Mechanical and Cellular Factors Regulating Tendon Development

A Dissertation
Presented in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy
with a
Major in Biological Engineering
in the
College of Graduate Studies
University of Idaho
by

Sophia K. Theodossiou

Major Professor: Nathan R. Schiele, Ph.D.
Committee Members: Deborah L. Stenkamp, Ph.D.; Benjamin D. Richardson, Ph.D.;
Bryn A. Martin, Ph.D.
Department Administrator: Ching-An Peng, Ph.D.

August 2020

Authorization to Submit Dissertation

This dissertation of Sophia K. Theodossiou, submitted for the degree of Doctor of Philosophy with a Major in Biological Engineering and titled "Mechanical and Cellular Factors Regulating Tendon Development," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

Major Professor: _____ Date: _____
Nathan R. Schiele, Ph.D.

Committee Members: _____ Date: _____
Deborah L. Stenkamp, Ph.D.

_____ Date: _____
Benjamin D. Richardson, Ph.D.

_____ Date: _____
Bryn A. Martin, Ph.D.

Department Administrator: _____ Date: _____
Ching-An Peng, Ph.D.

Acknowledgements

This work would not have been possible without the assistance of others at the University of Idaho and beyond. I would like to start by acknowledging the John F. Keegan Fellowship, which funded travel to many conferences and allowed me to complete and show my work to all who were interested. I would like to thank the Department of Biological Engineering for being an intellectually stimulating and supportive environment in which to complete my graduate studies, and all the dedicated faculty and staff members, without whom my progression throughout this degree would not have been possible. I am especially grateful to Dr. Ching-An Peng, Judy Vandegrift, and Debbie Foster, who make sure everything is signed and running as it needs to be.

Many of the chapters in this dissertation represent collaborative efforts within and outside the University of Idaho, and I am grateful to everyone involved for their hard work and support. Special thanks go to Abby Raveling, John Tokle, Jordan Becker, Jett Murray, Nick Pancheri, LeeAnn Hold, Jeff Courtright, and Annie Carper, whose co-authorship and countless hours in the lab made many of our publications possible. I would also like to thank our collaborators at Idaho State University, especially Dr. Michele Brumley, Aimee Bozeman, Alleyna Martes, Nicholas Burgett, and Hillary Swan.

I would like to gratefully acknowledge my committee members: Dr. Nathan Schiele, Dr. Deborah Stenkamp, Dr. Ben Richardson, and Dr. Bryn Martin. Thank you for everything you have taught me through your classes, seminars, comments on my papers and grants, casual conversations, and your work as researchers. Without your time, feedback, and letters of recommendation, the postdoc adventures ahead would not be happening. Finally, I am immensely grateful to my PhD advisor, Dr. Nathan Schiele. Without his vision, patience, leadership by example, and mentoring, none of our research would be possible. Thank you for offering me the dream job I didn't even know I wanted, and for teaching me to be an independent researcher, to ask and answer the right questions, to write, and - most importantly - to do good work and have fun.

Some PhDs take a non-academic village; this one certainly did. Thank you to those of you who encouraged me to take the plunge and leave the world of my "real" job and pursue this adventure, especially Ashley, Karen, Kristine, Shelby, and Brian. Courtney and Jeremy, thank you for our endless and fascinating conversations about science, for adopting us, and for teaching me to ski powder. This is me tagging you both in to get your own PhDs, it's time. Get on it! Grandma C, thank you for backyard fires, Christmas briskets, and being a part of this journey. Thank you to Ann, Margrit, Jeannie, and Cindy: you paved the way for this generation, and we won't forget that. Thank you to Kate for patiently listening to my schemes and encouraging me to pursue them, and for my

favorite piece of desk flair. To the members of the headlamp o'clock running club: thank you for getting up long before dawn and far below freezing to run away the stress of grad school. That means you, Becky, Eloise, Courtney, and Kelli. To the EMTs, medics, firefighters, and admins who keep Moscow Ambulance going and who offered support at 3 am and at 3 pm, and everywhere in between: thank you for inspiring me to never stop working. Special thanks to John, Greg, Karin, Deb, Dan, Rick, Faith, Shannon, Bob, Case, and Brad – Crew Delta is on duty. Extra thanks to Greg for running the starving grad student meal service on more Wednesdays than I can count. Thank you to all the "peer mentors" who supported me and each other throughout this process, especially AJ, Stephanie, Janneke, and Brooke. Special thanks go to Team Garfield: to Dr. Bartrem, for never canceling a skin-track date, for being the definition of a true friend, for finding my ski that one time, and for being the only person I know who can look dignified with toilet paper up their nose on Moscow Mountain at 6 am; to Paaayum, for sticking all the inspirational quotes on my desk when I almost bailed, for the pencil toppers, and for many, many other things along the way - I sure am glad you caught me looking at those 3rd degree burn photos, because life would be a lot different if you hadn't; and to Pilot Steve, for keeping planes in the air, rangers on the road, and the driest of humor in every conversation. Thank you to Maria and Rosie for being shining examples of fighting for things that are worth it, taking life in stride, and always being there for me. Thank you to Gikas, Aleksei, Jessie, Donna, John, Chris, and Kim, for being the kind of family I don't just have to hang out with, but actually want to. Thank you to my parents and grandparents, who taught me that there will never be a substitute or shortcut for hard work. Special thanks to my mom, who flew out to Idaho more times than she ever imagined she would, and for always cheering on my smallest and biggest dreams. Finally, the biggest thanks of all goes to Zack: I could not have done this without you. Thank you for realizing this was crazy (like many of our endeavors) and supporting me anyways. I can't wait to develop those wicked Boston accents together.

Dedication

To everyone who was so busy thinking they couldn't, they didn't realize they did.

And, to Mom.

Table of Contents

List of Tables

List of Figures

Statement of Contribution

Data analyzed in chapters 6 and 7 of this dissertation were collected at Idaho State University in Pocatello, Idaho, by our collaborators in Dr. Michele Brumley's lab, between 2017 and 2020. The researchers collecting the data are named as co-authors of these chapters. Undergraduate researchers in Dr. Nathan Schiele's lab also participated in some data collection and analysis, and are named as co-authors of these chapters. I share first authorship of chapter 3 with another student, as we both contributed equally to the publication. Data analyzed in chapters 4, 5, and 8 were collected primarily by me, with assistance from undergraduate researchers in Dr. Nathan Schiele's lab. The students assisting with data collection and analysis are named as co-authors on these chapters. A portion of the review article that makes up chapter 3 was authored by an undergraduate researcher in Dr. Nathan Schiele's lab, and he is listed as a co-author of this chapter. I had the primary responsibility of data collection, analysis, and interpretation, and manuscript drafting, and so I am the primary author on every chapter in this dissertation. Any errors in this dissertation are my own.

Chapter 1: Introduction

Tendons are collagenous musculoskeletal tissues that transfer forces from muscle to bone to facilitate movement. Tendons are frequently injured, and the incidence of tendon injuries is increasing [1, 2]. They have limited intrinsic healing capacity, which results in poor healing and long-term loss of function [3]. Clinical treatments for tendinopathies are limited and their efficacy varies from patient to patient. Tissue engineered regenerative strategies using mesenchymal stem cells (MSCs) are promising for the treatment of tendon injuries [4-8]. However, more information is needed in order to establish tissue engineered and regenerative approaches as viable clinical options, as these approaches are challenged by the difficulties of precisely controlling stem cell differentiation. Specifically, there is a limited understanding of the appropriate mechanical and cellular cues to guide tenogenesis of stem cells (differentiation toward tendon), which has hindered widespread clinical use of stem cell-based therapies.

The tissue engineering paradigm has emerged as a systematic approach for developing replacement tissues and regenerative therapies. The tissue engineering paradigm centers around applying three main factors: cells, signals, and scaffolds (Figure 1.1). Signals are factors that may regulate cell behavior and can be mechanical, including cyclic loading or elastic modulus, as well as chemical, including growth factors or inhibitors. Cells are important for developing and maintaining new tissue, and cells can endogenously produce mechanical and chemical signals. Finally, the scaffold is typically a temporary biomaterial structure that supports cell growth and tissue formation. In a number of systems, the mechanical and chemical environments can also be manipulated via the scaffold. Identifying the appropriate composition and combination of cells, signals and scaffolds to induce tenogenesis is a major challenge. Therefore, we look to the developing embryonic and postnatal tendon environment for inspiration to guide the selection of tenogenic factors. Recapitulating this early tenogenic environment in a regenerative therapy is highly desirable, as normal tendon development is the ideal model of tendon formation. This *developmentally inspired* approach aims to incorporate the mechanical and chemical cues regulating tendon development into tissue engineered and regenerative approaches for the treatment of tendon injuries. However, a limited understanding of the factors involved in normal tendon development is a challenge for *developmentally inspired* tendon tissue engineering strategies. A deeper understanding of the factors influencing stem cell fate and the ability to precisely guide their differentiation are needed before stem cells can be used therapeutically in tissue engineering strategies and regenerative treatments for tendon injuries. Chapter 2 provides an extensive overview of existing engineered models of tendon

development and disease. These models address various aspects of the molecular factors regulating tendon development, as well as the mechanical and biochemical factors underlying tendon disease. However, despite these advances, there remains a significant gap in knowledge of the mechanical and cellular factors regulating tendon development, during both early tenogenesis and postnatal tendon formation.

To address this gap in knowledge, *the overall goal of this dissertation was to explore the developmentally inspired mechanical and cellular factors involved in tendon formation and tenogenic stem cell differentiation.*

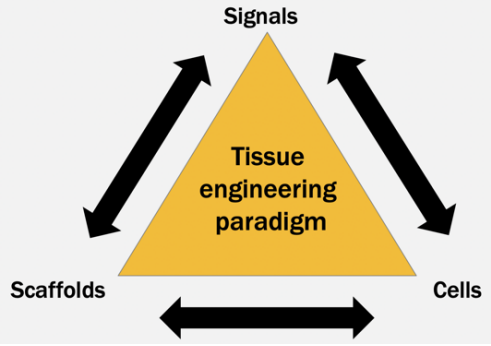


Figure 1.1 Schematic representation of the tissue engineering paradigm of "Cells – Signals – Scaffolds"

Mechanical stimuli are one potential factor driving tenogenesis of stem cells [7, 9, 10]. Mechanical loading during embryonic tendon development appears to regulate the structural and mechanical formation of tendon [11, 12], but the mechanisms by which mechanical stimuli exert their influence on developing tendon and stem cells remain largely unidentified. Therefore, the overall goal of Aim 1 was to identify how tendon formation is mechanically regulated. Aim 1 was assessed in both a neonatal rat model of early-stage tendon formation (Chapters 6 and 7), and in a 3-dimensional (D) MSC culture model to mechanically stimulate cells *in vitro* (Chapter 4).

In addition to mechanical stimuli, biochemical and cellular cues may drive tenogenic differentiation of stem cells. One growth factor in particular had been identified in embryonic tendon development and appeared useful for inducing tenogenic differentiation of stem cells: transforming growth factor beta (TGFβ)2 [13]. However, there was limited information on how TGFβ2-induced tenogenesis regulated the levels of cell-cell junction proteins (e.g., cadherins and connexins) (Chapter 5). Cell-cell junctions are of particular interest in tendon development due to the presence of cadherins and connexins in embryonic, postnatal, and mature tendons, and their ability to regulate

cellular communication and tissue formation. To further explore the potential roles of cadherins and connexins in tenogenesis, an extensive overview of prior studies examining their impact on tendon development and disease is presented in Chapter 3. This review highlights the many unknown ways in which cadherins and connexins may regulate tendon development, and underscores the need for further research. Additionally, it was unknown which signaling pathways were activated during TGFβ2-induced tenogenesis. Therefore, the overall goal of Aim 2 was to identify how TGFβ2-induced tenogenesis in MSCs impacts cell-cell junctions (Chapter 5) and identify the signaling pathways that regulate TGFβ2-induced tenogenesis (Chapter 8).

The research aims of this dissertation are summarized below:

Aim 1: Identify how mechanical loading from the onset of locomotion influences the postnatal development of tendon mechanical properties. Hypothesis: The onset of locomotor behavior in the developing neonate regulates the formation of functional tendon mechanical properties. Aim 1a developed and evaluated a small-scale mechanical load frame, and mechanical bioreactor to mechanically evaluate neonatal tendons and mechanically stimulate cells in culture (Chapter 4). Aim 1b assessed the onset of locomotor behavior development and evaluated how mechanical properties of Achilles and tail tendons formed in the first 10 postnatal days in a rat model (Chapter 6). Aim 1b determined how disruption of mechanical loading in early postnatal development affects mechanical property development in Achilles and tail tendons (Chapter 7).

Aim 2: Determine how TGFβ2 impacts the tenogenesis of MSCs. Hypothesis: TGFβ2-induced tenogenesis in MSCs is accompanied by changes in levels of cadherins and connexins, and involves canonical and non-canonical signaling pathways. Aim 2a assessed how tenogenic markers and proteins levels of N-cadherin, Cadherin-11, and Connexin-43 are impacted in tenogenically differentiating MSCs (Chapter 5). Aim 2b evaluated how TGFβ2-induced tenogenesis in MSCs was regulated by the PI3K/Akt/mTORC1/P70S6K and Smad3 signaling pathways (Chapter 8).

Results of the studies outlined in the following chapters contributed to our understanding of tendon development. With completion of these Aims, we *enhanced understanding of how mechanical stimuli and cellular signals regulate tendon differentiation and development*, with the ultimate goal of therapeutically targeting the mechanisms involved to generate novel regenerative therapies for tendinopathies.

Chapter 2: Models of Tendon Development and Injury

Theodossiou SK, Schiele NR. Models of tendon development and injury. *BMC Biomedical Engineering* 1(2019) 32.

Abstract

Tendons link muscle to bone and transfer forces necessary for normal movement. Tendon injuries can be debilitating and their intrinsic healing potential is limited. These challenges have motivated the development of model systems to study the factors that regulate tendon formation and tendon injury. Recent advances in understanding of embryonic and postnatal tendon formation have inspired approaches that aimed to mimic key aspects of tendon development. Model systems have also been developed to explore factors that regulate tendon injury and healing. We highlight current model systems that explore developmentally inspired cellular, mechanical, and biochemical factors in tendon formation and tenogenic stem cell differentiation. Next, we discuss *in vivo, in vitro, ex vivo,* and computational models of tendon injury that examine how mechanical loading and biochemical factors contribute to tendon pathologies and healing. These tendon development and injury models show promise for identifying the factors guiding tendon formation and tendon pathologies, and will ultimately improve regenerative tissue engineering strategies and clinical outcomes.

Introduction

Tendons transfer forces from muscle to bone and are essential for movement. Unfortunately, tendons are frequently injured [14], and their poor healing ability results in long-term loss of function [3]. Medical interventions, including surgical and non-surgical treatments, physical therapy, steroid injections, and anti-inflammatory medications have limited efficacy, and re-rupture is common [15]. These poor outcomes motivate the search for alternative treatment strategies aimed at preventing tendon injury, improving regenerative healing, and developing engineered tendon tissue replacements from stem cells. A major challenge for developing regenerative approaches has been a limited understanding of the factors that regulate tendon formation, injury, and healing.

Normal embryonic and postnatal tendon development are perfect models of tendon formation, but have been poorly understood. However, over the past 20 years, significant progress has been made in identifying underlying cellular, biochemical, and mechanical factors that regulate tendon formation during early development, and these important findings have been discussed in other recent reviews [9, 10, 16-27]. Using this new information, developmentally inspired

approaches have recapitulated aspects of embryonic tendon cell differentiation and tendon formation *in vitro*. Here, we first focus on cell and explant tissue culture and tissue engineered model systems that have explored the cellular, biochemical and mechanical aspects of tendon development. In the second part of this review, we highlight model systems that may inform future clinical interventions for adult tendon injury. Specifically, we discuss *in vivo*, *in vitro*, and *ex vivo* models of tendon injury. In addition to experimental models, we highlight recent computational models that explore factors involved in tendon degeneration, injury, and healing.

Models of Embryonic and Postnatal Tendon Development

Tendon formation is initiated in early development as the musculoskeletal and connective tissues differentiate from embryonic mesoderm [26]. A few specific markers have been identified to distinguish tenogenesis (differentiation toward the tendon lineage) of progenitor and stem cells. Scleraxis, a transcription factor, is an early marker and regulator of tenogenesis [28-31]. Scleraxis regulates expression of tenomodulin, a late stage tenogenic marker [32, 33]. Mohawk is another transcription factor and regulator of tendon differentiation and formation [34]. The increased presence of collagen type (Col) I also indicates tenogenesis [35], but collagen production alone is not indicative of tenogenic differentiation since it is a major component of other musculoskeletal tissues such as bone and skin. However, the development of an aligned collagen structure and mechanical function can indicate appropriate tenogenesis and tendon formation. Taken together, this set of tenogenic markers (scleraxis, mohawk, tenomodulin, collagen content and organization, and mechanical properties) has led to advancements in understanding tendon development. To determine regulators of tenogenesis, *in vitro* and engineered model systems have been developed to incorporate the key cellular (cell organization and environment), biochemical (growth factors and extracellular matrix), and mechanical (tissue elastic modulus and dynamic loading) cues that are characteristic of developing tendons (Table 2.1).

