- mutant mice exhibit gross abnormalities in patellar tendons including irregular collagen color and vibrancy, decreased tendon length, and abnormal patella shape and size. Upon inspection, 18% of the patellar tendons were found to be ruptured. Mechanical test results showed 33% and 31% reductions in linear stiffness and modulus, respectively, in the mutants in comparison to the control animals. Histological evaluation confirmed the gross anatomical observations, including elongated and flattened patella and excessive scar tissue formation in the ruptured tendons. This study, in concert with previous developmental studies, suggests that Mkx is critical in regulating tendon tissue formation and maturation and influences the accumulation of normal tendon properties into adulthood. Therefore, future studies should investigate the role of mohawk homeobox following tendon injury and identify downstream targets for manipulation in tissue engineering strategies.
INTRODUCTION

Tendons and ligaments are commonly injured musculoskeletal tissues that continue to pose a burden on the U.S. economy\(^1\). Tissue engineers seek to improve healing outcomes by incorporating cells, matrix material, and in vitro pre-conditioning protocols to create tissue engineered constructs (TECs); however, we have failed to successfully and repeatedly restore native tissue properties following repair. Previous attempts in our laboratory have benchmarked success on 1) exceeding peak forces previously recorded in vivo and, 2) matching normal tangent stiffness beyond in vivo force levels with an associated safety factor\(^{17}\). We now know that to achieve these mechanical properties, we need to understand the biological processes that ultimately give rise to these desired mechanical outcomes.

Normal tendon development presents an opportunity to understand the mechanisms that function to form tendon throughout embryogenesis and early post-natal growth. Collaborating with developmental biologists at Cincinnati Children’s Hospital Medical Center, we have begun to identify novel molecular targets and signaling processes that regulate successful tendon formation, differentiation, and maturation and translate these findings to improve our tissue engineering approaches. To date, little is known about the molecular events that initiate and contribute to successful tendon tissue formation. We are only now beginning to determine the spatiotemporal expression profiles of tendon-related markers during development and understand the role they play in differentiation and maturation.

Scleraxis (Scx), a basic helix-loop-helix transcription factor, was identified in 2001 as a tendon cell marker activated during embryogenesis\(^{53}\). Further, it is modulated by mechanical loading and has been found to regulate collagen type I and tendomodulin expression into
adulthood. More recently, mohawk homeobox (Mkx), a gene that encodes an atypical homeodomain-containing protein exhibiting transcriptional repressor activity, has been identified as a key regulator of tendon morphogenesis during embryogenesis and early post-natal stages. Members of the homeobox gene superfamily function to pattern and spatially organize the embryo throughout development. Mkx contains three amino acids between helix I and helix II of the homeodomain furthering classifying it as a three-amino-acid loop extension (TALE) member of the atypical homeobox genes. During mouse embryogenesis, Mkx expression is first observed approximately nine days post conception in the developing somites and later expression becomes localized to the testis cords of the developing male, early chondrogenic mesenchymal cells, the kidney, and the immature tendon cells of the limbs and tail. In early tendon tissues, Mkx expression is observed to co-localize with Scx expression; however, studies have shown they act independently of one another to regulate tendon cell differentiation and maturation. Loss of Mkx gene expression resulted in irregular collagen fibril sizes and distribution, abnormal tendon sheath formation, and decreased expression of collagen type I, fibromodulin, and tenomodulin evaluated up to twenty-one days following birth. Furthermore, gross inspection showed abnormal tendon color and vibrancy. It is unknown how loss of Mkx function impacts long-term tendon mechanical properties and biological function in the adult animal or if it plays a role in tendon healing following injury.

Thus, the objective of this study was to evaluate the effects of knocking out Mkx expression on the long-term morphological and biomechanical outcomes in the adult murine patellar tendon. We hypothesized that compared to the heterozygous littermate controls, Mkx-/mutant mice would exhibit 1) decreased patellar tendon size and 2) decreased mechanical properties.
RESULTS

Mkx-/ Mutant Mice Show Abnormal Tendon Morphology

Gross observation of the Mkx-/ patellar tendons showed several abnormalities compared to the C57BL/6 and Mkx+/ control strains (Fig. 24a). Mkx-/ patellar tendons 1) appeared short in length, 2) were difficult to discern from the medial and lateral retinaculum and, 3) lacked the typical collagen vibrancy and color observed in the wildtype and heterozygotes (Fig. 24c). Mkx-/ patellas also had an observable phenotype appearing large, and irregularly shaped (Fig. 24c). Furthermore, 18% (2 out of 11) of the Mkx-/ PTs evaluated were found to be ruptured prior to sacrifice and therefore not included in the biomechanical testing (Fig. 24d).

Fig. 24: Gross observations of Mkx+/ and Mkx-/ patellar tendon and surrounding tissue. C57BL/6 patellar tendons have an average length of 3 mm, have well-defined medial and lateral borders, and ‘glistening’ white collagen (a). Mkx+/ PTs have average length and collagen vibrancy along with well-defined borders, not qualitatively different from the wildtype control (b). Mkx-/ PTs were short, lacked collagen color and vibrancy, difficult to discern medial and lateral PT borders, and abnormally large patella (white arrow) (c). Two out of the eleven specimens were found to be ruptured as indicated by proximal translocation of the patella (top white arrow), lengthening of the PT, and the appearance of the femoral chondyle through the midsubstance of the PT (bottom white arrow) (d).

Mkx-/ Mice Exhibit Decreased Structural and Material Properties Compared to Mkx+/ Controls
Mkx/- PTs were significantly shorter compared to Mkx+/− tendons (2.59 ± 0.46 mm vs. 3.15 ± 0.48 mm; p=0.014); however, intact width, intact thickness, and tested width were not different (p=0.35, p=0.43, p=0.83, respectively) (Table 27).

**Table 27:** Patellar tendon dimensions for Mkx+/− and Mkx/- PTs mouse strains (mean±SD)

<table>
<thead>
<tr>
<th></th>
<th>Intact Length (mm)</th>
<th>Intact Width (mm)</th>
<th>Cut Width (mm)</th>
<th>Thickness (mm)</th>
<th>Cross-Sectional Area (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mkx+/− (N=11)</td>
<td>3.15±0.48</td>
<td>0.96±0.23</td>
<td>0.55±0.09</td>
<td>0.33±0.04</td>
<td>0.18±0.02</td>
</tr>
<tr>
<td>Mkx/- (N=9)</td>
<td>2.59±0.46a</td>
<td>0.83±0.16</td>
<td>0.55±0.11</td>
<td>0.28±0.06</td>
<td>0.15±0.04</td>
</tr>
</tbody>
</table>

*a significantly different than Mkx+/− PT

Mkx+/− PT mechanical outcomes were not different from previously tested C57BL/6 values (Fig. 25). In terms of structural outcomes, the Mkx/- PT was significantly less stiff compared to Mkx+/− (Fig. 26, Table 28; 6.79 ± 2.55 N/mm vs. 10.14 ± 1.80 N/mm; p=0.003) but ultimate strength was not significantly different (3.54 ± 1.44 N vs. 4.56 ± 1.34 N; p=0.12). Material analysis of Mkx/- tendon tissue showed a significant reduction in modulus (Fig. 26, Table 28; 124.81 ± 60.93 MPa vs. 180.91 ± 34.32 MPa; p=0.02), but no significant difference in maximum stress (22.54 ± 10.83 MPa vs. 25.75 ± 6.83 MPa; p=0.43). Mkx/- displayed a 22% increase in strain at failure compared to the controls (Fig. 26).
**Fig. 25:** Load-displacement failure curves for native 20-week old C57BL/6 and Mkx+/- patellar tendon. There were no significant differences in structural properties between the two strains (p>0.05). Error bars represent SEM.