Table 2.1 Summary of developmental tendon models

Developing Tendon Characteristics	Model Characteristics	Model Outcomes	References
High cell density and low collagen content (Ansorge 2011) [39] (Chaplin 1975) [36] (Richardson 2007) [42] (Schiele 2015) [37]	Self-assembled cellular fibers	Upregulated expression of scleraxis and tenomodulin with loading; potential for scaffold-free, cellular self-assembly for single tendon fibers	Mubyana 2018 [45] Schiele 2013 [9]
	Embryonic tendon cells in fibrin gels	Upregulated tendon genes and collagen synthesis; improved tendon formation in fibrin gel vs collagen gels models	Kalson 2010 [46] Kapacee 2010 [64] Yeung 2015 [75] Breidenbach 2015 [76]
Cell-cell junction proteins (Cadherin-11 & N-Cadherin) (Richardson 2007) [42]	Chick tendon explants, fibroblasts, and mouse MSCs	Possible regulators of early tendon tissue formation; N-cadherin and cadherin-11 levels decreased with tenogenic induction	Richardson 2007 [42] Schiele 2013 [9] Theodossiou 2019 [13]
TGFβ2&3	Mouse embryonic tendon progenitor	TGFβ2 increased scleraxis and tenomodulin expression	Pryce 2009 [31] Brown 2014 [48]

(Pryce 2009) [31] (Kuo 2008) [47]	cells and fibroblasts, and MSCs		Brown 2015 [49] Havis 2014 [53] Havis 2016 [52] Chien 2018 [55]
	Mouse MSCs	TGFβ2 increased scleraxis and tenomodulin production; decreased N-cadherin and cadherin -11 production	Theodossiou 2019 [13]
	Human BM-MNCs and MSCs in fibrin gels	TGFβ3 increased collagen fibril synthesis, and upregulated TGFβ3, Col I, and Smad2	Kapacee 2010 [64]
Scleraxis and mohawk (Schweitzer 2001) [28] (Liu 2015) [57] (Otabe 2015) [58] (Shukunami 2018) [62]	Scleraxis knockdown in equine embryonic stem cells, and fetal and adult tendon cells	Decreased expression of Col I, COMP, and Sox9, and reduced cell survival in embryonic stem cells and fetal tendon cells with scleraxis knockdown; adult tendon cells unaffected	Bavin 2017 [63]
	Scleraxis knockout in mice, and scleraxis knockdown in isolated rat tendon cells	Decreased or absent tenomodulin expression at P1 in scleraxis $^{-/-}$ mice; tenomodulin expression reduced to 17% of control by scleraxis knockdown in rat tendon cells	Shukunami 2018 [62]

	Mohawk knockout in rats via CRISPR/Cas9	Heterotropic mineralization of Achilles tendons and tendon hypoplasia in 3 and 4-week-old rats; increased expression of Col II, Runx2, Aggrecan, COMP, and osteopontin in patellar tendon cells	Suzuki 2016 [59]
	Overexpression of mohawk and scleraxis in mouse MSCs and cell sheets	Increased expression of Col I, biglycan, Col III, Col V, Col XIV, decorin, fibromodulin, tenascin C, tenomodulin, and scleraxis via binding to the TGFβ2 promoter	Liu 2015 [57]
	Overexpression of mohawk in human and mouse bone marrow-derived MSCs	Increased expression of Col I, tenomodulin, tenascin C, tensascin XB, scleraxis	Otabe 2015 [58]
	Overexpression of scleraxis in human MSCs in a silk-collagen scaffold	Increased expression of tenogenic genes, cell alignment, and fibril diameter	Chen 2014 [61]
FGF4	Mouse MSCs and chick limb explants	Species-specific scleraxis expression: decreased in mouse or increased in chick	Havis 2014 [53] Havis 2016 [52]

(Edom-Vovard 2002) [50] (Brent 2005) [51] (Havis 2014) [53]	Mouse embryonic tendon progenitor cells and MSCs	No changes or decreased scleraxis expression	Brown 2014 [48] Brown 2015 [49]
BMPs (Lorda-Diez 2014) [73] (Liu 2015) [57] (Otabe 2015) [58]	Chick progenitor mesodermal cells	Transient gene expression determines response to BMP isoforms	Lorda Diez 2014 [73]
	Human bone marrow-derived MSCs	BMP-12 increased mohawk, scleraxis, Col I, tenascin XB, and decorin expression	Otabe 2015 [58]
LOX and Mechanical Stimuli (Marturano 2013) [30] (Pan 2018) [12]	Embryonic chick tendon and limb explants	Paralysis decreased elastic modulus and LOX, hypermotility increased LOX and elastic modulus, LOX inhibition decreased elastic modulus	Pan 2018 [12]
Lengthening/slow stretching (Hamburger & Hamilton 1951) [96]	Embryonic chick tendon cells in fibrin gels stretched 2 mm/day	Increased collagen fibril diameter, packing volume, and stiffness	Kalson 2011 [84]
Elastic modulus	RGD-functionalized alginate gels with	Scleraxis and Col I gene expression was regulated by elastic modulus	Marturano 2016 [85]

(Marturano 2013) [40]	embryonic-mimicking elastic modulus		
Progressive mineralization of tendon to bone attachment (Thomopoulos 2010) [23]	FEA model of cell- and tissue-level stress concentrations	Cell-level stresses much higher than tissue-level stresses; higher stresses may drive enthesis formation	Liu 2014 [103]

Cellular Cues

Embryonic and early stage postnatal tendon is highly cellular and collagen content is relatively low, compared to adult tendon [36-41]. For example, collagen content of Achilles tendons from postnatal day (P)4 mice is less than 3% of the dry weight [39], and in 1 week old sheep, cells account for nearly 33% of the tendon volume [38]. High cell density and cell organization in developing tendons may contribute to the organized and aligned collagen fibrils found in mature tendons. Based on scanning electron microscopy (SEM) imaging of embryonic tendon, it was proposed that embryonic tendon cell condensation and alignment of the cell's plasma membrane channels, where collagen fibrils may be released into the extracellular space by the cells, regulate collagen fibril alignment [42]. The cell-cell junction protein cadherin-11 was demonstrated to play a role in embryonic tendon cell organization. When cadherin-11 was knocked down in isolated and cultured whole chick metatarsal tendons at embryonic day (E)13 using small interfering RNA (siRNA), the cells appeared to move apart, and plasma membrane channels and collagen fibrils were disrupted [42]. In a different study, serial block face-SEM was used to visualize cells in embryonic, neonatal, and postnatal mouse tail tendons [43]. Throughout development, the number of cells per unit volume decreased, but direct cell-cell contacts were maintained [43]. A study in E8 to 11 chick calcaneal tendons showed that the tendon progenitor cells formed an aligned and organized actin cytoskeleton network that appeared to be continuous between adjacent cells (Figure 2.1 A) [37]. Disrupting the actin cytoskeleton with blebbistatin in E10 calcaneal tendons decreased tendon elastic modulus. Similarly, the elastic modulus of embryonic tendon cell-seeded alginate gels decreased with blebbistatin treatment [37]. These findings suggest that the actin network of embryonic tendon cells contributes to the mechanical properties of the developing tendon. Taken together, these developmental studies underscore the role of tendon progenitor cells in tendon tissue formation, and suggest that their content and organization are important considerations in engineered models.

A few *in vitro* engineered model systems have been developed to mimic the high cell density of embryonic and neonatal tendons. A scaffold-free approach used directed cell self-assembly to recapitulate the high cell density and low collagen content associated with embryonic tendon [44]. 3-dimensional (3D) channels were laser micromachined into agarose gels, which were lined with a thin coating of fibronectin and seeded with neonatal fibroblasts. The channels directed cell self-assembly into single fibers with high cell density, and an organized and aligned cell structure [44]. Cells in the fibers contained cadherin-11, the cell-cell junction protein found in embryonic tendons [42]. In a different study, uniaxial cyclic tensile loading of the cellular fibers for 1, 3, and 7 days improved tendon fiber formation [45]. The fibroblasts forming the fibers had aligned and elongated cell nuclei

and actin filaments (Figure 2.1 B). Scleraxis and tenomodulin gene expression increased in loaded fibers on day 1, and tenomodulin increased between day 1 and 7. Interestingly, none of the unloaded control fibers survived past day 3 [45]. In this model, loading appeared to counteract the self-generated static tension that arises in the cellular fibers. It is possible that only the loaded cellular fibers had established enough structure to support long-term fiber formation. These cell-based, scaffold-free models offer the advantage of combining high cell density with mechanical stimulation, making them a useful system for investigating key cellular aspects of early tendon development in a controlled *in vitro* environment.

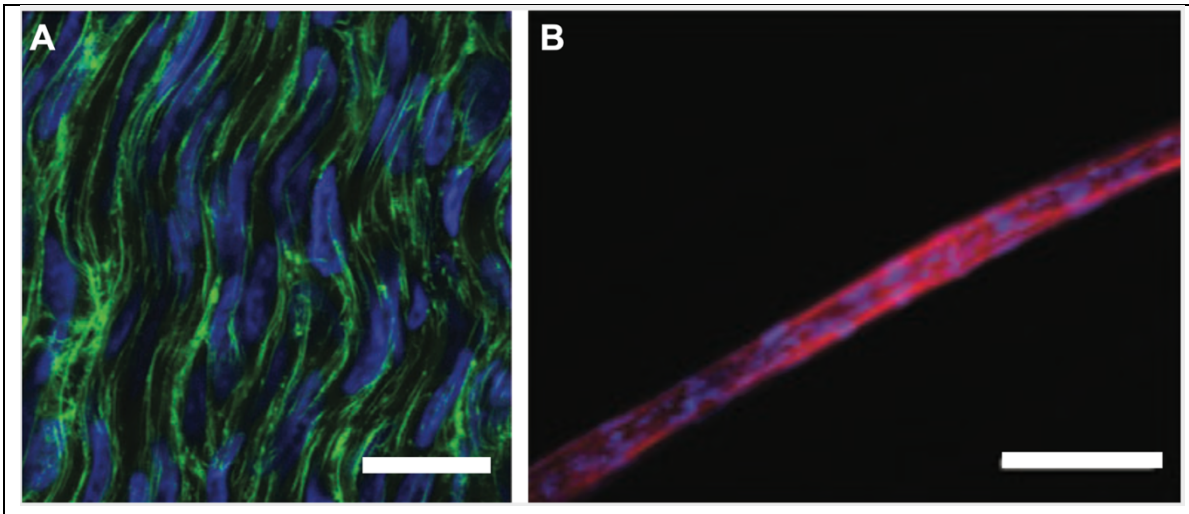


Figure 2.1 Embryonic tendon and a cellular fiber model.

A) E11 chick calcaneal tendons have high cell density and an organized actin cytoskeleton network. Actin cytoskeleton (green) and cell nuclei (blue) show actin filaments in embryonic tendon that appear to form a continuous network between adjacent cells. Scale bar = 10 μm. B) A self-assembled cellular tendon fiber to mimic the high cell density of embryonic tendon, following 7 days of mechanical loading in vitro. Actin cytoskeleton (red) and cell nuclei (blue) show high cellularity, actin stress fiber organization and nuclear elongation. Scale bar = 100 μm. Figure 2.1 A reprinted with permission by Wiley Periodicals, Inc. from Schiele et al 2015 [37]. Figure 2.1 B reprinted with permission by Mary Ann Liebert, Inc. from Mubyana & Corr 2018 [45].

Fibrin gels have also been used as *in vitro* model systems to explore what roles cells may be playing in embryonic tendon formation. Cell encapsulated in fibrin gels, formed from thrombin and fibrinogen crosslinking, can mimic the soft, 3D structure, and high cell density representative of embryonic tissues, without introducing exogenous collagen matrix. E13 chick metatarsal tendon cells seeded into fibrin gels at ~1.5 million cells/mL and cultured for up to 42 days resulted in tissue constructs that appeared similar to embryonic tendon, with newly synthesized collagen fibrils aligned along the axis of tension [46]. This embryonic-mimicking model system was then used to explore

how contraction by the embryonic tendon cells may regulate mechanical development. When actin cytoskeleton-mediated cell contractility was disrupted for 24 h using cytochalasin D and blebbistatin, the mechanical properties of the tissue constructs failed to increase, even though collagen production was not altered [46]. This model implies that development of tissue mechanical properties may depend on contractility of the embryonic tendon cells. Taken together, engineered models have revealed the contributions of cell contractility, the actin cytoskeleton, and cell-cell junctions to tendon formation. However, the mechanisms by which cells regulate tendon development remain an ongoing area of study. Alongside these cell-level contributions, biochemical and mechanical cues may also guide tenogenesis.

Growth Factors and Biochemical Factors

A number of growth factors have been identified in embryonic tendon development, but transforming growth factor beta (TGFβ) has emerged as a critical tenogenic regulator. TGFβs and their receptors (TGFβR1 and TGFβR2) have been found in embryonic chick [47] and mouse [31] tendon. Chick calcaneal tendons from E13 to 16 were evaluated for TGFβ1, 2, 3, TGFβR1 and TGFβR2 using immunohistochemistry [47]. TGFβ2 and 3, and TGFβ receptors were detected at all ages in the tendon midsubstance, but TGFβ1 was not observed. In embryonic mice, TGFβs were found to regulate scleraxis expression and tendon formation [31]. No tendons formed in the limbs, trunk, tail, and head of TGFβ2 and TGFβ3 double knockout mice at E14.5, even though tendon progenitor cells were present, indicating that TGFβ signaling is required for maintenance of the tendon phenotype [31]. Taken together, TGFβs are critical to embryonic tendon formation *in vivo*.

Based on these findings in developing embryos, a number of studies have explored TGFβs in developmental and tissue engineered *in vitro* models. Mouse embryonic fibroblasts and mouse mesenchymal stem cells (MSCs) (C3H10T1/2 cells) both increased scleraxis expression when treated with TGFβ2 in culture [31]. In another study, mouse tendon progenitor cells, isolated from the limbs and axial skeleton at different ages (E13 to 17, and P7), were treated with either TGFβ2, cyclic tensile loading (1% strain, 0.5 Hz), or fibroblast growth factor (FGF)4, a member of the FGF/ERK/MAPK signaling pathway [48]. TGFβ2 treatment enhanced scleraxis gene expression across all ages in both axial and limb tendon progenitor cells. When E16.5 tendon progenitor cells were treated with combinations of TGFβ2, FGF4, and cyclic loading, scleraxis gene expression was upregulated in all treatment groups that included TGFβ2 [48]. In a similar study, E14 mouse tendon progenitor cells were compared directly to adult mouse bone marrow-derived MSCs [49]. MSCs had increased scleraxis gene expression with TGFβ2 treatment alone, and when TGFβ2 was combined with loading. FGF4 treatment alone decreased scleraxis [49], even though FGF4 had been identified in early stage

embryonic mouse and chick tendon development [50, 51]. As before, scleraxis gene expression by embryonic tendon progenitor cells was upregulated in all treatment groups that included TGFβ2 [49].

To further assess the ability of TGFβ2 and FGF4 to drive tenogenesis, E3-4 chick forelimbs were grafted with beads containing FGF4, TGFβ2, FGF4 with a Smad2/3 inhibitor (SIS3), or TGFβ2 with a FGF/ERK/MAPK inhibitor (PD184352) [52]. Both FGF4 and TGFβ2 treatment increased scleraxis expression, and the Smad 2/3 and FGF/ERK/MAPK pathways regulated tenogenesis independently, as neither inhibitor downregulated scleraxis expression. When evaluated in chick and mouse limb explant cultures, FGF4 upregulated scleraxis expression in chick limbs, but downregulated scleraxis in E9.5 mouse limbs. TGFβ2 upregulated scleraxis in both animal models [52]. With scleraxis, tenomodulin was also upregulated in chick forelimb explants from E6.5 and 7.5 treated with TGFβ2 and FGF4. Additionally, E5.5, 6.5, and 7.5 chick limbs paralyzed during explant culture using decamethonium bromide (rigid paralysis) and pancuronium bromide (flaccid paralysis) had downregulated expression of scleraxis and tenomodulin [52]. FGF4 restored scleraxis expression in paralyzed chick limbs [52]. FGF4 was not tenogenic for mouse limb cells, where it inhibited scleraxis expression [52], in agreement with other *in vitro* models [48]. In the absence of FGF4, TGFβ2 was sufficient to maintain scleraxis and tenomodulin expression in immobilized chick limbs [52]. Taken together, these studies suggest variations in TGFβ and FGF signaling during embryonic tendon development between species, with only TGFβ2 able to induce tenogenesis in both mouse and chick. These results also indicate that TGFβ2 and FGF4 signaling may be initiated by mechanical stimuli from muscle contractions, to induce and maintain tenogenesis.

TGFβ2 was also used to explore tenogenic differentiation in mouse MSCs [53]. TGFβ2 treatment upregulated tenogenic genes via the Smad2/3 pathway, as a Smad 2/3 inhibitor (SIS3) eliminated TGFβ2-induced scleraxis expression [53]. In the same study, chemically blocking TGFβ receptors prevented tenogenic gene upregulation. A transcriptomic analysis of developing E11.5 to 14 tendons showed upregulation of several FGF ligands during differentiation, but downregulation of MAPK signaling [53]. The role of FGF signaling was then assessed in mouse limb explants [53]. A FGF/ERK/MAPK inhibitor (PD184352) activated scleraxis expression in explants from E9.5 or later, while activation of the FGF pathway downregulated scleraxis, consistent with prior studies [48]. Taken together, the results of these *in vitro* mouse and chick models suggest multiple growth factor-mediated pathways through which tendon development is initiated, modulated, and maintained, but highlight the pro-tenogenic impacts of TGFβ2.

Genetically manipulated cells have been utilized in other *in vitro* models of tendon development to investigate the role of Smad signaling in TGFβ2-induced tenogenesis. In addition to

Smad2/3, TGFβ may drive differentiation of tendon and cartilage through Smad4 [54]. To explore Smad4 in tenogenesis, 3D fibrin gels were seeded with mouse embryonic fibroblasts modified by adenovirus-Cre-mediated floxing to knockout Smad4 [55]. Smad4 knockout cells still showed enhanced tenogenic differentiation with TGFβ2 treatment, but without TGFβ2-induced proliferation [55], suggesting that regulators of tendon cell proliferation are important to consider. While scleraxis expression remained higher in Smad4 knockout cells treated with TGFβ2 than wild type controls, untreated Smad4 knockout cells stained more strongly for glycosaminoglycans (GAGs), suggesting potential chondrogenic differentiation [55]. This *in vitro* developmental model demonstrated the role of TGFβ2 and Smad4 in regulating tenogenesis.

Tenogenic induction via TGFβ2 was also explored in mouse MSCs over 21 days *in vitro*. TGFβ2 treated cells showed fibroblastic morphology and enhanced proliferation, while protein levels of scleraxis increased at day 14 and 21, and tenomodulin increased at day 21 [13]. Cell-cell junction protein levels of N-cadherin and cadherin-11 decreased at all timepoints, and connexin 43 increased before trending downwards [13]. This study further showed that TGFβ2 may be useful in tenogenic induction of MSCs, and that cell-cell junctions found in embryonic tendon (cadherin-11, N-cadherin, and connexin-43) [42, 56], may also be regulated during tenogenesis.

While TGFβ2 appears to regulate tenogenesis, recent work has focused on identifying regulators of TGFβ2. Mohawk was found to bind to the TGFβ2 promoter, indicating mohawk directly influences TGFβ2 gene expression [57]. Overexpression of mohawk in cell sheets cultured from mouse MSCs increased gene expression of scleraxis, tenomodulin, biglycan, decorin, fibromodulin, tenascin C, and Col I, III, V, and XIV [57]. Ectopic expression of mohawk and scleraxis both individually decreased the osteogenic and adipogenic potential, as well as the self-renewal capacity of MSCs, while neither transcription factor affected the chondrogenic capacity of the cells [57]. Finally, mohawk was found to more efficiently promote tenogenesis compared to scleraxis ectopic expression, as ectopic mohawk expression resulted in a higher upregulation of fibromodulin, tenomodulin, and Col I, III, and V, as well as larger Col I fibril diameters within the cell sheets [57]. In a different study, mohawk overexpression in human bone marrow-derived MSCs upregulated expression of tenomodulin, tenascin C, tenascin XB, and Col I after 7 days, compared to controls [58]. Early growth response (EGR) 1, a recently identified tenogenic transcription factor [35], has also been explored as a potential regulator of TGFβ2. However, despite evidence that mohawk directly drives TGFβ2 expression [57], overexpression of both mohawk and scleraxis *in vitro* failed to increase expression of EGR1 and 2 [58], indicating another mechanism may be responsible for TGFβ2 regulation via EGRs. Collectively, mohawk appears to influence tenogenesis alongside scleraxis, and acts via TGFβ2

signaling, though additional studies are needed to determine how TGFβ2 is regulated during tendon development.

The role of mohawk in tenogenic differentiation was further demonstrated *in vivo*. mohawk knockout rats generated via CRISPR/Cas 9 gene editing showed heterotopic ossification of the Achilles tendon at birth, and at 3 and 4 weeks of age [59]. This is an interesting finding, especially considering that heterotopic mineralization is frequently observed in human tendinopathies [60]. Furthermore, mohawk knockout rats had systemic hypoplasia of tendons, similar to mohawk knockout mice [34]. Cells derived from the patellar tendons of 3 week old Mohawk knockout rats had upregulation of chondrogenic and osteogenic genes, compared to cells from 3 week old mohawk $^{+/+}$ rats [59]. In the same study, overexpression of mohawk via retroviral transduction of patellar tendon-derived cells from the knockout rats suppressed chondrogenic, osteogenic, and adipogenic differentiation, consistent with similar findings in mouse.

Overexpression and knockdown of scleraxis have also been used to explore tenogenesis. Overexpression of scleraxis in human embryonic stem cell-derived MSCs seeded onto knitted silk-collagen scaffolds increased tenogenic gene expression, cell alignment, and collagen fibril diameter, compared to control cells [61]. Disruption of scleraxis negatively impacts tenogenesis. Scleraxis-null mice have tendon hypoplasia, complete loss of some tendons, and diminished tenomodulin expression [28, 30, 62]. Tendon cells isolated from P7 and P14 rats and treated with siRNA to knockdown scleraxis had tenomodulin expression that was reduced to 17% of the expression levels in control cells [62]. Another study knocked down scleraxis expression in equine embryonic stem cells, and adult and fetal tendon cells [63]. Scleraxis knockdown in fetal tendon cells significantly reduced Col I, cartilage oligomeric matrix protein (COMP) and Sox9 (a cartilage marker) gene expression, and reduced cell survival and tissue formation in 3D culture [63]. Interestingly, adult tendon cells were not affected by scleraxis knockdown. Overall, scleraxis appears necessary to maintain tenogenic differentiation, possibly through regulation of tenomodulin. Since tenogenesis is mediated at least in part by TGFβ2, the relationship between scleraxis, mohawk, TGFβ2, and tenomodulin requires further investigation.

TGFβ3 treatment and cell type were explored in an embryonic-like tendon formation model *in vitro* using human bone marrow-derived MSCs and bone marrow-derived mononuclear cells (BM-MNCs) [64]. Only MSCs produced embryonic tendon-mimicking collagen fibrils and fibropositors (cell structures that assemble fibrils) when cultured in fibrin gels under static tension for 7 days. TGFβ3, Col I, and Smad2 were upregulated in MSCs, and MSC contractility was prevented when treated with a Smad2 inhibitor (SB431542). TGFβ3 treatment increased collagen fibril synthesis, and

upregulated TGFβ3, Col I, and Smad2 in MSCs and BM-MNCs, illustrating a potential role for TGFβ3 in augmenting the tenogenic potential of human stem cells. In other studies, TGFβ3 is chondrogenic [65], but these results suggest the tenogenic or chondrogenic effects of TGFβ3 may depend on factors such as cell type, tension generated by cell contractility, or characteristics of the engineered matrix.

Other growth factors have been explored in model systems of tendon development *in vitro*. Bone morphogenetic proteins (BMPs), members of the TGFβ family, are involved in musculoskeletal tissue and tendon development [66-70] and induce tenogenic differentiation. Human bone marrow-derived MSCs treated for 5 days with BMP-12 increased expression of mohawk, scleraxis, Col I, tenascin XB, and decorin, compared to control cells, but tenomodulin levels were not impacted [58]. BMP-12 was also found to increase tenogenic gene expression in adipose-derived [71] and bone marrow-derived [58, 72] stem cells, making BMP-12 useful for inducing tenogenesis across multiple cell lines.

The role of other BMP isoforms in limb development was examined via *in situ* hybridization of chick limb autopods from E6.5 and 8, and *in vitro* micromass culture of E4.5 chick progenitor mesodermal cells isolated from limb buds [73]. Cells in micromass culture were treated throughout 12 days with exogenous BMP-2, 4, 5, and 7, as well as growth and differentiation factor (GDF)-5. In 2-day cell cultures treated with BMP-2 for 6 h, and in 4-day cell cultures treated with BMP-2 for 6 h, scleraxis expression was downregulated. Inhibition of BMP-2 upregulated scleraxis in 2-day cultures, but surprisingly, scleraxis was downregulated in 4-day cultures treated with a BMP inhibitor (AB204). These findings indicate that the cellular response to available BMPs depends on transient gene expression occurring in the target cells at the time of BMP signaling, and can vary based on culture day [73]. Understanding the variable cell responses to the same signaling pathway during differentiation provides new opportunities for understanding the spatiotemporal regulation of tenogenesis.

In addition to growth factors, several *in vitro* models have examined potential biochemical contributions of the extracellular matrix (ECM) during tenogenesis [74]. When E14 chick metatarsal tendon cells were cultured in fibrin or collagen gels, the gene expression profiles of cells in fibrin were most similar to native embryonic tendons, whereas cells in collagen gels had expression profiles more similar to cells in 2D culture, with an overall reduction in mechanotransduction-associated gene expression [75]. In addition to an ellipsoid cell morphology and parallel alignment, cells in fibrin constructs secreted their own *de novo* collagen matrix, which occurs in normal development [75]. Similarly, tendon and ligament progenitor cells from E17.5 scleraxis-GFP mice displayed increased

collagen alignment and linear region elastic modulus when seeded in fibrin gels, compared to collagen gels. Cells in fibrin gels also had increased scleraxis, tenascin C, and fibromodulin expression after 14 days in culture [76]. Based on these studies, embryonic tendon may be better represented by *in vitro* models that incorporate minimal collagen matrix, which mimics the low collagen content found in developing tendons [39, 40].

Embryonic tendon cells produce matrix metalloproteinases (MMPs), enzymes that can degrade collagen and other proteins that may regulate the cell's local biochemical environment. MMP-2, membrane type (MT)1-MMP, and MT3-MMP are present within tendon during embryonic development [77-79], and these MMPs may play a role in tendon tissue formation. Based on its presence in embryonic tendon, MT1-MMP was explored in an *in vivo* rat rotator cuff injury model [80]. Fibrin glue seeded with bone marrow-derived MSCs genetically manipulated to overexpress MT1-MMP was injected into a supraspinatus tendon injury. Tendons repaired with MT1-MMP overexpressing MSCs had improved mechanical properties and more fibrocartilage at 4 weeks post-injury, compared to control MSCs, suggesting that MT1-MMP augmented the healing process [80]. Based on these findings, developmentally inspired MMPs deserve further study in models of tendon formation.

Overall, model systems have applied growth factors and biochemical cues identified in embryonic tendon development to influence tendon formation *in vitro*. TGFβ2 has been increasingly explored, as it appears to induce tenogenesis across a range of *in vitro* systems. Future model systems need to identify how TGFβ2 is produced and controlled to direct tendon formation. Interactions between biochemical cues (ECM and growth factors) are complex and may vary based on the cell type and species used, the timing and concentration of each biochemical cue, and the presence of mechanical loading. Such interactions need to be further explored in isolation and combination.

Mechanical factors

Elastic modulus

Elastic modulus, the measure of a material's resistance to elastic (i.e., non-permanent) deformation, is a factor that may guide stem cell differentiation [81, 82], and a few studies have measured the elastic moduli of embryonic tendons. Tensile testing showed that elastic moduli of E13 to 18 chick tendons range from approximately 200 kPa to over 20 MPa [46, 83, 84]. Nanoscale and microscale elastic moduli of chick calcaneal tendons from E5.5 to 17, measured by force volume-

atomic force microscopy, increase nonlinearly from 7 to 21 kPa, and from 5 to 108 kPa, respectively [40]. These increases in elastic modulus occur simultaneously with differentiation of tendon progenitor cells, and may be an important tenogenic factor that several model systems have explored.

To identify the impact of elastic modulus on tenogenesis, alginate hydrogels were designed to mimic the elastic modulus of embryonic tendon at specific developmental stages [85]. Alginate hydrogels functionalized with arginyl-glycyl-aspartic acid (RGD), to enable cell attachment, were tuned using a combination of alginate concentration and calcium crosslinking density to have nanoscale elastic moduli from 3.4 to 20.1 kPa, representing the nanoscale elastic moduli of embryonic chick tendon from prior to E5.5 and up to E17 [85]. Tendon progenitor cells isolated from E11 chick calcaneal tendons were encapsulated in the 3D alginate hydrogels and cultured for 7 days *in vitro*. Scleraxis and Col XII gene expression increased at the highest elastic modulus (representing late stage embryonic tendon). Col I expression was downregulated at elastic moduli representing middle and later embryonic stages, whereas tenomodulin and Col III were not affected by elastic modulus [85]. This model suggests that embryonic tendon mechanical properties impact tenogenic markers, but additional factors may be needed, as late stage tendon markers (tenomodulin) were not affected. It is also possible that embryonic magnitudes of elastic moduli are not fully representative of the tenogenic environment. Tendon formation continues throughout postnatal development with increases in differentiation markers [86], collagen content, and mechanical properties [38, 39]. For example, linear region elastic modulus of postnatal mouse Achilles tendon increases from approximately 87 MPa at P4 to 544 MPa at P28, and toe region elastic modulus increases from 25 MPa to 72 MPa [39]. Elastic modulus of postnatal tendon can serve as a template for models aiming to mimic the complete developing tendon environment. As the stress-strain relationship in tendon is non-linear [87], the elastic modulus (e.g., toe region or linear) that impacts tenogenesis needs to be explored. Furthermore, tendon material properties can be evaluated at nano- and microscales (e.g., atomic force microscopy) or bulk scale (e.g., uniaxial tensile test), but how each scale impacts cells is unknown and challenging to uncouple. Model systems exploring the effects of bulk and cell-level material properties on tenogenesis are needed.

Static and dynamic tensile loading

Mechanical loading is a critical factor in tendon development, and has been highlighted in recent reviews [9, 10, 26]. In the developing embryo, quasi-static or static loading may result from limb lengthening or the contractile forces generated by the tendon cells themselves, while dynamic loading results from skeletal muscle contractions. *In vitro* bioreactor systems have been developed to apply mechanical stimuli [55, 88-90], with loading enhancing tenogenic markers [4, 5, 91], collagen production [92], and mechanical properties [46, 93-95] of engineered tissues. Here, we discuss developmentally mimicking tendon models that investigate the effects of static and dynamic loading.

Tendon cells isolated from adult human semitendinosus and gracilis tendons and cultured in fibrin gels under self-generated static tension produced embryonic-like tendon tissue, with increased collagen fibrillogenesis and deposition of aligned collagen fibrils [92]. After 10 days of culture, force-displacement curves displayed the characteristic toe and linear regions of tendon [92]. The cells produced Col I, III, XII, and XIV, fibronectin, integrin α5, and small-diameter collagen fibrils and fibropositors, all components found in embryonic tendon [92]. With the right environment and self-generated static tension, adult tendon cells may behave as embryonic tendon cells, and develop an embryonic tendon-like tissue. However, in a different study, fibrin gel contraction by embryonic tendon cells occurred at a faster rate than adult tendon cells [46]. While adult tendon cells may form embryonic-like tissues *in vitro*, the ability for embryonic tendon cells to rapidly modify their microenvironment by contraction may result in functionally distinct tissues and should be considered when evaluating cell types for *in vitro* developmental models.

Slow stretching has been explored in a model of tendon formation, based on the observed increase in limb length during development [96]. Specifically, lengthening of the third metatarsal in chick from E10 to 14 was proposed to stretch the developing metatarsal tendon. To mimic this, a slow continuous stretch was applied to embryonic chick metatarsal tendon cells seeded in fibrin gels [84]. Slow stretching (2 mm/day over 4 days to double the construct length from 8 to 16 mm) increased collagen fibril diameter, fibril packing volume, and stiffness, all characteristics of more mature tendon (Figure 2.2) [84]. Unstretched controls resembled early stage embryonic tendon. Extrinsic stretch can be effectively applied to mimic *in vivo* stretch experienced by the developing tendon, but the appropriate magnitudes and timing for each tendon need further characterization.

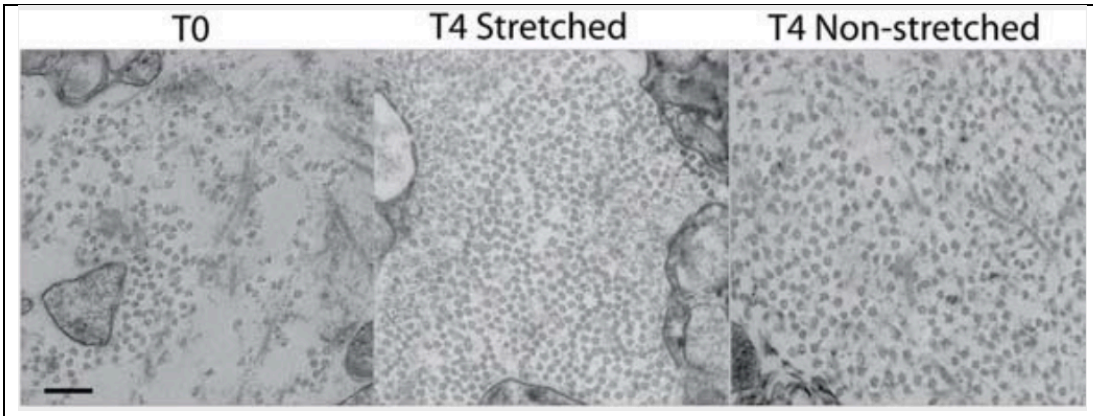


Figure 2.2 Stretch influences collagen fibril formation in an embryonic tendon model.

Transmission electron microscopy images of fibrin gel tendon constructs seeded with embryonic chick metatarsal tendon cells at day 0 (T), and after four days (T4) with and without stretching. Slow stretching (2 mm/day) increased collagen organization and collagen fibril packing volume in this *in vitro* model of embryonic tendon formation. Scale bar = 250 nm. Figure reprinted with permission by Wiley Periodicals, Inc. from Kalson et al 2011 [84].

Dynamic movement in the embryo is facilitated by developing muscles, whose concomitant development alongside tendons provides both mechanical and biochemical cues that drive tenogenic differentiation. Pax3 knockout mice ($Pax3^{Spd/Spd}$), which lack skeletal muscle, show that tenogenesis is initiated even in the absence of normal myogenesis [11]. However, while initial tenogenic induction is independent of muscles, tendons are unable to elongate and are subsequently lost by E13.5 in $Pax3^{Spd/Spd}$ muscle-less mice [11], similar to prior studies in muscle-less chick limbs [50, 97, 98]. When muscles were intact, but genetically altered via a muscular dysgenesis (*mdg*) mutation to limit movement, tendon progenitors in the embryonic mouse forelimb were maintained at E12.5 [11]. However, tendons from *mdg* mice at E16.5 were smaller than in wild-type mice, though they were not diminished to the same extent as tendons from $Pax3^{Spd/Spd}$ muscle-less mice [11]. While muscles may not be required for tenogenic induction, several previous studies suggest that muscles and subsequent mechanical stimuli are needed for continued tendon development [11, 12, 50, 97, 98]. This was further demonstrated in a chick model. Chick embryos subjected to systemic rigid paralysis (using decamethonium bromide) for 48 h had reduced calcaneal tendon elastic modulus at E17, whereas hypermotility (using 4-aminopyridine) increased elastic modulus [12]. Lysyl oxidase (LOX), an enzyme involved in collagen crosslinking and embryonic tendon mechanical property development [99], was also assessed in calcaneal tendons in embryos and limb explant cultures from paralyzed and hypermotile chicks at E19 [12]. In embryos, paralysis reduced LOX activity, and when LOX was inhibited, hypermotility no longer increased elastic modulus. Overall, embryonic movements may

regulate the formation of tendon mechanical properties through LOX-mediated collagen crosslinking. Based on these studies, *in vitro* models exploring mechanical loading may consider LOX-mediated mechanisms of tissue formation. Furthermore, exogenously applied LOX increased ultimate tensile strength and modulus in an engineered tissue model [100], suggesting that LOX can be successfully used to enhance tissue mechanical properties *in vitro*.

In vitro, cyclic loading representing contracting muscles during development has the potential to impact tenogenesis. For example, cyclic loading of mouse MSCs seeded in collagen gels increased scleraxis and Col I gene expression, over static controls [91]. Scleraxis also increased as a function of strain magnitude and number of loading repetitions. Similarly, cyclic loading enhanced tendon tissue formation and tendon gene expression in self-assembly models that captured embryonic tendon cellular cues [44, 45]. However, appropriate levels of loading (e.g., strain magnitude, frequency, rate, duration, etc.) for tendon formation are still unknown. The *in vitro* models reviewed here may be employed to determine these loading parameters in bioreactor systems isolated from other confounding factors associated with *in vivo* models. Determining the timing, intensity, and duration of tenogenic mechanical stimuli is a challenge for tendon tissue engineering, and will require additional *in vivo* and *in vitro* studies.