**Table 28:** Structural and material properties for Mkx+/- and Mkx-/- patellar tendon properties (mean±SD)

<table>
<thead>
<tr>
<th></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>Mkx+/- (N=11)</td>
<td>4.56±1.34</td>
<td>10.14±1.80</td>
<td>25.75±6.83</td>
<td>180.91±34.32</td>
</tr>
<tr>
<td>Mkx-/- (N=9)</td>
<td>3.54±1.44</td>
<td>6.79±2.55(^a)</td>
<td>22.54±10.83</td>
<td>124.81±60.93(^a)</td>
</tr>
</tbody>
</table>

\(^a\)significantly different than Mkx+/- PT
**Fig. 26:** Loss of mohawk homeobox expression produces abnormal patellar tendon mechanical properties in the adult animal. Mkx-/- animals showed a 33% reduction in linear stiffness (a; p=0.003), a 31% reduction in modulus (b; p=0.02), and a 22% increase in failure strain compared to Mkx+/- mice. Error bars represent SEM.

**Both Mkx+/- and Mkx-/- Display Atypical Entheses Formation**

Toluidine blue O staining of Mkx-/- and Mkx+/- tendons revealed abnormal patellar tendon phenotypes. Gross examination of the Mkx-/- showed two distinct phenotypes, which we have examined histologically (Fig. 27). The first phenotype showed a significantly decreased tendon length (Fig. 27a) and elongated and flattened patella shape (Fig. 27a). The second phenotype resulted from a rupture occurring at some point during the animal’s 20-week life. This was characterized by excessive scar tissue formation, proximal shift of the patella, complete loss of normal tendon architecture, and increased cellularity (Fig. 27d). Histologic examination of the Mkx+/- patellar tendon revealed normal patella insertion (Fig. 28a) and collagen architecture (Fig. 28b); however, it appears the fibrocartilage region at the tibia extends up into the midsubstance of the PT, as indicated by the stacked cuboidal cells along the collagen fibers (Fig. 28c).
Fig. 27: Mmx-/ animals exhibit two distinct phenotypes (a,d). Toluidine blue O staining shows regions of patellar tendon structure including tendon midsustance (light purple), unmineralized region (dark purple), and mineralized region (gray-purple). Cell nuclei stain dark purple and can be observed along the length of the patellar tendon embedded in the collagen. For the first phenotype (a), tendons were shorter compared to Mmx+/ and displayed increased patella length and flattened, as opposed to rounded, shape. The orientation of the collagen fibers with the patellar and tibial tidemark appears normal (b,c); however, there was no visible tibial tuberosity (red arrow, c). The second phenotype observed was classified as a rupture occurring at some unknown time during the 20 weeks of the animal’s life (d). This was characterized by excessive scar formation, tissue thickening, fat pad enlargement, a proximal translocation of the patella, hypercellularity, and unidentifiable tendon tissue.
Fig. 28: Mkx+/- shows normal patella insertion (a) and collagen architecture (b). The tibial insertion was abnormal, displaying increased fibrocartilage region length characterized by cuboidal, stacked chondrogenic cells, extending into the midsubstance of the patellar tendon (c, inset).

DISCUSSION

Investigating normal tendon development provides the opportunity to identify novel molecular targets and pathways for future tissue engineering studies. Previous work by our group, and others, indicates that Mkx is a key regulator in successful tendon formation and early stages of maturation following birth\textsuperscript{55-57}. In this study, we show that ablating Mkx expression produces morphological abnormalities and biomechanical deficiencies in the adult patellar tendon.

Consistent with developmental studies\textsuperscript{55-57}, gross observations of the adult Mkx-/- mice showed collagen discoloration, poorly defined patellar tendon borders, and a reduction in tendon size compared to the controls. Further, we found a 18% patellar tendon rupture rate in the Mkx-/- mutants. These gross observations were coupled with 33% and 31% reductions in linear stiffness and modulus, respectively, in comparison to the heterozygote control. Ito et al. measured the tensile properties of the Achilles tendon in 3-month old Mkx null mice and reported a 40% reduction in linear stiffness; however, there was no difference in modulus
between the homozygous and heterozygous animals and suggested the mechanical outcomes were a result of the decreased tendon size\textsuperscript{56}. They also reported no differences in ultimate load or maximum stress.

The homeobox genes are a large class of genes that function during early developmental stages to direct the formation of tissues and limbs and/or provide cues for differentiation. These genes encode a homeodomain-containing transcription factors that control specific target gene expression. Previous studies suggest that the function of Mkx is to regulate tenocyte differentiation and maturation\textsuperscript{55}. In this study, we showed abnormalities associated with mineralization/ossification at both the patellar and tibial insertions, resulting in decreased tendon length. In the homozygous animals, we found the patella to be abnormally shaped, being flatter and longer than controls (Fig. 27a). Additionally, the tibial tuberosity failed to form properly (Fig. 27c). In the heterozygous animals, we found increased extension of the tibial fibrocartilage region into the midsubstance of the patellar tendon (Fig. 28c). By knocking out Mkx function, tenocyte differentiation and function could be altered in such a way that results in a failure of these cells to adequately orchestrate the molecular events forming the tendon entheses.

This study is not without limitations. 1) We did not measure gene expression for tenogenic markers. Liu et al. demonstrated that knocking out Mkx expression resulted in decreased expression of collagen type I, fibromodulin, and tenomodulin up to 21 days following birth\textsuperscript{55}. Decreased expression of these matrix components early on during post-natal development suggest that they may be downstream targets of Mkx-mediated transcriptional regulation, which would contribute to the decreased mechanical properties observed in the adult. We need to incorporate gene expression analysis in future work to gain insight to the effects of Mkx ablation on the tenogenic nature of the tissue. 2) We did not investigate collagen fibril
diameter and distribution. Based on the abnormal tendon color and vibrancy, one would expect there to be abnormalities in the collagen structure, perhaps altering collagen binding site structure or collagen fibril fusion\textsuperscript{263, 264}. Liu et al. found the process of collagen fibrillogenesis to be impaired in the Mkx\textsuperscript{-/-} animal as indicated by the lack of a bimodal distribution of both small and large collagen fibrils by 21 days post birth. While it is still unknown what specific role Mkx plays during tendon development and maintenance; it is hypothesized to regulate the process of collagen fibrillogenesis through modulation of proteoglycans or tenocyte maturation\textsuperscript{55, 56}. To expand upon this work, we should include an analysis of collagen fibril diameter distribution within the Mkx\textsuperscript{-/-} tendons to determine if developmental findings hold true in the adult.

Abnormal expression of other collagen types, particularly collagen type III, could also be a contributing factor to the Mkx\textsuperscript{-/-} phenotype. Collagen type III is a fibrillar collagen often found co-localized with collagen type I. It is highly expressed in the developing embryo\textsuperscript{265}. During the early stages of chick development, small diameter collagen types I and III fibrils are co-distributed throughout the metatarsal tendon\textsuperscript{266}. As the chick matures, both collagen type I expression and fibril diameters increase while collagen type III expression decreases\textsuperscript{266}. In a mature, healthy tendon, collagen type I predominates. Interestingly, the ratio of collagen type I and collagen type III expression levels following tendon injury shows an increased concentration of collagen type III accompanied by decreased fibril diameters\textsuperscript{78, 267}. One potential explanation for the Mkx\textsuperscript{-/-} phenotype is a skewed collagen I/collagen III ratio, with an overexpression of collagen type III due to poor collagen type I formation; however, further work is needed to better characterize the collagen composition of the adult Mkx\textsuperscript{-/-} tendon.

This study represents an attempt on our part to employ the approach we laid out in Chapter 3 for defining and evaluating biological success criteria. Previous developmental work
identified Mkx as highly involved in the formation of tendon. We have now proceeded to the next step in the strategy and assessed how manipulation of this parameter affects the mechanical outcomes in the adult. Clearly, loss of Mkx function greatly affects the maturation of the patellar tendon based on the morphological deficiencies, reduced mechanical properties, and the increased rate of spontaneous rupture compared to controls. We should now investigate the role of Mkx following injury to ascertain how improper activation of this gene during the healing process may be negatively affecting the accumulation of mechanical properties and/or contributing to scar formation.

**MATERIALS AND METHODS**

**Ethics Statement**

All animal protocols were approved by the University of Cincinnati and Cincinnati Children’s Hospital Medical Center Institutional Animal Care and Use Committees.

**Experimental Design**

Patellar tendon dimensions, structural and material properties, and failure locations were measured in 20-week old Mkx-/- (n=11) mice generated as previously described and Mkx+/- littermates (n=11) served as the control. Overall tissue morphology was assessed using hematoxylin and eosin staining (n=3 per group). Twenty week-old mice were used because the mice have reached skeletal maturity and the size of the patellar tendon permits for biomechanical evaluation.

**Biomechanical Testing**

Animals were sacrificed at 20 weeks of age and frozen at -20°C until the day of testing. Prior to testing, animals were thawed, skin and muscle was dissected away, and the knee joint was flexed to 45°. In the limb, the medial and lateral portions of the patellar tendon were
removed leaving the patella-central PT-tibia unit (0.55 ± 0.10, mean ± SD). The central-PT tissue unit length and width was measured by taking a digital image with a ruler in plane. The patella-PT repair tissue-tibia unit was then placed in a custom grip and fixed in place using polymethylacrylate (Dentsply International, York, PA) with the tibia cemented into the grip and secured with a staple to prevent slipping. The grip with the test tissue was then loaded into a materials testing system (100R; TestResources, Shakopee, MN) and lowered to position the patella into the conical-shaped bottom grip. PT repair tissue thickness was measured by taking a digital image of the pre-loaded tissue with a ruler in plane. The tissue was tested in a 37°C PBS bath by applying a preload to 0.02N, preconditioning for 25 cycles with 0-1% strain, and then failing in uniaxial tension at 0.1% of total tendon length/second. The applied load (N) and grip-to-grip displacement (mm) were recorded throughout the testing period.

Ultimate load, failure displacement, stress, and strain were recorded during the testing period. A linear regression algorithm was used to calculate stiffness and modulus from the linear portion of the load-displacement and stress-strain curves, respectively. The failure location was also recorded following the completion of the mechanical test.

**Histology**

After sacrifice, limbs were dislocated at the femoral joint, the skin and foot removed, and samples were fixed in 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) for 24 hours. Samples were then decalcified with 0.5M EDTA/PBS for 7 days at 4°C. Following decalcification, samples were further trimmed and embedded in O.C.T. compound (Andwin Scientific Tissue-Tek™, Schaumburg, IL) for frozen sectioning. Serial, longitudinal sections were cut along the length of the tendon at 3 different levels using a Leica CM3050S cryostat (Leica, Wetzlar, Germany). Twenty-micron thick sections were taken using cryofilm (Cryofilm
Type 2C, Section-lab, Hiroshima, Japan) at each level for histological analysis. Sections were affixed to glass slides and allowed to dry for 48 hours at room temperature. Sections were then hydrated in dH$_2$O for 15 minutes and then incubated in 0.025% toluidine blue (T161-25; Fisher Scientific, Waltham, MA) solution for 1 minute. Sections were rinsed in dH$_2$O and then mounted in 30% glycerol (in dH$_2$O). Sections were imaged to assess overall tissue morphology.

**Statistical Analysis**

All data were confirmed to be normal and homoscedastic preceding statistical testing. Tendon dimensional measurements and biomechanical values were compared between strains using a two-tailed student’s t test (p<0.05). The IBM SPSS Statistics 2.1.0 software (Chicago, Illinois) was used to perform all statistical testing.

**Acknowledgements**

We gratefully acknowledge the National Institutes of Health for providing research and student support (R01 AR056943). The authors would like to thank Dr. Lou Soslowsky for assistance with biomechanical test setup and analysis, and Steve Gilday and Hodari James for technical assistance.
Developing a Murine Model of Collagenase-Induced Patellar Tendon Injury

Andrea L. Lalley, B.S.\textsuperscript{a}, Cynthia Gooch, B.S.\textsuperscript{a}, Jason T. Shearn, Ph.D.\textsuperscript{a}

\textsuperscript{a}Biomedical Engineering Program, College of Engineering and Applied Science, University of Cincinnati, Cincinnati, Ohio

\textsuperscript{1}This manuscript is currently in preparation for the Journal of Orthopaedic Research.
ABSTRACT

The prevalence of degenerative tendon conditions requires the development of novel therapies to improve healing outcomes. Unfortunately, there are few murine models currently available that offer the opportunity to study degeneration. This study presents preliminary results to guide future decisions regarding the development of a collagenase-induced tendon injury model. We conducted a set of preliminary experiments to develop a murine model of collagenase-induced tendon injury. We hypothesized that injecting collagenase into the patellar tendon would result in decreased mechanical properties in the short-term, with a return to native properties by 5 weeks post-injection. Following a dose-series experiment evaluated at 24 hours post injection, we concluded the optimal concentration to be between 15000IU and 20000IU, based on a reduction of mechanical properties and reasonable rupture rate. Further study indicated that 15000IU and 20000IU collagenase injections resulted in decreased mechanical properties in the short-term, but a restoration of native mechanical properties by 5 weeks. While we failed to produce a sustained model of tendon degeneration, we are now interested in identifying and characterizing the processes that led to the restoration of native properties. Future work will incorporate histological evaluation of the repair tissue to fully characterize the healing response and begin to investigate the mechanisms contributing to this healing response.
INTRODUCTION

Injuries to the musculoskeletal system, particularly to tendon and ligaments, greatly affect the U.S. population, with 110 million patients reporting an injury in 2008\(^1\). Overuse injuries constitute a large majority, with 50\% of all job-related complaints resulting from an overuse or repetitive motion injury\(^268\). Further, it is estimated that 97\% of all tendon injuries exhibit characteristics of degeneration\(^269\). The prevalence of these injuries, coupled with a lack of effective clinical intervention, necessitates further investigation to develop an adequate animal model of tendinopathy to test novel therapies\(^270, 271\).