To explore mechanical loading parameters, computational models may be a good alterative, but have only been used for evaluating enthesis formation. The enthesis is a progressively mineralized fibrocartilage interfacial tissue that extends from the tendon to the bone insertion and is impacted by loading [22, 23, 101, 102]. A computational model of mineralization during enthesis formation was developed based on histological data from mice at P7, 10, 14, 28, and 56 [103]. The mineralization gradient was predicted to be driven by cell-level stress rather than tissue-level stress, which may allow for relatively small tissue-level stresses to drive mineralization via the larger effect exerted on individual cells [103]. Cell-level local stresses predicted by the model at early time points almost reached adult physiological levels, likely stimulating mineralization [103]. The development of this complex interface tissue has been explored *in vivo* [104-106], but future engineered systems and computational models may be useful for understanding the mechanical and biochemical factors involved in enthesis and tendon formation.

Models of adult tendon injury

Adult tendon contains a dense network of aligned and continuous collagen fibrils that are responsible for force transmission [107, 108]. Unfortunately, the incidence of tendon ruptures is increasing [14, 109, 110], and tendon heals as disorganized scar tissue that does not regain mechanical function [3, 111]. A major challenge has been a limited understanding of the numerous

factors that influence tendon injury (e.g., tendinopathy and ruptures) and healing. Model systems and computational models have been developed to explore impacts of mechanical loading, biochemical factors, and inflammatory cytokines on adult tendon injury and healing (Table 2.2).

Table 2.2 Summary of tendon injury models

Injured Tendon Characteristics	Model Characteristics	Model Outcomes	References
Overuse injury	Downhill running in rats	Induced overuse injury in the supraspinatus	Soslowsky 2000 [119] Archaumbault 2006 [120]
	Bipedal downhill running in rats	Reduced stiffness and tensile strength; localized disintegration of collagen bundles	Ng 2011 [121]
	Uphill running in rats	Achilles tendons adapted to loading; no observable pathology	Heinemeier 2012 [116] Dirks 2013 [118]
Transection/Acute injury	Neonatal and adult mouse Achilles tendons	Regeneration observed in neonates, but not adults	Howell 2017 [157]
	Mouse supraspinatus tendons with full and partial transections	Different cell populations involved in	Moser 2018 [147] Yoshida 2016 [148]

		healing of full versus partial injury; distinct cell lineages participate in healing response	
	Rat Achilles tendon partial transection repaired with scaffolds	Cells in scaffolds expressed mohawk during repair	Otabe 2015 [58]
	Mouse Achilles tendon full transections repaired with MSC sheets overexpressing mohawk	Mohawk-overexpressing MSC sheets resulted in increased collagen fibril diameter, visible crimp, increased stiffness, elastic modulus, maximum force and stress, and energy absorbed	Liu 2015 [57]
	Canine digital flexor tendons	Following injury, IL-1β upregulated	Manning 2014 [153]

		4,000-fold, MMP-13 upregulated 24,000-fold	
IL-1β treatment	E15 and P7 mouse tendon cells treated with IL-1β	Higher expression of IL-6, TNFα, COX2, MMP-3 and MMP-13 in P7 compared to E15	Li 2019 [160]
	Human patellar tendon fibroblasts treated with IL-1β and strain	IL-1β and 8% strain upregulated MMP-1, COX2, and PGE2; IL-1β and 4% strain downregulated expression of MMP-1, COX2, and PGE2 compared to 8% strain	Yang 2005 [154]
	Adult and fetal equine tendon cells, and equine embryonic	Adult and fetal tendon cells upregulated MMP-	McClellan 2019 [158]

	stem cells treated with IL-1β	1, -2, -3, -8, -9, and -13, tenascin-C, Sox9, and downregulated scleraxis and COMP, compared to embryonic stem cells	
Genetic knockouts	Tenomodulin knockout mice with transected and repaired Achilles tendons	Downregulation of Col I, tenascin-C, thrombospondin 2, and TGFβ1; upregulation of scleraxis, COMP, and proteoglycan 4	Lin 2017 [161]
	GDF-5 knockout mice subjected to Achilles tendon injury	Delayed healing and increased adipocytes in knockouts	Chhabra 2003 [162]
	Decorin-null and biglycan-null mice subjected to full thickness, partial	Smaller diameter collagen fibrils, decreased cell density, and	Dunkman 2014 [165] Dunkman 2014 [166]

	width patellar tendon injury in adult and aged groups	altered cell shape and collagen alignment in knockouts; biglycan influenced early healing, decorin influenced late healing	
Chronic Injury/Induced Tendinopathy	Transection or Botox-unloading of rat Achilles tendon	Irreversible loss of scleraxis expression with transection; partial loss and return of scleraxis with Botox	Maeda 2011 [167]
	Immediate or delayed repair of rat rotator cuff injury	Delayed repair had worse outcomes than immediate repair	Killian 2014 [168]
	TGFβ1 injection to rat Achilles	Warburg pathway, hypoxic, angiogenic, and	Sikes 2018 [171]

		glycolytic metabolism gene activation	
	Collagenase injection in rat Achilles tendon	Increased IL-6 and MMP-9 in senescence-accelerated rats compared to senescence-resistant rats	Ueda 2019 [151]
	Carrageenan injection in rat patellar tendon; treatment with IL-1 receptor antagonist	Carrageenan decreased tendon length, and increased MMP activity and inflammation. Inflammation absent with IL-1 receptor antagonist	Berkoff 2016 [159]
	Stress deprivation in rat tail tendons	Increased MMP-13 expression	Arnoczky 2007 [112]

Ex vivo Loading			
	Stress deprivation in rat tail tendons	Stress deprivation decreased TIMP/MMP ratio; loading increased TIMP/MMP ratio	Gardner 2008 [135]
	Fatigue loading of rat flexor digitorum longus tendon loaded at low (6.0%–7.0%), moderate (8.5%–9.5%), and high (11.0%–12.0%) tensile strain	Isolated fiber deformations at low strain; fiber dissociation and localized rupture, decreased stiffness, and increased hysteresis at high strain	Fung 2009 [124]
	Equine flexor and extensor tendon cells subjected to 10% biaxial cyclic loading	Collagen synthesis, proliferation, COMP expression as a function of tendon type	Goodman 2004 [128]

	Equine superficial digital flexor tendon fascicles cyclically loaded from 2-12% uniaxial strain and 1800 cycles	Increased expression of IL-6, COX2, C1, C2, and MMP-13	Thorpe 2015 [126]
	Bovine deep digital flexor tendons cyclically loaded from 1-10% strain	Collagen fiber disruption, kinks, and interfascicular network damage, and expression of IL-6, COX2, MMP-1, 3, and 13	Spiesz 2015 [127]
	Mouse patellar tendon cells isolated from 3-week old mohawk knockouts and subjected to 4% cyclic tensile loading	Increased chondrogenic gene expression (Col II, Aggrecan, COMP)	Suzuki 2016 [59]
	Cell- and tissue-level responses to strain simulated via Hill functions	Tissue-level response similar at low and high strain conditions	Mehdizadeh 2017 [179]

Computational models	Hill-type equations of human Achilles-soleus unit	Proteolytic damage leads to collagen fiber shortening; mechanical damage lengthens fibers	Young 2016 [178]
	Regression model of healing	Multiple differential predictors of early development and early developmental healing; however, no differential predictors of late development and late developmental healing	Ansorge 2012 [180]
	2D FEA simulation of "jumper's knee" in Patellar tendon	Highest localized strain predicted successfully	Lavagnino 2008 [174]

	Agent-based model of collagen fibril alignment with applications in tendon loading during healing	Peak collagen alignment occurs at lower strain level than peak deposition; peak deposition occurs above damage threshhold	Richardson 2018 [181]
	Multiscale OpenSim model of cellular responses to various loading parameters	Single set of cellular response curves explained tendon behavior observed in several different experiments	Chen 2018 [182]
	Empirical model of patellar tendon response to aging and injury	Effects of aging and injury on patellar tendon mechanical properties predicted by damage models	Buckley 2013 [184]

	Empirical model of Achilles tendon response to decorin and biglycan knockout in aging mice	Model predicted changes in dynamic modulus resulting from decorin and biglycan knockout	Gordon 2015 [185]

Mechanical Loading

Overuse injury

Adult tendon injury may be influenced by mechanical loading [112-115]. Models to explore overuse injury have induced uphill and downhill treadmill running in animal models. Adult rats running on a 10° incline treadmill (1 h/day, 5 days/week) over 12 weeks had no observable Achilles tendon damage, compared to controls [116]. Elastic modulus and the ratio of failure stress to body weight increased in Achilles tendons from the running group. Running upregulated expression of Col III and insulin-like growth factor (IGF)-I, but downregulated TGFβ1, connective tissue growth factor (CTGF), and ECM components fibromodulin and biglycan, with no impact on Col I. Notably, these gene expression profiles are not observed in human tendinopathies [117]. The increased mechanical properties coupled with these changes suggest that tendons adapted to increased mechanical stimuli and exercise may maintain or improve tendon health, but this did not produce an overuse injury model [116]. These results were consistent with a study that found no histological evidence of tendon injury with uphill running in rats [118]. In contrast, downhill running on a 10° decline (17 m/min, 1 h/day, 5 days/week) for 4, 8, or 16 weeks induced an overuse injury in the supraspinatus tendon of the rotator cuff in adult rats [119]. Compared to unexercised controls, downhill running increased cellularity and rounded cell-shape, and decreased collagen fiber alignment, cross-sectional area, maximum stress, and elastic modulus [119]. In a follow-up study, 2 and 4 weeks of downhill running increased cartilage-associated gene expression for Col II, aggrecan, and Sox9 in the rat supraspinatus tendon, compared to nonrunning controls [120]. These rat models of tendon overuse demonstrate that some tendons can adapt to mechanical loading, while others display pathology, suggesting that specific tendons are more prone to overuse injuries, an important consideration for selecting an appropriate model system.

A potential limitation of rat models in overuse tendon injury is the difference in locomotion between bipedal humans and quadrupedal rats. To address this, a custom treadmill was used to allow adult rats to run downhill bipedally on a 20^{o} decline (1h/day, 7 days/week) for 8 weeks. Achilles tendons of the running group had increased cell proliferation, a more ovoid cell morphology, and less organized ECM, with localized disintegration of collagen bundles. Bipedal running also reduced stiffness and ultimate tensile strength, compared to controls [121]. Achilles tendons did not appear to adapt to the increased loading demands with this magnitude of bipedal running, but appeared pathogenic, making this a potentially good model of Achilles tendon overuse injury. However, bipedal running in a normally quadrupedal animal may be a confounding factor. Model systems to mimic human adaption or overuse injuries in tendon are needed, and also must consider other

potential factors including age, gender, systemic inflammation, co-morbidities, prior injuries, and lifestyle. Specific loading parameters such as duration and intensity also need to be explored as studies in human Achilles tendon show adaption as a function of strain magnitude during loading [122, 123].

Ex vivo models have examined damage in tendons resulting from repetitive loading. Fatigue damage in isolated adult rat flexor digitorum longus tendons was assessed at low (6.0%–7.0%), moderate (8.5%–9.5%), and high (11.0%–12.0%) peak levels of clamp-to-clamp tensile strain [124]. Samples were cycled between 1 and 16 N at 0.75 Hz until the desired strain magnitude was reached. Stiffness decreased and hysteresis increased, but only at high strain. Low strain led to isolated collagen fiber damage, but as strain increased, fiber dissociation and localized rupture were observed, and damaged fiber areas increased. This model expanded the range of strains that must be considered when assessing tendon damage, but used a relatively high strain magnitude (12%), which may account for the differences observed between strain magnitudes. Interleukin (IL)-1β, an inflammatory cytokine, and MMP-13 may also be impacted by strain magnitude applied to tendon [125]. Adult female rat patellar tendons were cyclically loaded *in vivo* between 1 and 35 N at 1 Hz until reaching 0.6% or 1.7% strain. Following 1 and 3 days of recovery, tendons elongated to 1.7% displayed microstructural damage and upregulated expression of MMP-13 and IL-1β, compared to the 0.6% group, which downregulated expression of both MMP-13 and IL-1β [125].

Other *ex vivo* models applied mechanical loading to isolated tendons and tendon fascicles. Equine superficial digital flexor tendon fascicles cyclically loaded from 2 to 12% uniaxial strain for 1800 cycles had increased levels of inflammatory mediators, IL-6 and cyclooxygenase 2 (COX2) [126]. Collagen degradation markers, C1 and C2, and MMP-13 activity were also increased with cells appearing rounder and less elongated. Although these markers of tendon damage were increased, overall levels were relatively low, indicating a possible low-level inflammatory response. Low-level inflammation with loading may have implications for long-term tissue health, rather than inducing an acute injury. Similar results were obtained when bovine flexor tendons were cyclically loaded from 1–10% strain [127]. Loaded tendons had collagen fiber disruption and kinks, and interfascicular network damage, as well as expression of IL-6 and COX2, which were absent from non-loaded controls (Figure 2.3). MMP-1, 3 and 13 were detected in interfascicular regions of loaded tendons, but only minimally detected in controls [127]. The interfascicular tissue involvement in the loading response is a novel finding of this model, and highlights a possible role in tendon pathology.

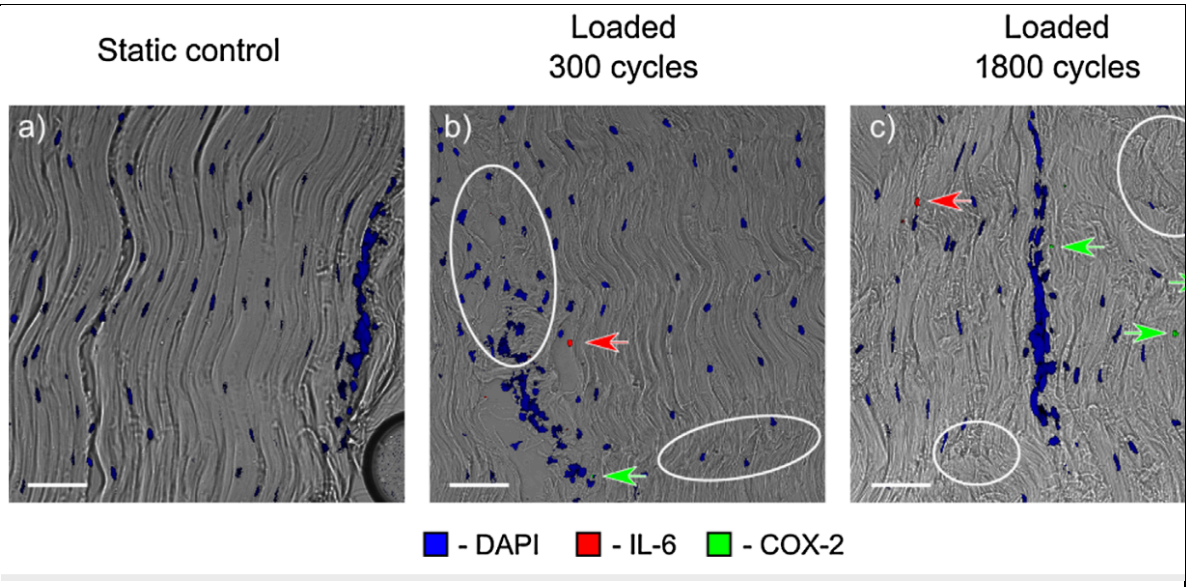


Figure 2.3 Mechanical loading impacts *ex vivo* tendon damage.

Ex vivo static a) and cyclically loaded (b, c) bovine flexor tendon fascicles immunostained for inflammatory markers IL-6 (red) and COX-2 (green), and co-labeled for cell nuclei (DAPI, blue). Fascicles and the interfascicular matrix of the loaded samples show damage (white ellipses), with collagen fiber kinks and interfascicular matrix disruption. IL-6 and COX-2 are found in loaded samples only, with COX-2 expression increasing with cycle number. Scale bar = 10 μm. Figure reprinted under a Creative Commons Attribution License from Spiesz et al 2015 [127].

In vitro cell culture models have assessed effects of cyclic strain and growth factors on tendon cell behavior, as a function of tendon type. Equine tendon cells isolated from flexor and extensor tendons of fetal, P11, 8 month, and 4, 8, and 10 year old horses were cyclically loaded to 10% strain for 24 h, and treated with TGFβ1 or TGFβ3 [128]. TGFβ1, TGFβ3, and cyclic strain did not increase flexor tendon cell proliferation. Extensor tendon cell proliferation was increased by loading, but not by TGFβ1 or TGFβ3 treatment. TGFβ1 and TGFβ3 increased Col I and III production, incorporation of 3-hydroxyproline into the collagen, and COMP in both cell types regardless of whether cells were loaded, but when TGFβ1 or TGFβ3 were combined with loading, neither cell type had increased proliferation at any age. COMP and Col I and III synthesis was higher in flexor tendon cells from horses up to 8 months old, compared to flexor cells isolated from older horses. Interestingly, age had no effect on activity of extensor tendon cells. Tendon-specific responses to mechanical stimulation and aging emphasize the importance of controlling for tendon type in model systems.

In a different cell culture model, adult rat patellar tendon cells were loaded *in vitro* via hydrostatic pressure to 2.5 and 7.5 MPa [125]. Both loaded groups upregulated IL-1β and MMP-13

expression, compared to unloaded controls. siRNA knockdown of IL-1β partially suppressed loading-induced MMP-13 expression and activity [125]. MMP-13 has been associated with human tendinopathies [129], and this model shows that MMP-13 expression may be regulated by loading and resulting inflammatory cytokines. Using model systems, loading parameters to induce an adaptive rather than pathogenic response may be identified and provide opportunities for clinical interventions incorporating loading.