Our laboratory has investigated tendon healing using a full-length, full-thickness central patellar tendon injury model in the mouse and rabbit; however, this injury model is not clinically relevant and fails to display aspects of tendinopathy. Developing animal models that exhibit degenerative attributes continues to be a focus of the field; however, it has been difficult because the pathology and physiology of tendinopathy is not well understood\(^271\). In general, tendinopathy can be characterized as a loss of normal extracellular matrix composition and architecture, abnormal collagen fibril structure and distribution, cellular morphologic changes including rounding and apoptosis, and neovascularization, but definitions vary\(^60, 237, 272, 273\). These injuries are typically attributed to excessive, repetitive motions over an extended period of time; however, the molecular mechanisms regulating the processes are unknown\(^274\).

Unfortunately, animal models cannot fully replicate the human tendinopathic condition\(^275\). The majority of animals used to study tendon healing and repair are quadrupeds and thus experience different loading profiles and magnitudes compared to bipeds\(^275\). Another aspect to consider is the different metabolic rates between laboratory animals and humans, which can affect the rate and/or capacity of healing following injury\(^225, 276\).
Current animal models of tendinopathy are classified as either mechanically- or chemically-induced with studies conducted in various species including rat, rabbit, and equine, although each approach and model has limitations\textsuperscript{274}. Previous work in our laboratory indicates that, following a collagenase injection to a rabbit patellar tendon, the chronic pathology resolves by 12 weeks post-injury, with a return to near normal tendon architecture and cellular morphology (unpublished data). This approach could be viewed as a failure, as the insult did not maintain the chronic phenotype in the long-term; however, we have been interested in understanding the pathways that are activated to restore the tendon to its native structure. Characterizing the healing process following a collagenase-induced injury could provide insight towards improving tendon healing.

We have been developing a mouse model of collagenase-induced tendon damage to investigate an injury model that more closely resembles common clinical presentations. Furthermore, this injury model will add to our current knowledge base on tendon healing allowing us to investigate multiple injury types (‘acute’ vs. ‘chronic’), evaluate the effects of different injury types on the natural healing process (i.e. does an ‘acute’ injury heal differently than a ‘chronic’ injury?), and determine how potential therapeutic molecules might induce differential responses in contrasting injury model systems. To this end, we conducted a series of experiments investigating the effects of injecting increasing concentrations of collagenase (*Clostridium histolyticum*) into a mouse patellar tendon. We set out to identify a concentration range that would produce an initial decrease in mechanical properties in the short term (24hr) with a gradual restoration of properties over an extended period of time (2 and 5 weeks). This work will lay the foundation for future experiments investigating healing in knock-out/knock-in mouse strains characterizing the role of novel targets identified through normal development and
regenerative mechanisms throughout the healing process.

RESULTS

Biomechanical Outcomes at 24 Hours Post Injection

Overall, collagenase-injected tendons appeared hypervascular with increasing vascularity associated with increasing collagenase concentrations. Several collagenase-injected tendons were ruptured at the site of injection, characterized by abnormal collagen color, proximal translocation of the patella, and visible collagen fraying (Fig. 29). Of the tendons observed at the 24 hour time point, 0% of the saline, 2500IU, 5000IU, and 15000IU, 33% of the 10000IU and 20000IU, and 66% of the 30000IU, 40000IU, and 50000IU (not included in statistical analysis) were ruptured, precluding these samples from biomechanical assessment (Table 29).

![Gross morphology of patellar tendon following collagenase injection. Native, uninjured tendon appears white and vibrant (a). Twenty-four hours post injection, there is an influx of vascularity (yellowish color) and a longitudinal slit is observable in the middle of the tendon, presumably the route traveled by the collagenase following injection at the distal insertion (b). Ruptures were common following collagenase injection and were characterized by excessive vascularity, patella displacement, and visible collagen fraying (c).](image-url)

Fig. 29: Gross morphology of patellar tendon following collagenase injection. Native, uninjured tendon appears white and vibrant (a). Twenty-four hours post injection, there is an influx of vascularity (yellowish color) and a longitudinal slit is observable in the middle of the tendon, presumably the route traveled by the collagenase following injection at the distal insertion (b). Ruptures were common following collagenase injection and were characterized by excessive vascularity, patella displacement, and visible collagen fraying (c).
Table 29: Rupture rates following patellar tendon collagenase injections at 24 hours, 2 weeks, and 5 weeks post injury.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Limbs Assigned</th>
<th>Number of Limbs Ruptured</th>
<th>Rupture Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>4</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>24 Hour</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>4</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>2500IU</td>
<td>4</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>5000IU</td>
<td>4</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>10000IU</td>
<td>3</td>
<td>1</td>
<td>33%</td>
</tr>
<tr>
<td>15000IU</td>
<td>3</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>20000IU</td>
<td>3</td>
<td>1</td>
<td>33%</td>
</tr>
<tr>
<td>30000IU</td>
<td>3</td>
<td>2</td>
<td>66%</td>
</tr>
<tr>
<td>40000IU</td>
<td>3</td>
<td>2</td>
<td>66%</td>
</tr>
<tr>
<td>50000IU</td>
<td>3</td>
<td>2</td>
<td>66%</td>
</tr>
<tr>
<td>2 Weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>4</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>15000IU</td>
<td>10</td>
<td>3</td>
<td>30%</td>
</tr>
<tr>
<td>20000IU</td>
<td>10</td>
<td>4</td>
<td>40%</td>
</tr>
<tr>
<td>5 Weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15000IU</td>
<td>10</td>
<td>4</td>
<td>40%</td>
</tr>
<tr>
<td>20000IU</td>
<td>10</td>
<td>6</td>
<td>60%</td>
</tr>
</tbody>
</table>

In terms of ultimate load and linear stiffness, saline injected tendons were no different than normal tendons (Fig. 30a,b, p=0.65). The lower collagenase concentrations (2500IU, 5000IU, 10000IU and 20000IU) also produced no differences with respect to normal ultimate load and linear stiffness (Fig. 30a,b, p<0.05). The 15000IU injection resulted in a 33% reduction in ultimate load, significantly less than normal, uninjured values (p=0.004). Failure occurred at the distal insertion at or near the injection site. Collagenase concentrations larger than 20000IU produced substantially reduced properties; however, the high rate of rupture precluded these samples from further investigation.
Fig. 30: Ultimate load (a) and linear stiffness (b) represented as a percentage of native tissue at 24 hours post injection (mean ± SD). Findings from this preliminary work supported investigating 15000IU and 20000IU concentrations because structural properties begin to substantially decrease and show increased rupture rates beyond the 20000IU concentration.

Based on these preliminary results, we chose to expand the sample size and evaluate 15000IU and 20000IU injections at 2 and 5 weeks to determine the temporal healing trends following collagenase-induced injury.