While tendon over-loading may induce damage, under-stimulation also leads to pathology [112, 130-133]. MMPs have been explored as mediators of load-dependent tendinopathy in *ex vivo* models of stress deprivation. Adult rat tail tendons subjected to one week of stress deprivation *ex vivo* increased MMP-13 gene expression and enzymatic activity, and inhibiting MMPs improved ultimate stress, tensile modulus, and strain at ultimate stress [134]. Mechanical loading also stimulates tissue inhibitors of metalloproteinases (TIMPs), which inhibit MMPs [135]. *S*tress deprivation of rat tail tendons *ex vivo* decreased the TIMP-1 to MMP-13 ratio, compared to cyclically loaded controls [135]. When tail tendons were subjected to 1%, 3%, or 6% cyclic strain for 24 h, all groups increased the TIMP-1 to MMP-13 ratio [135]. Mechanically activating TIMPs may prevent MMP-mediated degradation. Mechanical stretch may also protect collagen fibers aligned along the axis of loading by hiding MMP-cleavable degradation sites within the collagen [136-141]. Based on these models, stress deprivation in tendon may stimulate MMP production, while also making collagen more susceptible to MMP degradation, but these compounded effects need further study *in vivo*.

A few injury models have explored how mechanical loading impacts tendon healing. When a supraspinatus injury was cast immobilized in adult rats, mechanical, compositional, and structural properties improved, compared to injured groups allowed cage activity or allowed to run at 10 m/min for 1 hr/day and 5 days/week [142]. However, immobilized groups had upregulated chondrogenic genes, while exercise upregulated tenogenic genes [142]. Another study investigated Achilles tendon injuries in mice [143]. Healing of a bilateral full thickness, partial width excisional injury was evaluated at 0, 1, 3, or 6 weeks. A fatigue test showed initial decreases in tangent stiffness, dynamic modulus, and hysteresis immediately following injury that were not improved after 6 weeks of healing [143]. In a follow-up study, the hindlimbs were cast-immobilized in plantarflexion for 1 or 3 weeks following Achilles tendon transection, and then assessed after 16 weeks [144]. Tendons immobilized for 1 week had lower joint stiffness in plantarflexion than tendons immobilized for 3 weeks, though both were increased compared to transected controls with normal cage activity. Stride width during walking, tendon cross-sectional area, and laxity (the tendency of the tendons to elongate under fatigue loading) increased in mice immobilized for both 1 and 3 weeks, compared to uninjured

controls. Secant stiffness remained at pre-injury levels, and tissues appeared histologically normal for both injured groups [144]. This model recreated immobilization periods consistent with conservative management of acute tendon injuries in humans, and showed some improvement in tendon mechanical properties. However, laxity may lead to joint dysfunction, and may be regulated by contractile tendon cells [145, 146], suggesting immobilization during healing did not return tendons cells to their normal function. Taken together, these injury models show that the mechanical environment may play a role in tendon healing.

Surgical injury models of the rotator cuff

Surgical models to induce injury have shown promise for identifying factors that influence rotator cuff healing. Partial and full detachment tears of the supraspinatus tendons in adult mice were induced by either insertion of a 26G needle through the central portion of the supraspinatus tendon into the insertion site at the enthesis, or a full transection and surgical repair using sutures [147]. Both injury models healed via scar formation, but the amount of scarring following full detachment and repair led to permanent impairments in gait and disruption of the architecture and organization of the enthesis. In the partial tear model, gait was not affected, but there was still considerable hypercellular scarring and increased cell density within the healing enthesis. In the same model, lineage tracing showed minimal scleraxis or Sox9 expression in the scar, suggesting that that the scar-forming cells were not predominantly derived from tendon, articular cartilage, or unmineralized enthesis [147]. Axin2-expressing cells (indicating resident stem cell lineage) were not found in the scar of the partial tear model, but were the majority of cells detected in the scar of the full tear. Sox9-expressing cells were detected in the articular cartilage of the humeral head, the unmineralized enthesis fibrocartilage, and near the insertion in both the full and partial tear models [147]. These results suggest that distinct cellular mechanisms may operate in response to partial or full tear injuries of the rotator cuff.

Another surgical model developed a full-thickness injury by detaching the central portion of the supraspinatus tendons of adult mice [148]. Healing was assessed at 1, 2, and 5 weeks post-surgery along with evaluating smooth muscle actin, proteoglycan-4, and aggrecan-expressing cells at the site of healing. 2 weeks post-surgery, proteoglycan-4 expressing cells were found in midsubstance and in the paratenon on the bursal side of the supraspinatus, as well as in the articular cartilage of the humerus and joint capsule, while smooth muscle actin-expressing cells were localized to the paratenon, blood vessels, and periosteum [148]. Aggrecan-expressing cells were found in the articular cartilage of the humerus, the unmineralized fibrocartilage at the supraspinatus tendon enthesis, and in the fibrocartilage cells of the acromioclavicular joint, but were not found elsewhere in the midsubstance, myotendinous junction, or paratenon [148]. The distal stump of the injured tendon

underwent minimal remodeling, as indicated by a lack of labeled cells, but cells from both the bursal and articular surfaces appeared to contribute to healing, a novel finding in rotator cuff injury models [148]. Together these models have implications for the type of surgical model used to investigate rotator cuff injuries (i.e. partial or full transection). The identification of multiple distinct cell lineages participating in the healing process is interesting and worth exploring in chronic models of rotator cuff injury.

Biochemical factors

Inflammatory cytokines

The inflammatory cytokines IL-6 and IL-1β have been implicated in tendinopathies [149, 150]. IL-6 and MMP-9 were upregulated in adult senescence-accelerated and senescence-resistant mice in response to collagenase type I injections in the Achilles tendon, compared to controls injected with saline [151]. Upregulation of IL-6 was higher in the senescence-accelerated mice compared to the senescence-resistant mice, suggesting the inflammatory response increases with age. IL-6 was also upregulated in tendon cells from bovine extensor tendon fascicles cyclically loaded to 30% and 60% of failure strain [152]. Compared to unloaded controls and fascicles loaded to 60% of failure strain, fascicles loaded to 30% of failure strain increased IL-6 and Col I expression and had no structural damage. Together, these findings suggest that IL-6 is involved in an adaptive response to loading and may be influenced by aging, but additional studies are needed to distinguish adaptive and pathological functions of IL-6.

IL-1β is a potent mediator of inflammation and is associated with tendon injuries [153]. IL-1β was upregulated 4,000-fold, 1 day after a laceration injury in canine forelimb flexor tendons, and remained elevated compared to uninjured controls for 9 days post-injury [153]. *In vitro*, human patellar tendon cells treated with IL-1β and cyclically loaded to 8% strain *in vitro* for 4 h upregulated expression of MMP-1, COX2, and prostaglandin (PGE)2, compared to cells treated with IL-1β and stretched to 4% strain [154]. When compared to unstretched controls, 4% strain and IL-1β downregulated expression of MMP-1, COX2, and PGE2, while 8% strain and IL-1β upregulated MMP-1, COX2, and PGE2 [154]. This *in vitro* model shows that mechanical stimulation and IL-1β may mediate markers of tendinopathy.

In vivo tendon injury models show that embryos and neonates retain greater regenerative capacity than adults [155, 156]. For example, an Achilles tendon transection in neonatal (P5) mice

showed regenerative healing, with a return to pre-injury mechanical properties and gait, while adult mice healed with scar and diminished mechanical properties [157]. IL-1β has been explored in model systems aimed at understanding the inflammatory responses in adult and fetal tendon cells. IL-1β treatment of adult equine tendon cells increased expression of MMP-1, 2, 3, 8, 9, and 13, as well as tenascin-C and Sox9 (a chondrogenic marker), and decreased expression of scleraxis and COMP, compared to IL-1β treated equine fetal tendon cells and tendon cells derived from equine embryonic stem cells [158]. Gene expression of tendon cells derived from embryonic stem cells was not altered with IL-1β, possibly due to lower expression of IL-1 receptors and increased expression of IL-1 decoy receptors. This model suggests that tendon cells derived from embryonic stem cells retain their reduced response to inflammatory cytokines (e.g., IL-1β). Additionally, blocking IL-1 receptors may limit adult tendon pathology [159]. Impacts of IL-1β were also explored in isolated E17 and P7 mouse tendon cells [160]. When directly compared to E15 cells, P7 cells treated with IL- 1β for 24 h upregulated inflammatory mediators, specifically IL-6, tumor necrosis factor (TNF)α, COX2, MMP-3 and MMP-13 [160]. Together, these *in vitro* models showed that postnatal and adult tendon cells have an inflammatory response to IL-1β, which may contribute to poor postnatal tendon healing and scar formation, and are intrinsically different from embryonic cells. Improved understanding of the pathways regulating scarless healing in embryonic and neonatal tendons may advance adult tendon healing strategies.

Knockout and overexpression models

Animal models have been developed to explore impacts of specific proteins on tendon injury and healing. Tenomodulin knockout mice and wild-type controls underwent Achilles tendon transection and surgical repair [161]. Col I, tenascin-C, thrombospondin 2, and TGFβ1 were downregulated in tenomodulin knockouts, but scleraxis was upregulated, along with chondrogenic genes, COMP and proteoglycan 4. Compared to wild-type controls, scar tissue in tenomodulin knockout mice was more disorganized and had increased adipocyte and blood vessel accumulation, apoptosis, and reduced tendon cell proliferation. These findings suggest that tenomodulin may be an important factor in regulating adult tendon healing.

Mohawk may be involved in tendon cell responses to loading and healing. Cells isolated from patellar tendons of 3 week old mohawk knockout rats and subjected to 4% cyclic tensile loading for 6 h *in vitro* had increased chondrogenic gene expression, compared to control cells from mohawk $^{+}/^{+}$ animals [59]. Cyclic loading of tendon cells from mohawk $^{+}/^{+}$ rats increased expression of the tenogenic genes, mohawk, and Col I and III [59], suggesting that mohawk plays a role in mechanoregulation. Partial transections of rat Achilles tendons repaired with scaffolds seeded with

bone marrow-derived MSCs had increased expression of mohawk, Col I, tenascin C, and tenomodulin, compared to defects repaired with a cell-free scaffold, suggesting that mohawk is expressed in MSCs during repair [58]. Another *in vivo* injury model repaired full transections of adult mouse Achilles tendon with cell sheets composed of mohawk overexpressing mouse MSCs [57]. After 4 weeks of healing, tendons repaired with mohawk-overexpressing cell sheets had collagen fibrils with increased diameter and a visible crimp pattern, and increased stiffness, elastic modulus, maximum force and stress, compared to repairs using cell sheets that contained wild type MSCs [57]. Overall, mohawk expression appeared to enhance tendon healing. As mohawk expression is suppressed in human tendinopathy [117], interventions regulating mohawk expression may have potential for preventing and treating tendon injuries.

GDF-5 has also been explored in tendon healing. GDF-5-null 8 week old mice with an induced Achilles tendon injury lagged 5 to 9 days behind wild-type mice in attaining peak values for normalized DNA, GAG, and hydroxyproline content [162]. Compared to wild-type controls, tendons of GDF-5-null mice had increased collagen fibril disorganization and adipose cells, and reduced collagen fibril area fraction and orientation [162]. However, despite the initial delay, at 12 weeks both groups had similar structural properties, suggesting that other factors may be able to promote healing in the absence of GDF-5 [162]. Redundancy and overlap in many signaling pathways are a persistent challenge in understanding the biochemical factors in tendon injury, but GDF-5 may regulate early tendon healing.

Decorin and biglycan, small leucine rich proteoglycans, have been implicated in the mechanical properties and aging of tendon [163, 164], and have been investigated in the response to injury. Biglycan-null and decorin-null mice were subjected to a full thickness, partial width patellar tendon injury at P120 [165]. At 3- and 6-weeks post-injury, all injured tendons contained smaller diameter collagen fibrils, compared to uninjured controls, but biglycan-null tendons had fewer of the largest diameter fibrils. Furthermore, decorin-null and biglycan-null tendons had decreased cell density, and altered cell shape and collagen alignment following injury [165]. Overall, this model suggested that early healing is influenced by biglycan, while healing 6 weeks post-injury is impaired in the absence of decorin. In a follow-up study, the same injury model was evaluated in P270 decorin-null and biglycan-null mice to determine impacts of age on patellar tendon healing [166]. At 3 weeks post-injury, tendon healing was delayed in both biglycan-null and decorin-null mice, compared to wild-type control tendons that had a higher dynamic modulus [166]. These findings contrast with injury at P120, where biglycan-null mice were deficient in healing at 3 weeks post-injury, while decorin-null mice healed more poorly at 6 weeks post-injury [165]. Together, these models show that

decorin and biglycan impact tendon healing differently depending on age, which highlights age as an important consideration in injury models.

Models of Chronic Injury

The models discussed above have featured mainly acute injuries. Chronic tendon injuries are challenging to develop in models due to the multitude of contributing and unknown factors and the long timescales associated with pathologies. Nevertheless, chronic injury models have been developed by altering mechanical loading or biochemical factors. An *in vivo* model used 10-week-old scleraxis-GFP mice to compare the chronic loss and gradual return of mechanical loading through botulinum toxin A (Botox), to an acute loss of mechanical loading (transection) [167]. At 3 days after Achilles tendon transection, 70% fewer tendon cells remained in the injury site (cell death was mainly via apoptosis), and scleraxis expression was irreversibly lost in most remaining cells [167]. However, when tensile loading was reversibly lost (via Botox) and gradually restored, there was still apoptosis, but a larger proportion of remaining tendon cells expressed scleraxis [167]. A TGFβ1 receptor inhibitor (SD208) prevented massive tendon cell death in transected tendons, suggesting loss of tension by transection resulted in TGFβ1 signaling that induced apoptosis. In the same study, when Achilles tendon cells were isolated and cultured *in vitro*, scleraxis expression decreased, but fluid flow-induced shear force restored scleraxis expression [167]. These *in vivo* and *in vitro* models showed that both chronic and acute loss of loading impact scleraxis expression and cell viability.

Another animal model evaluated healing of chronic and acute rotator cuff injuries [168]. Rat supraspinatus and infraspinatus tendons were transected and then surgically repaired after a delay of 8 or 16 weeks for the chronic case, or repaired immediately for the acute case. Compared to tendons injured and repaired immediately, tendons repaired after 8 weeks showed reduced toughness, elastic modulus, and stiffness when assessed at 4 weeks after reparative surgery [168]. Scar tissue formation and tendon retraction made surgery difficult in the delayed repair cases. Rats were not immobilized following injury, which may have led to larger tears and worse outcomes in the chronic injury groups [168]. This model showed the direct impact of a chronic versus an acute injury.

TGFβ1 is found in injured tendon, and may initiate inflammation via the hypoxia-inducible factor (HIF)1α pathway [169, 170]. Another chronic tendinopathy model was developed by injecting human TGFβ1 in adult mouse Achilles tendons [171]. This TGFβ1-injection model of tendinopathy also explored the role of glucose metabolism in tendon injury in both wild type and Adamts5$^{-/-}$ (TS5KO) knockout mice [171]. The production of lactate from glucose breakdown during hypoxia or

normoxia is implicated in chronic tendinopathy and may be a metabolic marker of tendon disease [172]. TS5KO mice have reduced or absent osteoarthritis following surgical joint injuries, since they lack the inflammatory aggrecanase ADAMTS5, and have a diminished response to inflammatory mediators such as TGFβ1. TGFβ1 injections in Achilles tendons *in vivo* and *ex vivo* upregulated several HIF1α, angiogenesis, and glycolytic metabolism associated genes in wild-type mice, but not in TS5KO mice. TGFβ1 injections activated the Warburg pathway, which generates lactate from glucose under normoxia rather than just hypoxia, inhibits mitochondrial energy production, and contributes to tendinopathy [171]. Taken together, this model showed that TGFβ1-induced glycolytic reprogramming contributes to pathogenic responses in tendons. Therapies aimed at blocking this metabolic shift may have clinical potential.

Computational models of tendon pathology

Computational models of tendon pathology have been used for assessing the causes, onset, and progression of tendon damage at both the cell and bulk tissue levels. Computational models provide insights that are otherwise difficult to obtain in an experimental setting, such as stress distributions in tendon. To understand stress distributions associated with injury, 2D finite element analysis (FEA) has been used [173, 174]. FEA was used to model stress concentrations in partial-thickness defects in the rotator cuff, and highlighted the importance of limiting mechanical loading to prevent worsening of partial tears [173]. Another 2D FEA model predicted locations of increased strain and isolated tendon fascicle damage in "jumpers knee," a common patellar tendon injury with previously unknown etiology [174]. Evaluation of the model using cadaveric patella-patellar tendon-tibia samples showed that the predicted loading conditions with the highest local strain induced tendon fascicle disruption in 3 of the 5 samples, at the anatomical location of reported pain [174]. This FEA model was later used to assess infrapatellar straps, a device used to reduce patellar tendon pain, and showed that strain was effectively decreased by the strap [175]. FEA models can be useful in assessing forces on tendon, and evaluating invasive and non-invasive interventions, but impacts on cell behavior cannot be easily integrated.

Injury alters the cellular, biochemical and mechanical characteristics of tendon. These changes can be challenging to express mathematically, but several tendon injury models are based on Hill equations, which are commonly used to model cellular responses, particularly secretion or degradation of molecules or ligands [176, 177]. A three-component Hill-type equation model was used to incorporate mechanical and strain-dependent proteolytic collagen fiber damage in a human

Achilles-soleus tendon unit [178]. The model predicted that proteolytic damage would result in collagen fiber shortening, while mechanical damage would result in overall fiber lengthening [178], thus showing that collagen fiber damage and resulting length after healing is modulated differently in overuse versus inflammation injuries. Predicting how collagen is altered by various damage and repair mechanisms will help guide treatments and prevent re-injury during rehabilitation.

ECM and inflammatory protein secretion by tendon cells has been modeled using a modified Hill equation [179]. Secretion profiles of IL-1β, MMP-1, Col I, and ADAMTS5 were predicted in response to tensile strain magnitude. A low (4%) and high (10%) strain applied to the tendon model both resulted in a damage response. A low tissue strain resulted in cell-level strain that was too low to elicit a cell response (e.g., underloading), and at high tissue strain (e.g., overloading), the collagen fibers ruptured and could no longer transfer localized strain to the cells, leading to ECM protein secretion profiles similar to the low strain condition [179]. Therefore, both low and high intensity loading increased inflammatory markers IL-1β and MMP-1, and decreased Col I. Based on these predicted cell expression profiles, quantitative thresholds for tendon mechanical under-stimulation (e.g., underuse) or overstimulation (e.g., overuse) were developed (Figure 2.4). Predicting tendon cell responses to various mechanical loads can guide therapies for promoting tendon homeostasis.