**Biomechanical Outcomes at 2 and 5 Weeks Post Injection**

At the 2-week time point, 0% of the saline, 30% of the 15000IU, and 40% of the 20000IU were ruptured and by 5 weeks, 40% and 60% of the 15000IU and 20000IU were ruptured, respectively (Table 29). Remaining limbs were assigned to either histology or biomechanics.

Biomechanical results show a decrease in structural properties at the 24 hour time point, with a return to native or above native properties by 5 weeks for both strains (Fig. 31a,b). There were no differences between the two concentrations at any time point, except for linear stiffness at 2 weeks, with the 20000IU dose achieving a higher percentage of normal (Table 30, Fig. 31b, p<0.05).
Table 30: Structural properties following 15000IU and 20000IU collagenase injection measured at 24 hours, 2 weeks, and 5 weeks.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ultimate Load (N)</th>
<th>Linear Stiffness (N/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15000IU</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24hr</td>
<td>2.90±0.48</td>
<td>7.33±1.26</td>
</tr>
<tr>
<td>2wk</td>
<td>3.80±0.70</td>
<td>7.36±0.96</td>
</tr>
<tr>
<td>5wk</td>
<td>5.58±0.34</td>
<td>10.21±0.38</td>
</tr>
<tr>
<td>20000IU</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24hr</td>
<td>3.59±2.24</td>
<td>6.75±3.03</td>
</tr>
<tr>
<td>2wk</td>
<td>3.70±0.65</td>
<td>10.36±1.72</td>
</tr>
<tr>
<td>5wk</td>
<td>5.56±0.41</td>
<td>9.79±1.31</td>
</tr>
<tr>
<td>Native</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.39±0.76</td>
<td>9.69±1.33</td>
</tr>
</tbody>
</table>

At 24 hours, ultimate load and linear stiffness values for the 15000IU dose were 66% and 76% of native, respectively, while ultimate load and linear stiffness values for the 20000IU dose were 82% and 70% of native, respectively. At two weeks, the ultimate load reached 86% of native for the 15000IU dose, while the 20000IU dose increased modestly to 84%. With respect to linear stiffness, there was no change for the 15000IU dose; however, the 20000IU dose increased by 37%, exceeding native linear stiffness by 7%. By 5 weeks, both dose levels had exceeded native values for both ultimate load and linear stiffness. The 15000IU group showed significant improvements between 2 and 5 weeks with respect to both ultimate load and linear stiffness, while the 20000IU group showed an improvement with respect to ultimate load. Due to the number of ruptures, the sample size was small and resulted in variability in the data set, particularly for the 20000IU dose at 24 hours.
Fig. 31: Ultimate load (a) and linear stiffness (b) represented as a percentage of native tissue at 2 and 5 weeks post injection (mean ± SD) for 15000IU and 20000IU collagenase injection. At 24 hours, there was a reduction in properties; however, by 5 weeks both concentrations had resolved the injury, resulting in properties no different than normal. Bars indicate significance with respect to time within the dose level (p<0.05).

DISCUSSION

Establishing a mouse model of collagenase-induced tendon damage has major implications for characterizing degenerative tendon phenotypes and identifying potential pathways for therapeutic development. This preliminary study was designed to establish a model of collagenase-induced injury that might exhibit some attributes of a degenerative tendon phenotype. We first identified a suitable collagenase concentration range that produced decreased mechanical properties in the short-term but minimized the number of tendon ruptures. Based on the preliminary work evaluated at 24 hours post injection, we chose to evaluate 15000IU and 20000IU concentrations at 2 and 5 weeks following injury. At 24 hours, there was a reduction in both ultimate load and linear stiffness for both concentrations compared to native properties; however, mechanical assessment at 2 and 5 weeks indicated the injury was resolving, with a return to native or above native properties by 5 weeks. This suggests that our strategy to
induce a degenerative phenotype was not effective. Successful models of degeneration should exhibit long-term, chronic impairment has indicated by reduced mechanical properties.

Despite this conclusion, findings from this study are of interest based on the ability of the mouse to functionally repair a collagenase-induced injury. Based on this work, we have generated a set of additional research questions that we plan to investigate with future studies: What are the differences between a chemical-induced injury and a tendon excision injury? Are the responses activated following these two types of injury the same or different? What mechanisms are activated/deactivated during the healing process following a collagenase-induced injury? Why do some tendon injuries heal while others do not? Answering these questions has the potential to greatly impact current therapeutic approaches to treating tendon injuries.

We expected the collagenase injections to induce a more substantial reduction of mechanical properties with the reduction in properties extending for a longer period of time. One potential explanation for the unexpected results could be a result of the murine metabolism. The basal metabolic rate per gram of body weight for a mouse is 7 times greater than humans. The half-life of collagenase ranges between 12-36 hours. It could be that the mouse is capable of metabolizing the drug at such a rate that prevents the collagenase from creating an immense amount of damage. To induce a more sustained degenerative phenotype, multiple collagenase injections over time may be needed. Previous work by our laboratory investigating degeneration following collagenase injection in a rabbit patellar tendon indicates a single injection fails to maintain reduced mechanical properties; however, the addition of a second collagenase injection 4 weeks following the primary injection results in a sustainment in reduced properties (unpublished data).
This study is not without limitations. 1) Due to the rate of tendon rupture, the sample sizes at 2 and 5 weeks were small. To build upon this preliminary work, we plan to increase the sample size to confirm that conclusions we have made are substantiated. 2) Histological evaluation of the healing tissue was not completed. We plan to conduct histology on the healing tissue at 24 hours, 2 weeks, and 5 weeks to investigate overall tissue morphology and cellular phenotype. Furthermore, we will evaluate the histologic outcomes using the Bonar scoring system, a method of assessing the tendinopathic nature of the healing tissue by assigning scores for tenocyte morphology, ground substance concentration, collagen organization, and vascularity using a formalized system\textsuperscript{272, 278}.

While our model of collagenase-induced injury failed to create a degenerative tendon phenotype in the long-term, we are now interested in understanding the healing response that produced a return to native properties by 5 weeks. Findings from future work investigating this response, coupled with previous work characterizing other tendon injury models (tendon excision and biopsy punch defect), will expand our current knowledge regarding the body’s natural response to various tendon injuries and allow us to identify successful and unsuccessful healing pathways.

MATERIALS AND METHODS

Ethics Statement

All protocols and procedures were reviewed and approved by the University of Cincinnati’s Institutional Animal Care and Use Committee.

Experimental Design

Gross observations and biomechanical outcomes were measured in 20 week-old male C57BL/6 mouse patellar tendons following collagenase (C6885; Clostridium histolyticum,
Sigma-Aldrich) injection. Initially, a broad range of collagenase concentrations were tested including 2500IU (n=4), 5000IU (n=4), 10000IU (n=3), 15000IU (n=3), 20000IU (n=3), 30000IU (n=3), 40000IU (n=3), and 50000IU (n=3) and biomechanically assessed at 24hr post injection (Table 31). Saline-injected patellar tendons (n=4) served as the control along with native, uninjured patellar tendons (n=4).

Based on results from the preliminary study evaluating a broad range of collagenase concentrations, we then expanded our assessment by evaluating the biomechanical outcomes following 15000IU (n=10) and 20000IU (n=10) collagenase injections at 2 and 5 weeks.

**Table 31:** Experimental design for collagenase injections evaluated biomechanically at 24 hours, 2 weeks, and 5 weeks post injection

<table>
<thead>
<tr>
<th></th>
<th>24 Hour</th>
<th>2 Weeks</th>
<th>5 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>2500IU</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>5000IU</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>10000IU</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15000IU</td>
<td>3</td>
<td>3-histology</td>
<td>3-histology</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7-biomechanics</td>
<td>7-biomechanics</td>
</tr>
<tr>
<td>20000IU</td>
<td>3</td>
<td>3-histology</td>
<td>3-histology</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7-biomechanics</td>
<td>7-biomechanics</td>
</tr>
<tr>
<td>30000IU</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40000IU</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50000IU</td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Surgical Procedure**

Animals were anesthetized through inhalation of 3% isoflurane and both hindlimbs were shaved and aseptically prepared using ethanol and betadine washes. Mice were placed on a sterile surgical table and incisions were made on each limb to expose the patellar tendon. A 0.4 μL collagenase injection was administered at the distal end of the patellar tendon just proximal to the tibial insertion using a 10μL Hamilton syringe (80030; Hamilton Company, Reno, NV) equipped with a Hamilton syringe repeating dispenser (14700; Hamilton Company, Reno, NV)
to assure accurate and consistent delivery of the collagenase volume. Incisions were closed using 5-0 prolene suture (Ethicon, Somerville, NJ) and animals were allowed unrestricted cage activity until the time of sacrifice.

**Biomechanical Testing**

Animals were sacrificed at appropriate time points and frozen at -20°C until the day of testing. Prior to testing, animals were thawed, skin and muscle was dissected away, and the knee joint was flexed to 45°. The medial and lateral portions of the patellar tendon were removed leaving the patella-central PT-tibia unit (0.58 ± 0.08, mean ± SD). The central-PT tissue unit length and width was measured by taking a digital image with a ruler in plane. The patella-central PT tissue-tibia unit was then placed in a custom grip and fixed in place using polymethylacrylate (Dentsply International, York, PA) with the tibia cemented into the grip and secured with a staple to prevent slipping. The grip with the test tissue was then loaded into a materials testing system (100R; TestResources, Shakopee, MN) and lowered to position the patella into the conical-shaped bottom grip. Patellar tendon tissue thickness was measured by taking a digital image of the pre-loaded tissue with a ruler in plane. The tissue was tested in a 37°C PBS bath by applying a preload to 0.02N, preconditioning for 25 cycles with 0-1% strain, and then failing in uniaxial tension at 0.1% of total tendon length/second. The applied load (N) and grip-to-grip displacement (mm) were recorded throughout the testing period.

Ultimate load, failure displacement, stress, and strain were recorded during the testing period. A linear regression algorithm was used to calculate stiffness and modulus from the linear portion of the load-displacement and stress-strain curves, respectively. The failure location was also recorded following the completion of the mechanical test.
**Histological Evaluation**

*At the present time, histological evaluation has not been completed, but will be processed using the following methods:*

After sacrifice, limbs were dislocated at the femoral joint, the skin and foot removed, and samples were fixed in 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) for 24 hours. Samples were then decalcified with 0.5M EDTA/PBS for 7 days at 4°C. Following decalcification, samples were further trimmed and embedded in O.C.T. compound (Andwin Scientific Tissue-Tek™, Schaumburg, IL) for frozen sectioning. Serial, longitudinal sections were cut along the length of the tendon at 3 different levels using a Leica CM3050S cryostat (Leica, Wetzlar, Germany). Thin (4µm) sections were taken using cryofilm (Cryofilm Type 2C, Section-lab, Hiroshima, Japan) at each level for histological analysis. Sections were affixed to glass slides and allowed to dry for 48 hours at room temperature and then stained for hematoxylin and eosin and alcian blue at pH of 2.5 following protocols for frozen sections to assess cell morphology, ground substance, collagen, and vascularity using the Bonar scoring system\textsuperscript{272, 278}. This scoring system is a semi-quantitative method of evaluating tendon degeneration using histological results as assessed by a group of blinded reviewers\textsuperscript{278}.

**Statistical Analysis**

All data sets were verified to be homoscedastic and normally distributed prior to statistical testing. Tendon mechanical values for structural properties (saline, 2500IU, 5000IU, 10000IU, 15000IU, 20000IU) were compared to native, uninjured values using a two-way Student’s t test at the 24 hour time point. Further statistical analysis was conducted for the 15000IU and 20000IU doses and analyzed via 2-way ANOVA with dose and time as fixed...
factors. The IBM SPSS Statistics 2.1.0 software (Chicago, Illinois) was used to perform all statistical testing.

ACKNOWLEDGEMENTS

We gratefully acknowledge the National Institutes of Health for providing research and student support (R01 AR056943). We also thank Brent Turner for his contributions to this project and Dr. Lou Soslowsky for his contributions to our current biomechanical assessment protocols.
Chapter 9

Conclusions and Recommendations

Principal Findings and Conclusions

In 2000, experts in the tissue engineering field proposed a new paradigm, functional tissue engineering\textsuperscript{17}. This strategy advocated defining mechanical design criteria by incorporating aspects of the native tissue loading environment and tissue mechanical failure properties to more effectively design tissue engineered constructs for load-bearing tissues. Using this approach, our lab has achieved improved mechanical outcomes compared to natural healing following a central patellar tendon defect in the rabbit model; however, we have yet to fully restore the native properties of the tissue\textsuperscript{25,279}.

My doctoral work is a part of our laboratory’s efforts to identify and evaluate potential biological success criteria to improve our methods of creating and assessing tissue engineered constructs for musculoskeletal applications. In collaboration with Dr. Christopher Wylie and Dr. Rulang Jiang at Cincinnati Children’s Hospital, our group has looked to normal development to identify novel markers that may prove to have utility with respect to tendon healing. My dissertation takes this approach a step further by incorporating a murine model that exhibits improved healing outcomes following injury to expand our list of potential targets for investigation.

As discussed in chapter three, we extended the concept of defining mechanical success criteria by proposing to include biological success criteria to improve the tissue engineering design process. To this end, we proposed a strategy to identify and evaluate potential biological design criteria by first adequately defining the native biological properties of tendon though
investigation of normal tendon development and tendon homeostasis in the adult. Next, we must identify and prioritize potential targets of interest, and finally, evaluate these potential targets by determining how altering expression affects the mechanical properties of tendon to prioritize the importance of each target. Applying this approach, we proposed three specific biological criteria which we believed to be essential for successful tendon repair: (1) scleraxis-expressing cells; (2) well-organized and axially-aligned collagen fibrils having bimodal diameter distribution; and (3) a specialized tendon-to-bone insertion site. Undoubtedly, the biological mechanisms regulating normal tendon development, maturation, and healing are complex and our laboratory will continue to investigate each of these processes to expand upon this list. Doing so will not only strengthen our understanding of tendon biology, but improve our approaches for creating and assessing successful tissue engineered therapies.