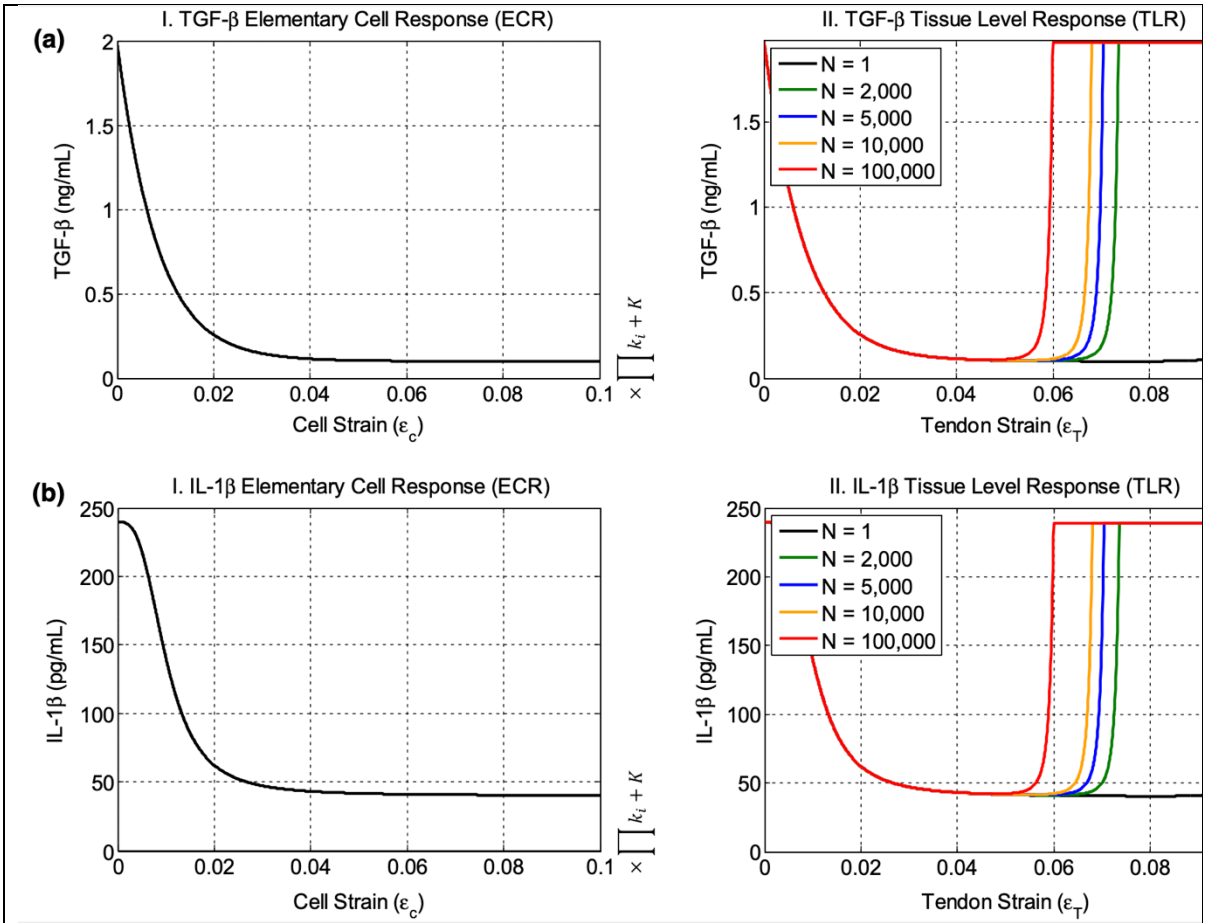


Figure 2.4 Computational modeling of cell- and tissue-level secretion profiles for inflammatory mediators in response to strain.

TGFβ1 (a) and IL-1β (b) profiles were predicted by a Hill-equation model for individual cells, the elementary cell response (ECR), and for cells in the whole tendon, tissue-level response (TLR). In the TLR, the secretion profile is U-shaped, as both low and high strain lead to a simulated "unloading" response of the tissue. Low tendon strain is "underuse" and high tendon strain leads to collagen fiber rupture and unloading of the cells. Figure reprinted with permission by Springer-Verlag Berlin Heidelberg from Mehdizadeh et al 2017 [179].

A regression model was developed to assess the mechanical properties of developing and healing Achilles tendons in mice with injuries induced at P7 or P21, and with 3 or 10 days of healing [180]. Proteoglycans were found to predict tendon elastic modulus during early healing, but not during later healing or during normal development (early or late) [180]. While multiple independent parameters predicted stress relaxation during normal development, only biglycan and collagen fibril diameter predicted the percent relaxation in the tendon during early healing [180]. Using regression analysis, it may be possible to predict outcomes based on specific measurable factors. In a different study, healing of a supraspinatus tendon injury with mechanical loading was evaluated using an agent-based computational model. The model predicted that collagen content would increase steadily

with increasing load, whereas collagen alignment would peak at an intermediate strain, and then decline at higher strain [181]. Peak collagen alignment occurred at a slightly lower strain level than peak collagen content. Notably, collagen deposition peaked after the damage threshold, suggesting that sub-damage threshold loading may be used therapeutically to optimize both collagen deposition and alignment. Tendon mechanical function after injury is largely determined by its underlying collagen structure, which may depend on the degree of mechanical loading experienced during healing, therefore understanding strain magnitude-dependent mechanisms of collagen remodeling is needed for developing therapies [181].

Various animal models of Achilles tendon healing have produced conflicting results, possibly due to differences in mechanical loading during healing. A multiscale computational model of rat Achilles tendon healing was developed to address this experimental variability and incorporate the loading environment to study impacts on cell behavior, collagen deposition, and scar formation [182]. The model generated a single set of cellular response curves that were able to explain observations of tendon behavior in several experimental studies with otherwise differing results [182]. The model successfully predicted cell-level behaviors from tissue-level strains, highlighting disparities in strains between cells and bulk tissues as a factor contributing to contradictory experimental results, and offering the possibility of reconciling these variances.

Empirical models have been developed to assess the progression of mechanical damage with injury and aging [183, 184]. In these models, damaged tendons are considered to be experiencing a lower strain than what is actually applied [183]. Based on this concept, mouse patellar tendons were evaluated as a function of age at P150, P300, and P570, and compared to P120 patellar tendons at 3- and 6-weeks after a full thickness, partial width injury [184]. Tendons were mechanically evaluated with a 10-cycle frequency sweep of 0.125% amplitude sinusoidal strain at frequencies of 0.01, 0.1, 1, 5, and 10 Hz superimposed onto a baseline offset strain (4, 6, or 8%). The equilibrium stress, dynamic modulus, and loss tangent were measured at each frequency and strain level, and an empirical model was used to develop a single damage parameter for each tendon group. The damage parameter was able to predict dynamic modulus and loss tangent for each tendon across frequency (0.01–10 Hz) and strain (4–8%). This model showed that the effects of aging and injury on patellar tendon mechanical properties could be described by the same damage model [184]. A similar strain-based empirical damage model was developed to examine impacts of decorin and biglycan knockout on Achilles tendons of P150, P300, and P570 mice [185]. The empirical damage model predicted the changes in dynamic modulus that resulted from the null phenotypes, and identified a correlation between

measured and predicted dynamic modulus based on genotypes and ages [185]. Overall, these models are useful tools for understanding and predicting tendon mechanics with age, genotype, and injury.

Alongside their considerable potential for providing insight into tendon injury and healing, computational models have inherent limitations. Most simplify multiple parameters of tendon responses to load and damage. Baseline values for tendon material properties, such as elastic modulus, are obtained from previous studies, but elastic modulus varies based on tendon and species [186]. Finally, as not all proteins involved in injury and healing are known, all models necessarily exclude some cellular responses to tendon injury. Nevertheless, computational models are proving useful as research tools and predictors of tendon responses to many physiological conditions. They will undoubtedly improve further as experimental studies continue to uncover mechanisms that regulate tendon development, injury and healing.

Conclusions and Future Directions

The high cell density, low collagen content, growth factors, and mechanical environment of embryonic tendon development have been incorporated into engineered model systems. Embryonic tendon becomes mechanically stronger, but differentiation and tissue formation continue postnatally, before maturation into adult tendon. Assessing the changes that postnatal tendons undergo through *in vitro* models remains an ongoing challenge. Furthermore, many biochemical and mechanical cues inevitably originate from surrounding tissues. The impacts of concurrent adjacent tissue formation (muscle and bone) on tenogenesis need to be explored, as simulating these tissues *in vitro* may facilitate more realistic tendon models. Few multi-tissue developmental models exist, but one study showed that 3D *in vitro* skeletal muscle-tendon constructs developed ultrastructural characteristics resembling *in vivo* muscle-tendon interfaces, when skeletal muscle constructs where co-cultured with self-organizing tendon constructs and explanted fetal rat tail tendon [187]. Such constructs can be supplemented with biochemical or mechanical factors to better mimic the developmental process. Furthermore, examining development of the musculoskeletal system as a whole will aid in understanding how tendon formation is regulated in coordination with adjacent tissues including muscle and bone.

An additional challenge with developmental models is that recreating the spatiotemporal sequence of embryonic or postnatal biochemical signaling alone may be inadequate for developing functional tissue [188]. Several models examine specific tenogenic factors in isolation, an understandable limitation given the complexity of tendon development. Future models will need to

assess the interplay between cell-level cues, mechanical loading, development of mechanical properties, and the biochemical factors involved in tendon formation.

In vivo, in vitro, ex vivo, and computational models have explored the impacts of mechanical loading and various biochemical factors on adult tendon injuries and healing. Few models have investigated human derived cells or isolated human tissues, mainly due to the understandable challenge of procuring tissues and working with human subjects. Recent studies in other tissue systems have developed humanized models (e.g. decellularized animal tissues seeded with human cells or humanized animal models) [189, 190], but this has not been as well explored in tendon. Advancing models of chronic tendon injuries are needed for exploring the factors that regulate tendon pathologies in human tissues and cells. Taken together, the developmental and injury models reviewed here have greatly improved our understanding of the numerous cellular, biochemical and mechanical factors that regulate tendon formation and health. Tendon models will ultimately improve clinical outcomes by offering novel insights into the mechanisms of how tendons develop and how they respond to injury and treatment.

Chapter 3: Cell-Cell Junctions in Developing and Adult Tendons

Theodossiou SK, Murray, JB, Schiele NR. Cell-Cell Junctions in Developing and Adult Tendon. *Tissue Barriers* 2019: 1695491.

Abstract

Tendons connect muscles to bones to transfer the forces necessary for movement. Cell-cell junction proteins, cadherins and connexins, may play a role in tendon development and injury. In this review, we begin by highlighting current understanding of how cell-cell junctions may regulate embryonic tendon development and differentiation. We then examine cell-cell junctions in postnatal tendon, before summarizing the role of cadherins and connexins in adult tendons. More information exists regarding the role of cell-cell junctions in the formation and homeostasis of other musculoskeletal tissues, namely cartilage and bone. Therefore, to inform future tendon studies, we include a brief survey of cadherins and connexins in chondrogenesis and osteogenesis, and summarize how cell-cell junctions are involved in some musculoskeletal tissue pathologies. An enhanced understanding of how cell-cell junctions participate in tendon development, maintenance, and disease will benefit future regenerative strategies.

Introduction

Tendons are musculoskeletal tissues that transfer mechanical forces from muscles to bones and are vital for skeletal movement. A major clinical challenge is the limited healing ability of tendon. If ruptured, tendon healing is characterized by the formation of scar tissue [26, 191] and inferior mechanical properties, compared to uninjured tissues [3]. Even with surgical repair, re-rupture rates range from 3.6% to 94% depending on the tendon, size of the tear, age and other factors [192-194]. In addition, tendon injury rates are climbing, with a 10-fold increase in the incidence of Achilles tendon ruptures from 1979 to 2011 [14]. The limitations of existing treatment options emphasize the need for tissue engineering and regenerative strategies to improve tendon healing and repair.

Regenerative tissue engineering approaches are challenged by a limited understanding of how tendon cells respond to mechanical and biochemical signals to enable initial tenogenic differentiation during embryonic development, and the formation and maintenance of tendon's highly organized extracellular matrix (ECM) [195]. The ECM in tendon is composed primarily of collagen type (Col) I that accounts for nearly 65-80% of the dry mass [196]. The collagen plays a critical role in

transmitting tensile forces and is hierarchically arranged, with collagen fibrils (nm scale) bundled into collagen fibers and fascicles (mm scale) [196]. In the adult tendon, tendon cells reside within the fascicles and interfascicular membranes, and these resident cells are thought to play a role in maintaining the collagen matrix [112] and the tendon length by cell-mediated contraction [146]. Though mature tendon is typically considered to have a relatively sparse distribution of cells, tendon cells are known to possess direct cell-to-cell connections [197].

Developing embryonic and postnatal tendons are characterized by a dense cell network [37, 198] with adjacent cells in direct contact [36]. These direct cell-to-cell adhesions in tendon cells occur through cadherins and gap junctions. For tendon, cadherins may be particularly relevant, as they are known mechanotransducers [199], and mechanical stimuli impact many aspects of tendon formation, injury, and homeostasis [4, 5, 9, 84, 91, 93-95, 106, 200-203]. This review examines the cadherins and gap junctions identified in developing and mature tendon, and discusses what role these cell-cell junction proteins might be playing in the formation, maintenance, and mechanoregulation of tendon. Like tendon, cartilage and bone are musculoskeletal tissues of mesenchymal lineage, but the cell-cell junctions in these tissues have been more extensively studied. Therefore, to inform future tendon studies, we also include a brief review of cadherins and gap junctions in chondrogenesis and osteogenesis, and in some musculoskeletal tissue pathologies. Understanding the specific role of cell-cell junctions in tendon cells and their impacts on cellular responses may ultimately advance strategies to prevent tendon injury and improve regenerative medicine and tissue engineering.

Cell-Cell Junctions in Developing Tendon

Embryonic tendon arises from a condensation of mesenchymal cells that are tightly packed and are in direct cell-to-cell contact [36]. These embryonic tendon cells are distinguishable from other musculoskeletal cells of mesenchymal lineage (e.g., cartilage and bone cells) by their expression of scleraxis [28], mohawk [34], and tenomodulin [32]. The transcription factor scleraxis is a regulator and early marker of tenogenesis [28-31], as is the transcription factor mohawk [34]. Scleraxis regulates tenomodulin, a late stage tendon marker [32, 33]. Tendon cells constitute a significant portion of the embryonic tendon structure [40]. The amount of DNA (as a measure of cell density) present in the calcaneus tendons of embryonic chickens from Hamburger-Hamilton stages (HH) 28 to 43 (e.g., embryonic day (E) 5.5 to 18) accounted for between 3 and 9% of the tendon dry mass, whereas hydroxyproline (a measure of collagen) accounted for less than 5% of the dry mass at HH43 [40].

The organization of embryonic tendon cells appears unique, with highly ordered cells tightly packed and aligned to the long-axis of the tendon, as observed in E13 chick metatarsal tendon, and E15.5 mouse tail tendon [42]. A different study found that cells in chick calcaneal tendon from HH34 to HH37 possessed a highly aligned and well-organized actin cytoskeleton network, with actin filaments that appeared continuous between cells (Figure 3.1 A) [37]. When the actin cytoskeleton of the embryonic tendon cells was disrupted with blebbistatin, a small molecule inhibitor of non-muscle myosin contraction, the elastic modulus of the tendon decreased significantly, suggesting that the cells contribute to the embryonic tendon mechanical properties [37]. Overall, embryonic tendon is highly cellular, with a well-organized and apparently interconnected network of cells. While inherent differences between avian and mammalian tendon development may exist, embryonic tendons across species as diverse as chick, mouse, and horse appear to share the characteristics of high cellularity, alignment and direct cell-cell contact [42, 204]. Given this high cellularity and cell alignment, cell-cell junction proteins are likely candidates as regulators of embryonic tendon cell organization. Therefore, we discuss the cell-cell junction proteins (mainly cadherins and connexins) that have been identified in developing tendons and explore their potential roles in tendon development.

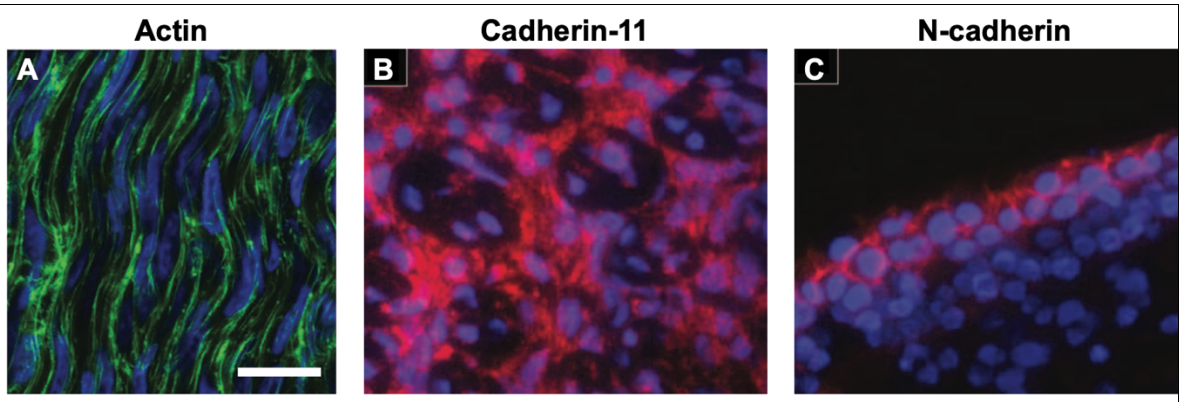


Figure 3.1 Embryonic tendons possess an organized actin cytoskeleton network, as well as cadherin-11 and N-cadherin cell-cell junctions.

(A) The high cell density and an organized actin cytoskeleton network is visible in the midsubstance of E11 chick calcaneal tendons. Actin filaments (green) in embryonic tendon appear to form a continuous network between adjacent cells. (B) Cadherin-11 (red) and (C) N-cadherin (red) are present in E13 chick metatarsal tendons, but N-cadherin is localized to the exterior of the midsubstance of the tendon, while cadherin-11 appears in the interior of the midsubstance. Cell nuclei are labeled with blue. Figure 3.1 A used with publisher's permission from Schiele et al 2015 [37]. Scale bar =10 μm. Figure 3.1 B and 3.1 C adapted with publisher's permission from Richardson et al 2007 [42].