Regeneration has long been the ‘holy grail’ for scientists and clinicians seeking to improve the lives of patients afflicted by injury or disease. While there are several animal models that show regenerative healing in response to injury, such as the urodele, there are few known mammalian models. Identifying and characterizing a mammalian model of regeneration is of particular interest because it would allow for more readily translatable application of findings to the human in terms of clinical therapy development. In 1998, the MRL/MpJ mouse was identified as a strain that showed a regenerative-like response to an identifying earhole punch and its injury response has since been characterized in a number of tissue types including heart, cornea, articular cartilage, and spinal cord\textsuperscript{105, 126, 129, 131, 280}. Despite this work, others have demonstrated the MRL/MpJ responds to injury no different than wildtype, suggesting the response may be tissue and/or injury mode dependent\textsuperscript{132, 134}. We became interested in the MRL/MpJ because, if it does indeed heal via a regenerative-like response, it offers a ‘positive
control’ for evaluating our tissue engineered therapies. We set out to assess the healing response of the MRL/MpJ using our well-characterized central patellar tendon defect model. 

Chapter Four: The MRL/MpJ shows improved mechanical properties following full-length patellar tendon injuries, but not as a result of a p21 deficiency.

In this study, we characterized the MRL/MpJ healing response to a central patellar tendon injury. We initially showed no differences in native C57BL/6 and MRL/MpJ mechanical and tenogenic expression profiles. Further, no difference in expression of the cyclin-dependent kinase inhibitor, p21, was observed. We hypothesized that we would not see differences in the healing response of these two strains based on similarities in the native tissues.

We found the progression of healing between the two strains at early post-surgical time points was not different; however, the MRL/MpJ response was characterized by increased cellular proliferation and decreased immunological response compared to the wildtype. The mechanical results were not different at 2 and 5 weeks; however, compared to the wildtype strain, which showed a plateau in properties following 2 weeks, the MRL/MpJ continued to heal, resulting in significantly improved properties between 5 and 8 weeks. This suggested to us that the MRL/MpJ response is not accelerated, rather prolonged, following tendon injury. Furthermore, the two strains showed no difference in p21 expression at early post-surgical time points, contradicting previous reports which claim a p21 deficiency is a contributor to the healing phenotype. 

Findings from this study suggest the MRL/MpJ employs an alternative healing response that results in superior healing outcomes, providing sufficient rationale for investigating this strain further. However, it remains unclear what mechanisms are driving this healing phenotype. We found 1) no differences in tenogenic expression patterns and 2) no difference in p21
expression between strains at any post-surgical time point. Based on these results, we surmised the healing response occurs due to a mutation causing activation/deactivation of some alternative pathway. To address this, we employed RNASeq, a deep-sequencing technology, capable of analyzing the entire RNA transcriptome of a given tissue sample.

Chapter Five: The MRL/MpJ healing response is characterized by a decreased inflammatory response and elevated expression of transcription factors implicated in developmental processes.

We created central patellar tendon defects in the C57BL/6, LG/J, and MRL/MpJ strains and conducted RNAseq analysis on the repair tissue at 3, 7, and 14 days post injury. We analyzed the results by conducting clustering analysis at each time point and differential analysis of transcription factor expression at 3 and 7 days post injury. Interestingly, the MRL/MpJ showed a substantially reduced immune response compared to the two other strains coupled with elevated expression of Hoxd13 and Pax9, two transcription factors implicated in normal developmental processes of the limb. Previous reports have suggested that a decreased immune response and a retention of embryonic features may contribute to the healing phenotype; however, it remains to be determined how or if these two processes interact to produce the superior healing response\textsuperscript{105,141}. It may be that, based on our findings from Chapter 4 showing the greatest mechanical differences between strains at 8 weeks, we need to evaluate the later healing time points to create a complete explanation for this strain’s healing outcomes.

While our laboratory’s primary focus is investigating tendon development and healing, we also understand the value of translational research and applying our findings across animal models and tissue types. Thus, we chose to evaluate the MRL/MpJ’s response to another
musculoskeletal injury, a critical-size femoral osteotomy, to determine if our findings from the tendon study would translate to another musculoskeletal tissue.

Chapter Six: The MRL/MpJ does not show improved healing outcomes following a critical-size femoral osteotomy.

While it remains unclear if the MRL/MpJ is a model of true regeneration following a tendon injury, findings from Chapter 4 and Chapter 5 support further investigation of this strain using other injury models. Our group has previously characterized a murine model of bone healing following a femoral osteotomy, and we demonstrated wildtype do not successfully repair a critical-size defect, resulting in the formation of a nonunion. We created critical-size defects in the MRL/MpJ mouse and compared the healing response to wildtype. Unfortunately, we found no differences in the repair outcome based on biomechanical measures. Immunostaining revealed increased expression of bone-forming and bone-remodeling markers present in the callus region up to 5 weeks following injury. This suggests that, similar to the response following tendon injury, the MRL/MpJ strain may employ a prolonged healing response compared to the C57BL/6.

The first three chapters represent work to address the first step in our strategy to develop biological success criteria by utilizing the MRL/MpJ as model to identify novel pathways and targets. As described previously, we have been collaborating closely with developmental biologists to identify unique molecular targets regulating tendon development. This collaboration has yielded exciting results, including the identification and characterization of indian hedgehog and mohawk homeobox, two targets found to be critical to normal tendon formation. Following our strategy, we chose to evaluate how manipulating expression of a target
identified from development might impact the mechanical and morphological parameters in the adult animal.

Chapter Seven: Loss of mohawk homeobox expression produces mechanical and morphological deficiencies.

Mohawk homeobox is a transcription factor that has been identified as a regulator of successful tendon development\textsuperscript{55-57}. Using our approach for establishing biological design criteria, we evaluated the effects of ablating mohawk homeobox expression in the adult patellar tendon. We discovered that the absence of mohawk homeobox during development and maturation produces significant mechanical and morphological deficiencies, with significant reductions in structural and material properties. Furthermore, 18\% of the observed patellar tendons ruptured sometime prior to the date of sacrifice, indicating the absence of mohawk homeobox led to severe alterations in the normal loading across the knee joint. The primary role of mohawk homeobox is not entirely clear; however, we, in concert with our collaborators in Developmental Biology, suspect it functions in tenocyte maturation.

Based on findings from this study, we know that mohawk homeobox is vital for tendon development and maturation to form a normally functioning tissue; however, we still do not know what role this marker has during tendon healing. Furthermore, we know that different types of tendon injuries employ different healing responses based on the type, severity, and location of the injury. This led us to a set of preliminary studies seeking to develop a mouse model of collagenase-induced tendon injury. Understanding the differential responses following various tendon injuries could provide insight to successful natural healing responses.
Chapter Eight: The creation of a collagenase-induced patellar tendon injury resulted in a return to native structural properties by 5 weeks post-injection.

Our group has used the central patellar tendon injury model over the past several decades to investigate tendon healing in both the mouse and rabbit models\textsuperscript{25,26,176,279}. Unfortunately, this model system is often criticized for not being clinically relevant, as patients generally sustain injuries resulting from degenerative processes. Thus, we chose to investigate a murine model of collagenase-induced injury in the wildtype, with the goal of establishing a model that exhibits aspects of common clinical presentations.