Cadherins in Embryonic Tendon

To identify possible cell-cell junction proteins, a gene microarray analysis of E13 chick metatarsal tendon was conducted [42]. Gene expression for cadherin-11, N-cadherin, R-cadherin, and connexin-32 and -43 was identified, but expression levels were highest for cadherin-11 [42]. The presence of cadherin-11 in embryonic tendon was also found using RT-PCR and immunofluorescence staining. Specifically, cadherin-11 was present in the fascicles and in the interfascicular space (Figure 3.1 B), as well as in the cell-cell contacts made by the embryonic tendon cells that had been isolated and cultured *in vitro* [42]. N-cadherin was also found by immunofluorescence, but appeared to be produced mainly by tendon cells on the tendon surface and not in the mid-substance of the tendon (Figure 3.1 C) [42]. When cadherin-11 expression was knocked down using siRNA, the embryonic tendon cells appeared to have disrupted cell-cell contacts and collagen fibril organization [42]. This study suggests that cadherin-11 may regulate tendon cell condensation and may even play a role in alignment of collagen fibrils. Cadherin-11 may also contribute to limb patterning, since cadherin-11 expression was shown by *in situ* hybridization to be restricted to the distal, but not proximal, regions of developing mouse limbs at E9.5 and E13.5 [205]. This same study demonstrated that mouse embryonic fibroblasts transfected with cadherin-11 cDNA adhered to other cadherin-11-transfected cells, but did not co-aggregate with cells transfected to express N-, E-, P-, or R-cadherin [205]. The proximal or distal restriction of cadherin expression and the timing of expression of multiple cadherins relative to cell condensation may ensure correct tissue patterning during development.

N-cadherin is a regulator of cell adhesion and connective tissue morphogenesis that has also been explored in patterning of the musculoskeletal tissues in the limbs. N-cadherin-null mice do not survive *in utero* unless rescued with transgenic expression of a cardiac cadherin [206]. While non-rescued N-cadherin-null mice survive to form forelimb buds at E9.5, they are not viable by E11-E12 due to cardiac malformations, and further limb development cannot be assessed [206]. To address this limitation, a follow-up study cultured forelimbs from rescued E10.5 N-cadherin-null mice *ex vivo* for 7 days (d), and found that the limbs developed and did not differ significantly from wild-type forelimbs in overall morphology, size, and cellular condensation of chondrogenic precursors [207]. Although N-cadherin expression was absent in the mutant limbs, expression of cadherin-11 was not affected, indicating that cadherin-11 and other cadherins may drive limb development in the absence of N-cadherin [207].

The cardiac, neural, and connective tissue malformations in N-cadherin-null mice are likely due to the role of N-cadherin in cell adhesion. Cell adhesion is necessary for patterning in early development, and is controlled upstream of the cadherins by T-box transcription factors [208]. In

mouse E16.5 forelimbs with deletion of the T-box transcription factor (Tbx)5, and E15.5 hindlimbs with deletion of Tbx4, muscle patterning was disrupted, and ectopic splitting of muscles of the zeugopod, the region of the developing limb encompassing the forearm but excluding the digits, was observed [208]. In the forearms of E15.5 Scleraxis-Green Fluorescent Protein (Scx-GFP)-expressing mice, Tbx5 deletion led to changes in tendon morphology. Specifically, there were fewer tendon fibers present, fibers were thinner than normal, and some fibers had fused with each other [208]. Despite the changes observed in the tendons, the muscles still made myotendinous attachments, and tendons developed entheses (tendon-to-bone attachments) on the forming skeleton, indicating that crosstalk between the developing muscles, bones and tendons was still intact [208]. The same study also found that N-cadherin expression was significantly lower in Tbx5 null mice [208], as was expression of β-catenin, a protein that couples with cadherins to facilitate cytoplasmic anchoring to the actin cytoskeleton and participates in both cell adhesion and signaling via the wingless/integrated (Wnt)/β-catenin pathway [209]. Although N-cadherin and β-catenin expression was reduced, expression of cadherin-11 and Tcf4, a downstream Wnt target, were unaffected, suggesting that Tbx5 deletion specifically affects N-cadherin and β-catenin, but does not globally disrupt cadherins or Wnt signaling [208]. These findings suggest that N-cadherin and regulation by Tbx5 are necessary for early embryonic tendon development and patterning, but more research is needed to understand how N-cadherin is participating in early tendon formation. In a different study, differentiation of dermal fibroblasts toward a myofibroblast phenotype was characterized by a transition from N-cadherin to cadherin-11 expression [210]. This process may occur when stronger bonds are needed between cells, as cadherin-11 bonds were found to have twice the strength as N-cadherin bonds [211]. Therefore, it is possible that tenogenically differentiating embryonic tendon cells express specific cadherins that have different bond strengths during specific developmental stages, though this will need further study. Taken together, both N-cadherin and cadherin-11 are found in embryonic tendons and appear to be involved in cell condensation and early tissue formation and patterning. A deeper understanding of how these cadherins contribute to tenogenic differentiation and ultimately functional tendon formation will be immensely valuable.

Other cadherins may also be regulating tendon development. The protocadherin Fat-1 is expressed in tissues of mesenchymal origin during early embryonic development [45]. Fat-1 controls cell proliferation during early musculoskeletal tissue development and cell condensation [212], and has been shown to regulate both transforming growth factor beta (TGFβ) [213] and Wnt/β-catenin signaling [214]. Genetic ablation and *in situ* hybridization in E12.5 mice showed Fat-1 is required in mesenchyme-derived connective tissue formation [212]. Conditional Fat-1 knockouts displayed abnormal morphology of the cutaneous maximus muscle and innervating motor neurons [212].

Muscle formation is needed for subsequent tendon development [11], but Fat-1 expression persisted in Pax3 $^{cre/cre}$ knockout mice, which lack skeletal muscle cells, suggesting that Fat-1 expression may be driven by mesenchymal or connective tissue cells, rather than muscle cells [212]. Effects of Fat-1 disruption were further shown to vary by cell and tissue type: extensive muscle-shape patterning defects resulting from Fat-1 ablation were seen in mouse mesenchymal cells, but not motor neuron cells [212]. *In situ* hybridization showed overlapping Fat-1 and scleraxis expression, and Fat-1 was required for the developing connective tissue precursors to interact with the cutaneous maximus muscle progenitors that were concurrently forming [212]. As tendon progenitors arise from embryonic mesenchyme [215], correct patterning of the mesenchyme and the contribution of Fat-1 to this patterning may be important for tendon development. Furthermore, the reciprocal signals exchanged between different progenitor populations (muscle, tendon, cartilage, bone) during limb patterning are essential for the correct development of the limb and associated connective tissues. Several distinct subtypes of connective tissue are derived from progenitors influenced by Fat-1 expression, including tendons, making Fat-1 a useful target for further research in tendon development.

Fat-1 expression may also contribute to proximodistal orientation during limb patterning. In E9.5-E13.5 mouse limb buds, proximal restriction of Fat-1, coupled with distal expression of cadherin-11, may ensure correct patterning by regionally inhibiting Wnt signaling [205]. In HH16-HH24 chick forelimb buds, whole mount *in situ* hybridization showed that Fat-1 expression was restricted to tendon primordia of proximal regions, but expression extended to the proximal and intermediate regions of the forelimb at HH27, and was detectable in mesenchymal cells between the developing digits at HH28 [216]. In contrast, hindlimbs had extended stretches of expression into the interdigit mesenchyme at HH27. Fat-1 was expressed diffusely in regions of developing tendon cells, but only proximally in developing chondrocytes, suggesting that expression of Fat-1 may be grouping specific cells together to form and maintain digit patterning and distinct musculoskeletal connective tissues [216].

A few potential regulators of cadherins during differentiation have emerged. Decorin, a proteoglycan involved in cell signaling [217], collagen fibrillogenesis [218], and aging of tendon [166], may be important for the cell aggregation phase of musculoskeletal tissue development, before various cell precursors have differentiated into tendon, muscle, cartilage, or bone. Micromass cultures of limb mesodermal cell precursors from HH25 chicks showed significant upregulation of Sox9 and cadherin-11, but no changes in scleraxis expression when cells were treated with human decorin for 1 d, and gene expression was analyzed at 2 d [219]. Cadherin-4, -7, and -13, and N-cadherin, were

unaffected. Interestingly, there was no upregulation of Sox9, cadherin-11, and scleraxis when decorin was added after 4 d of culture and gene expression analyzed at 5 d [219]. While Sox9 is considered a chondrogenic marker, it promotes the common cell aggregation step necessary for the cartilage/tendon/ligament specification that takes place in early embryonic development [220]. In this case, the upregulation of Sox9 may be due to decorin influencing the cell aggregation and condensation step that precedes differentiation [219]. The same study showed via *in situ* hybridization of whole limb mounts from HH20-31 chicks that the expression patterns for decorin, cadherin-11, and TGFβ2 overlapped at HH30 in the joint and tendon blastemas of the embryonic digits [219]. Addition of TGFβ2 to HH22 limb bud explants upregulated decorin, but increased decorin expression was lost when TGFβ2 signaling was blocked using the Smad2 inhibitor SB431542 [219]. Finally, in decorin-silenced mesenchymal cells, expression of Sox9 was significantly decreased, and expression of scleraxis was significantly increased, compared to control cells with functional decorin, though cadherin-11 expression was not examined [219]. Collectively, these results highlight overlapping expression of cadherin-11, TGFβ2, and decorin during differentiation of musculoskeletal tissues. The increase in decorin expression seen with the addition of TGFβ2 suggests that decorin and cadherin-11 are downstream targets of TGFβ2 signaling. Lack of decorin may lead to tendon pathologies via cadherin reduction. Decreased decorin expression has been proposed as a cause of abnormal collagen formation and tendon hypoplasia in mohawk-null mice [34]. The observed disruptions of the collagen matrix and gross morphology may be due to disruption of cadherin-11-mediated cell aggregation driven by the decorin deficiency. More research is needed to understand how decorin responds to TGFβ2 signaling and mediates expression of cadherin-11 and, potentially, other cadherins in developing tendons.

The cytoplasmic anchor of the cadherins, β-catenin, may also be involved in tendon development. β-catenin facilitates the formation of cell-cell junctions by modulating actin cytoskeleton-cadherin coupling through association with α-catenin and vinculin [221, 222]. β-catenin was found to be necessary for this process, as the α-catenin/vinculin complex is only capable of binding the actin cytoskeleton directly, and thus requires β-catenin to form linkages with cellular cadherins and the actin cytoskeleton [221]. β-catenin regulates cell growth through the Wnt pathway, and is constitutively expressed in the cytosol, where it is regulated via Axin, casein kinase (Ck)1, and glycogen synthase kinase (GSK)3β [223, 224]. In E13 chick metatarsal tendon, relatively high levels of β-catenin gene expression were identified by microarray analysis, along with cadherin-11 [42]. β-catenin upregulation was observed in tendon cells adjacent to a 14-gauge needle puncture injury in the Achilles tendon of 6-week old rats [225]. Interestingly, tendon cells had decreased gene expression for scleraxis, mohawk, and tenomodulin when Wnt/β-catenin signaling was chemically

activated during *in vitro* cell culture [225]. The same study found that activation of Wnt/β-catenin signaling decreased cellular levels of Smad2 and 3, and that Wnt/β-catenin activation suppressed tenogenic genes [225]. However, in a different study, equine bone marrow-derived mesenchymal stem cells (MSCs) encapsulated in collagen gels *in vitro* upregulated tenomodulin and decorin gene expression with β-catenin activation [226]. Taken together, β-catenin interacts with cadherins and regulates cell behavior, and may be both pro- and anti-tenogenic, depending on specific culture conditions or cell phenotypes. Future studies are needed to investigate how β-catenin may be regulating cadherins in tendon and how β-catenin impacts tenogenic differentiation and tendon formation.

Connexins in Embryonic Tendon

In addition to cadherins, gap junction proteins (connexins) are expressed in embryonic tendon [42]. The involvement of connexin-43 and another gap junction protein, connexin-32, in the differentiation of multiple stem cell lineages, and particularly in bone development and homeostasis, has been highlighted in recent reviews [227, 228]. Connexin-32 and -43 have been found in murine [197], rat [229], avian [42, 230], equine [204], and ovine tendons [231]. However, there is limited information on the role of connexins in tendon development. While both connexin-32 and -43 are expressed in tendon, their localization is different. In adult rat digital flexor tendons, connexin-43 has been found in the tips of cell processes and between cell bodies, while connexin-32 appears to be confined between the bodies of adjacent cells [197]. Though the functional significance of this pattern is not fully understood, it is possible that connexin-32 and connexin-43 enable a differential response to mechanical loading [230]. As embryonic tendon responds to mechanical stimuli [9], gap junction-mediated mechanotransduction may be involved, but the effects of blocking this communication during embryonic development have not been explored.

To further elucidate the role of gap junctions in developing tendons, connexin-32 and -43 expression during development has been examined by several studies. Using immunofluorescence staining, one study identified connexin-32 and -43 in the superficial digital flexor tendon (SDFT) and common digital extensor tendon (CDET) of fetal and mature horses [204]. Expression of connexin-32 and -43 peaked in developing tendons, and decreased in the first 6 months of life. Fetal tendons had significantly larger areas of labeled connexin-32 and -43, compared to tendons from young (1-6 months), young adult (2-7 years), and old (18-33 years) horses (Figure 3.2). There were no differences in expression of either connexin between the SDFT and CDET at any age [204]. While

both connexins were present throughout development, their patterns of expression and localization differed between ages, with more widely distributed expression and overall larger expression areas observed in fetal tendons compared to all older stages [204].

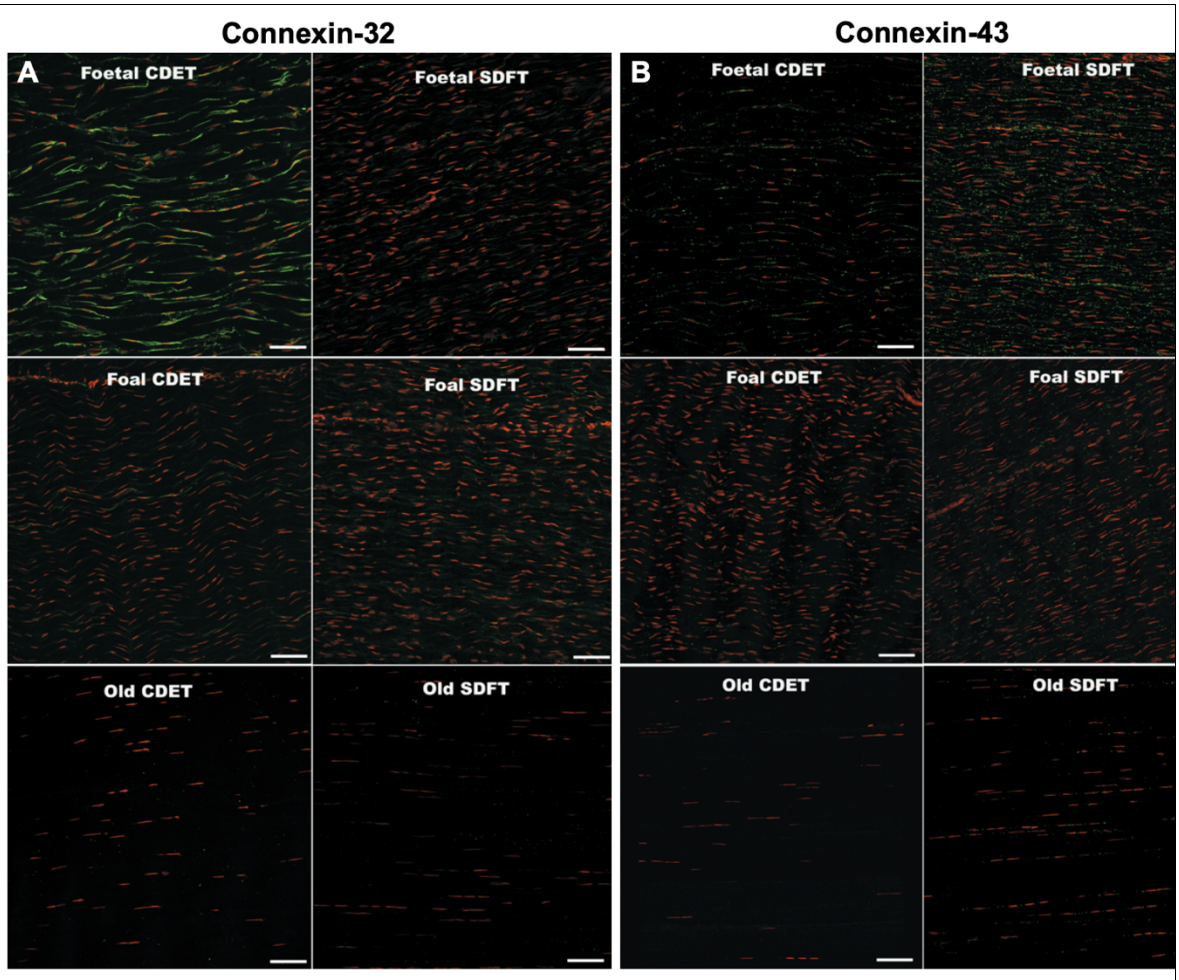


Figure 3.2 Longitudinal cryosections from equine CDET and SDFT tendons labeled for connexin-32 and connexin-43.

(A) Connexin-32 (green) labeled in tendons from fetal, foal, and mature horses. (B) Connexin-43 (green) labeled in tendons from fetal, foal, and mature horses. Cell nuclei are labeled with red. Scale bar = 80 µm. Figure used with publisher's permission from Stanley et al 2007 [204].