We first identified an appropriate concentration by evaluating a broad dose range at 24 hours post injection. The concentrations of 15000IU and 20000IU were chosen and we then measured the mechanical outcomes at 2 and 5 weeks. By 5 weeks, the injuries were fully resolved based on mechanical measures for both concentrations. While this approach did not induce a chronic, degenerative condition, we are now interested in identifying the pathway that produced this healing response.
Recommendations for Future Directions

Findings from this work have laid the foundation for implementing our approach for identifying and evaluating biological design criteria, incorporating both normal development and models that exhibit superior healing outcomes. There are several gaps in knowledge that remain as a result of the findings detailed in this dissertation. In this section, I propose a set of questions arising from this work and then present recommendations for future directions to address these questions.

Fig. 32: Flowchart for dissertation findings and future studies
What are the similarities that exist between normal development and regeneration?

Our laboratory has been collaborating with Cincinnati Children’s Hospital Medical Center Department of Developmental Biology (Dr. Chris Wylie and Dr. Rulang Jiang) to better understand the processes regulating successful tendon formation during development. This collaboration has influenced our work significantly, making us mindful of the orchestrated biological events that must occur for successful tissue formation. To build upon this collaboration, we should investigate the similarities that exist between normal development and regenerative responses to injury. For example, in Chapter 5, we present differential transcription factor analysis following tendon injury in the MRL/MpJ compared to the LG/J and C57BL/6 which showed increased expression of Hoxd13, a homeobox gene highly involved in regulating patterning during early developmental stages. This leads us to ask, does the MRL/MpJ reactivate developmental processes following injury? If so, then identifying commonalities between these two processes could establish the most suitable biological design criteria for assessing tissue engineered therapies. However, previous findings suggest regeneration may not simply be a reactivation of developmental pathways, as the injury environment is substantially different from the embryonic environment.\textsuperscript{281,282} Identifying and characterizing the similarities and differences between these two processes could greatly improve the field of regenerative medicine.

Does inhibiting aspects of the immune response lead to improved tendon healing outcomes?

While much work remains to be done characterizing the genetic profile of the MRL/MpJ following injury, results from this work suggest a decreased immunological response is likely involved in the healing phenotype. It is difficult to speculate as to whether or not this abnormality is the driver of the healing phenotype; however, investigating genetic knock-outs for
immuno-deficiencies could provide insight in determining the impact of how the immune response may impede healing.

Fetal wound healing has been investigated to shed light on how a scarless response can be activated in the event of injury. Fetuses possess a scarless response during the first trimester of development; however, as development continues, this capacity diminishes\textsuperscript{283}. Interestingly, the onset of the diminished healing capacity correlates with an increase in immune system development. Investigators have postulated the components of the immune response may impede healing even at these early developmental stages\textsuperscript{283}.

Based on previous reports showing improved skin repair in neutrophil-depleted mice, Ueno et al investigated corneal healing in both the MRL/MpJ strain and neutrophil-depleted mice\textsuperscript{105}. They postulated infiltrating neutrophils might slow the reepithelialization process, leading to poor healing outcomes in the wildtype animal. Both the MRL/MpJ and neutrophil-depleted mice showed enhanced reepithelialization and healing outcomes compared to wildtype; however, the MRL/MpJ’s response was accelerated compared to the neutrophil-depleted mice, suggesting the phenotype is not solely a result of a decreased immunological response\textsuperscript{105}.

Findings presented in this dissertation, along with the work briefly outlined above, suggest the immune response may indeed negatively impact the healing response. If so, evaluating murine strains that display diminished immune responses could shed light on the aspects of the system that interfere with repair. This could aid tissue engineers in identifying potential therapies such as targeted knockdown of specific pathways or molecules.

*Is the MRL/MpJ strain the optimal model to investigate regeneration?*

The MRL/MpJ mouse strain presents us with an exciting opportunity to characterize a system that has been shown to exhibit regenerative-like qualities following injury; however, it
has yet to be classified as a true model of regeneration. Previous findings by others in the field, along with work presented in this dissertation, indicate the MRL/MpJ response is variable, showing disparities in healing outcomes dependent on the injury and tissue type. It is still unclear what mechanism is driving the MRL/MpJ response. The genetic background is complex, with mutations found on 20 loci located on 7 different chromosomes, making it difficult to parse out what aspects truly contribute to the healing phenotype. If we plan to use regeneration as a model system to guide tissue engineering, investigating ‘true’ models of regeneration, such as the urodele, could be a better approach to understanding regenerative processes. With the advent of more robust genetic and molecular tools, it is becoming easier to study more obscure species, such as the urodele, with the same genetic power available in the mouse model. Seeking collaborations with experts in the field of regeneration offers the opportunity to further refine our list of biologic success criteria and strengthens our approaches to investigating mammalian models of regeneration.

**Why does healing following different modes of tendon injury result in variable outcomes?**

One interesting result of this work is the wildtype strain’s differential reparative responses to two types of tendon injury. We have previous characterized the central patellar tendon defect model, finding the mechanical properties plateau at 2 weeks and fail to improve beyond that time point. Results reported in this dissertation (Chapter 4) confirm this conclusion. To improve the clinical utility of our tendon injury model, we sought to develop a collagenase-induced tendon injury in Chapter 8 with the goal of producing a ‘degenerative’ tendon phenotype. Interestingly, the wildtype strain showed a decrease in mechanical properties at 24 hours; however, successfully restored structural properties to near or above native levels by 5 weeks post injection. Why is it that the healing outcomes are different between these two
injuries? Obviously, one injury is excisional and the other is chemically-induced, but the question becomes whether or not the mechanisms that are activated in response to each injury are the same or different. If the mechanisms are different, then can we apply aspects of the successful healing response (collagenase-induced) to the poor healing response (excisional) to improve the repair outcome? If the activated mechanisms are similar, then at what point does the response fail in the excisional injury? Answering these questions could have huge implications to the field of regenerative medicine and orthopedic surgery, as medicine becomes more personalized to each individual injury and patient.

*When we do create a tissue-engineered therapy, when is the appropriate time for clinical intervention?*

While the focus of this dissertation was primarily on identifying and characterizing potential design criteria, the ultimate goal of this work is to improve tissue engineering approaches. A question that arises from the findings presented here involves the optimal time frame for implanting a tissue-engineered replacement or augmentation. Typically when in vivo tissue engineering studies are conducted, the tissue engineered construct is implanted immediately following the creation of the tendon defect and the animal is then allowed to heal for some set amount of time. This may not be the best approach. The general response to tendon injury has been shown to include an initial inflammatory/immune response, matrix production, followed by matrix organization and remodeling. Implanting a construct into a tendon defect immediately following injury could, in fact, impede initial native reparative mechanisms. Furthermore, the early wound environment is chaotic, with a significant amount of cell death and matrix degradation occurring. It might be more appropriate to implant constructs at later time points, such as two weeks, where we observe the native healing response beginning
to subside. Based on what has already been discussed, perhaps implanting a construct immediately following injury creation that is composed of targeted, inhibiting molecules designed to reduce aspects of the immune response could prove to be a useful tool for improving repair outcomes.
Bibliography


194