Another study examined the expression of connexin-32 and -43 during ovine tendon development. Ovine calcaneal tendons at two different fetal stages (mid-gestation and late gestation, when the tendons were 14 cm and 40 cm long, respectively) had a more developed endotenon, as well as differences in cell shape [231]. Cells from mid-fetal tendons had a rounded morphology, which became fusiform and regionally aligned in late-gestation cells. Adult cells were elongated and

displayed a parallel arrangement along the longitudinal axis of the tendon [231]. Only cells from mid-fetal stage tendons expressed regenerative growth factors and pluripotent stem cell markers [231]. Cellularity was higher in mid-fetal tendons compared to both late-fetal and adult tendons, with increased cell proliferation compared to the two later stages. Connexin-32 and -43 were produced at similar levels during both fetal stages, but decreased significantly in adult tendons. Gene expression revealed significant decreases in Col I, Col III, scleraxis, tenomodulin, thrombospondin 4, osteocalcin, and TGFβ1 between the mid-fetal tendon cells and both the late fetal and adult tendon cells [231]. Taken together, these results highlight a progressive and rapid decrease of tendon marker gene expression throughout development, and show that this decrease begins *in utero* in an ovine model. The decreased production of connexins is noteworthy, since gap junction communication may be needed during development to coordinate the response to mechanical load and other stimuli driving tendon formation. The decrease in connexins with age may reflect a reduced ability to regenerate adult tendon, as embryonic tendon is known to heal scarlessly [191]. Embryonic tendon expresses both connexin-32 and -43, and this expression rapidly decreases following birth. However, it is unknown if changes in connexin expression are consistent across species or tendons (e.g., calcaneus vs digital flexor). It is also unknown how these gap junctions may be regulating tenogenic differentiation.

Connexin-43 expression was found to overlap with growth/differentiation factor (GDF)-5 expression during embryonic mouse limb development [232]. GDF-5 and connexin-43 expression were localized to the condensing digit and long bones of the hindlimb at E12.5, the perichondral regions of the forelimb at E13.5, and to the elbow and hip joint surrounding the femoral head from E12.5 to E14.5 [232]. Colocalized expression of GDF-5 and connexin-43 was also detected in tendons of the hip at E14.5, and around the tendons of each toe at E15.5, indicating that GDF-5 and connexin-43 are both present in embryonic tendon [232]. Together these results highlight that connexin-32 and -43 gap junction proteins are present in embryonic tendon. However, the specific contributions of connexins to embryonic tendon development remain unknown, and future studies correlating connexin expression with functional cellular communication and tenogenesis are needed.

Cell-Cell Junctions in Postnatal Tendon

In the developing mouse, early postnatal tendon appears to resemble embryonic tendon structure, with high cell density and low collagen content [198]. For example, the Achilles tendon of a mouse at postnatal day (P)4 contains less than 3% collagen and by P28 is still only 36% collagen [39]. In an ovine model, tendon cells accounted for 33.4% of the digital flexor tendon volume after 1

week of development, whereas at 40 weeks tendon cells accounted for less than 6% of the volume [38]. This high cell density in postnatal tendon suggests that the tendon cells have the potential to maintain direct cell-to-cell connections. Postnatally, mechanical forces significantly increase from the development of weight-bearing locomotor behavior [233], as do tendon mechanical properties and tenogenesis. Gene expression for scleraxis and tenomodulin was found to peak at P7 in the Achilles tendons of mice, when compared to P1 or later ages [86]. However, relationships between postnatal tendon formation and cell-cell junction proteins have not been extensively investigated. One study conducted a proteomic analysis of P1 mouse tendon cells from the flexor digitorum longus to determine the proteins in the pericellular regions. Potential cell-cell junction proteins were identified and include Fat-4, protocadherin-15, cadherin-13, and catenin alpha-1, a protein involved in linking cadherins to the actin cytoskeleton [234]. Additional studies are needed to understand how these proteins change throughout development and contribute to postnatal tendon formation.

Though few studies have examined cell-cell junctions in the early postnatal stages, connexins have been shown to decrease postnatally in horses. As described above, connexin-32 and -43 were significantly reduced from the fetal to the postnatal (1 to 6 months) stage in horse SDFT and CDET tendons, though these gap junctions were still present at all ages [204]. However, the reduction in connexin-32 and -43 from the fetal to the postnatal (e.g., foal) stage is noteworthy, since tendon development continues after birth and is exposed to increasing mechanical stimuli from the onset of weight-bearing locomotion.

Taken together, tendon cells express gap junction proteins and a range of cadherins during embryonic and postnatal development (Table 1), but more work is needed to identify their specific contributions to tendon formation. Further studies are also needed to identify novel cell-cell junction proteins that may be participating in tendon development, and to elucidate their tenogenic influence. Looking to other developing musculoskeletal tissues may provide potential proteins to explore. However, tendons have so far been found to lack pannexins, the large transmembrane channels that link the cytosol and extracellular matrix, even though they are present in bone and cartilage cells [228]. Based on the overlapping expression of cell-cell junction proteins and various growth factors [219, 232], and the apparent redundancy of signaling pathways previously thought to be necessary for limb development [206], other proteins may be participating in tendon development, and characterizing them will provide new targets for treating tendon injury and disease.

Table 3.1 Cell-cell junctions in developing tendon

Cell-Cell Junction Type		Possible Functions In Developing Tendon	References
Cadherins	N-cadherin	Cellular condensation, cell adhesion, limb patterning	Hasson 2010 [208] Luo 2005 [207] Luo 2001 [206] Richardson 2007 [42]
	R-cadherin	Unknown role in embryonic tendon, but expression identified in E13 chick metatarsal tendon	Kimura 1995 [205] Richardson 2007 [42]
	Cadherin-4	Unknown role in embryonic tendon, but expression identified in mesodermal cell precursors in chick at HH25	Lorda-Diez 2014 [219]

	Cadherin-7	Unknown role in embryonic tendon, but required for cell condensation and migration in chick limb mesenchymal cells	Kim 2009 [267]
	Cadherin-11	Cellular condensation, distinction of cartilage and tendon precursors, limb patterning, collagen fibril alignment	Kimura 1995 [205] Richardson 2007 [42]
	Cadherin-13	Unknown role, but identified in tendon cells at P1	Smith 2012 [234]
	Protocadherin-15	Unknown role, but identified in tendon cells at P1	Smith 2012 [234]
	Fat-1	Cell proliferation, patterning of muscle and tendon precursors, overlapping expression	Helmbacher 2018 [212] Smith 2007 [216]

		domain with scleraxis, limb patterning.	
	Fat-4	Unknown role, but identified in tendon cells at P1	Smith 2012 [234]
Connexins	Connexin-32	Mechanotransduction, stimulation of collagen production	McNeilly 1996 [197] Russo 2015 [231] Stanley 2007 [204] Wagget 2006 [230]
	Connexin-43	Mechanotransduction, inhibition of collagen production, separation of cartilage and tendon precursors, cell condensation, tissue patterning	Coleman 2003a [232] Coleman 2003b [277] McNeilly 1996 [197] Russo 2015 [231] Stanley 2007 [204] Wagget 2006 [230]
Tight Junctions	ZO-1 and Claudin-1	Epithelium surrounding tendons - containment of tendon cells within tendon and prevention of tendinopathic adhesions	Taylor 2011 [256]

Cell-Cell Junctions in Adult Tendons

While not as abundant as embryonic tendon cells, adult tendon cells are found within the tendon fascicles and interfascicular membranes. Adult tendon cells respond to mechanical and biochemical stimuli, and may play a role in tendon homeostasis [112, 146]. Various signaling mechanisms beyond cell-cell junctions may be involved, and these have been summarized in a recent book chapter [235]. Here, we examine the current understanding of cell-cell junction proteins in adult tendon (Table 2), with an emphasis on the response to mechanical load.

Table 3.2 Cell-cell junctions in adult tendon

Cell-Cell Junction Type		Possible Functions In Adult Tendon	References
Cadherins	N-cadherin	Response to mechanical load and growth factors, coupling with α-catenin	Barry 2014 [291] Desai 2013 [292] Keller 2011 [237] Ralphs 2002 [236]
	Cadherin-11	Unknown role in adult tendon, but associated with strong cell adhesions in myofibroblasts	Pittet 2008 [211]
Connexins	Connexin-26	Mechanotransduction; other functions unknown	Maeda 2012 [241]

	Connexin-32	Mechanotransduction, stimulation of collagen production; other functions unknown	Maeda 2012 [241] Ralphs 1998 [229] Wagget 2006 [230]
	Connexin-43	Intercellular communication, movement of small molecules between cells in response to strain, coupling with actin cytoskeleton to facilitate mechanotransduction, tissue maintenance, suppression of collagen production, downregulation of IL-1β during inflammatory response	Banes 1999 [245] Maeda 2012 [241] Maeda 2015 [242] Maeda 2017a [242] Maeda 2017b [244] Ralphs 1998 [229] Wagget 2006 [230] Wall 2007 [235]
Tight Junctions	ZO-1 and Claudin-1	Membrane surrounding tendon; adhesions form following disruption of	Taylor 2011 [256]

		these junctions after tendon injury	

Cadherins in Adult Tendon

Only a few studies have investigated the presence of cadherins in adult tendon cells. Immunofluorescence staining showed that digital flexor tendons from adult chickens possess N-cadherin *in vivo* [236]. N-cadherin appeared to mirror the distribution of actin filaments and vinculin, which form parallel rows along the tendon long axis. In the same study, isolated adult tendon cells grown in culture produced N-cadherin, as observed by immunofluorescence and western blotting. When cells were cyclically strained (75 millistrain, 1 Hz, 8 h/d for 4 d), N-cadherin protein levels increased significantly compared to static controls [236], suggesting that mechanical stimulation may impact the cell-cell adhesions in tendon cells. However, how cadherins may regulate or impact the tendon cell response to loading is unknown.

N-cadherin in adult tendon cells responds to growth factor treatment. Tendon cells isolated from the Achilles tendons of young adult (10-week-old) rats and treated with 1, 10, and 1000 ng/mL GDF-5 for 4 d had significant decreases in N-cadherin gene expression at all GDF-5 concentrations, compared to untreated controls [237]. However, when treated with 100 ng/mL of GDF-5 over 12 d, N-cadherin gene expression was increased at 3, 6, and 9 d compared to untreated controls, though at 12 d gene expression levels were similar to controls [237]. GDF-5 is known to impact tendon formation [238] and healing [162], but N-cadherin has been shown to decrease during tenogenic differentiation of mouse MSCs [13]. These variations suggest that N-cadherin expression may depend on the timing and intensity of GDF-5 signaling, but more studies are needed to assess how N-cadherin responds to growth factors in adult tendon cells.

Connexins in Adult Tendon

Connexins have been identified in adult tendon cells from a range of species [197, 204, 229, 230]. Although connexin levels decrease with increasing age [204, 231], their presence in adult tendons suggests they are facilitating communication between cells, and may be involved in maintaining the tissue. Using fluorescence recovery after photobleaching (FRAP) to quantitatively evaluate the diffusion-dependent redistribution of a gap junction-permeable fluorescent dye, one study showed that adult tendon cells from human hamstrings formed functional gap junctions with connexin-43 in both 2 and 3 dimensional (D) *in vitro* culture, and rapidly redistributed calcein acetoxymethylester (AM), a non-fluorescent dye that is converted to green-fluorescent and membrane-impermeable calcein by intracellular esterases [239]. HeLa cells, which do not communicate via gap junctions, did not redistribute calcein AM. Dye redistribution was impaired in

tendon cells when gap junctions were chemically inhibited using 18 β-glycyrrhetinic acid and carbenoxolone, indicating that intercellular communication was disrupted [239]. Additionally, isolated tendon cells with fewer cell-to-cell contacts recovered more slowly from the photobleaching and could not redistribute the calcein, compared to cells that formed many cell-to-cell contacts, suggesting that adult tendon cells depended on their gap junction-linked network to rapidly move small molecules [239].

Gap junction communication may also modulate inflammatory and catabolic responses in adult tendon, including the response to overuse and exercise. Adult rabbit Achilles tendon cells subjected to 30 minutes of heat (37 °C, 41 °C or 43 °C, to simulate the heating of tendon that can occur following intense exercise) had decreased cell viability and increased expression of the proinflammatory markers matrix metalloproteinase (MMP)-1, interleukin (IL)-1β and IL-6, and decreased expression of Col I, 24 h after heat stimulation [240]. Connexin-43 expression was not affected by heat treatment, but inhibiting gap junctions using 18 β-glycyrrhetinic acid resulted in further increases in expression of MMP-1, IL-1β, and IL-6, compared to cells subjected to heat but with functional gap junctions [240]. Interestingly, when connexin-43 was overexpressed, expression of Col I and IL-1β was significantly lower, and MMP-1 and IL-6 expression trended lower in the heated tendon cells, compared to cells heated without overexpression of connexin-43 [240]. Taken together, these findings show that connexins may influence inflammatory markers in tendon cells following intense exercise that results in heat.

In addition to heat, gap junctions are sensitive to mechanical signals [199], and respond to mechanical loading. In one study, gap junctions were quantified in the tail tendon fascicles of adult rats using FRAP and calcein AM [241]. Fascicles were statically loaded to 1 N and FRAP was carried out after 10 minutes or 1 h of loading to assess junction permeability between groups [241]. A mathematical compartment model was also generated to calculate a permeability constant for intercellular communication. Loading for 10 minutes did not change the permeability, while loading for 1 h significantly reduced intercellular permeability, compared to unloaded controls [241]. Strong gene expression of connexin-26 and connexin-43 was observed following 1 h of loading, while connexin-32 was undetectable. Connexin-43 gene expression increased significantly after 24 h of loading, while no differences were seen in connexin-26 levels [241]. Protein production of connexin-26 and connexin-43 was observed following 1 h of loading, though production of connexin-43 was decreased, and the localization of connexin-43 changed from the contact points of neighboring cells in the same row to a more discrete, punctate pattern [241]. In contrast, neither the protein levels nor the localization of connexin-26 were affected by the loading. Connexin-32 was not found in loaded or

unloaded fascicles [241]. The decrease in connexin-43 protein production is interesting given the increase in connexin-43 gene expression, and suggests that tendon cells attempt to remodel their gap junctions in response to load. However, actual production of gap junctions may be compromised with loading, and hence overall permeability is suppressed, further inhibiting intercellular communication. Overall, functional gap-junction intercellular communication may influence tendon cell behavior by propagating mechanical signals throughout cell networks to stimulate a coordinated response to loading.

In addition to the duration of loading, gap junctions may also be sensitive to the magnitude of mechanical strain. Adult tendon cells from rabbit Achilles tendons showed that intermediate tensile strain (4%) for 1 h enhanced intercellular communication, illustrated through increased transport of fluorescent tracer molecules between cells [242]. High strain (8%) significantly decreased intercellular gap junction communication, while neither 4 or 8 % strain affected intracellular communication, which was mediated by diffusion instead of by gap junctions [242]. Connexin-43 was localized to the cytoplasm and cell-cell boundaries in the unstrained cells, and was also detected near the nucleus in the 4% strain condition, while overall connexin-43 production was reduced at 8% strain [242]. Gap junction communication appears to be enhanced by moderate strain, but inhibited by high strain, possibly reflecting the physiologically relevant range of strain magnitudes [243]. Higher strain magnitudes may mechanically disrupt the cell-cell junctions and lead to the increased inflammation that follows gap junction inhibition [240]. Strain magnitudes were further assessed in a follow-up study. Cells from adult rabbit Achilles tendons were left unstrained, or subjected to 4% or 8% strain for 24 h, and an intercellular diffusion coefficient was used to quantify gap junction communication [244]. The intercellular diffusion coefficient was significantly increased following application of 4% strain for 1 h, compared to 0% strain controls, and its level was maintained for 6 h, but returned to pre-strain levels at 24 h [244]. This was accompanied by a transient increase in connexin-43 gene expression and localization at the cell membrane and within the cell bodies at 1 h and 6 h following 4% strain [244]. In contrast, application of 8% strain reduced the intercellular diffusion coefficient to the levels of the unstrained control group or below at 6 h, and inhibited connexin-43 expression, but did not change connexin-43 localization [244]. Moderate mechanical loading may be therapeutically useful for preventing or repairing tendon injuries [112, 133, 142], while the effects of high strains on gap junctions may explain the presentation of certain tendon pathologies. Taken together, the results of these studies suggest that disruption of cell-to-cell communication by tensile loading may account for some of the damage tendons can sustain from mechanical loading. Gap junctions are part of a complex cellular communication mechanism in tendon cells that responds to load and other stimuli, such as heat, by altering permeability of the cells

and enhancing or inhibiting production of connexins, and possibly modulating the message the cells receive.

In addition to mechanotransduction, gap junctions in adult tendon cells may be actively involved in the mechanoregulation of collagen production. Blocking gap junctions with octanol in adult chicken flexor digitorium profundus tendons *ex vivo* inhibited the increase in DNA and collagen synthesis that was observed following 3 d of cyclic loading to 0.65% strain, and was reversible when the octanol was removed [245]. Gap junctions may be initiating the cellular communication that coordinates collagen production in response to mechanical loading. The unequal distribution of connexins within tendons may reflect the different magnitudes and modes of mechanical loading associated with specific regions. In the rat, the distal Achilles tendon midsubstance, sesamoid fibrocartilage, and enthesial fibrocartilage regions were shown to have different expression of connexin-32 and -43 [229]. Immunofluorescence revealed prominent connexin-32 and -43 junctions in the tendon midsubstance, with both connexins localized between cells and rows, and connexin-43 predominantly occurring between lateral cell processes [229]. Connexin-32 formed plaques between cells with many small foci, while connexin-43 displayed a more punctate labeling pattern. Within the enthesis, both connexin-32 and -43 were significantly reduced. Connexin-43 was not detected in the enthesis, but connexin-32 was detected in the cells that stopped forming rows, and adopted a rounded morphology associated with mineralized fibrocartilage [229]. Finally, connexin-32 and -43 labeling was weak in the sesamoid fibrocartilage, with connexin-32 labeling localized mainly to the points of contact between cells, and with no visible connexin-43 labeling [229]. In addition to variations in connexin expression, cells in the different regions displayed distinct morphologies, with elongated, narrow cells observed in the midsubstance, and more rounded cells with fewer opportunities for cell-cell contact seen in the enthesis and sesamoid areas [229]. Cell shape affects the contacts cells can make with one another, and may enhance or suppress expression of certain gap junction proteins. It is worth noting that some bone cells in the calcified enthesis fibrocartilage expressed connexin-43 [229], demonstrating the wide-ranging distribution of connexin-43 throughout multiple musculoskeletal tissues. The variations in expression patterns of connexins between bone, tendon, and fibrocartilage may also help guide the differentiation process to ensure correct formation of separate tissues.

Gap junctions may also couple with the actin cytoskeleton and together play a role in mechanoregulation of tendon cells. Connexin-43 was found to colocalize with actin filaments in both avian and human tendon cells. While only ~4% of avian tendon cells had colocalization of actin and connexin-43 in an unloaded control group, cells subjected to 5% cyclic strain had significantly

increased colocalization of actin and connexin-43 [246]. The same study also investigated COS-7 cells (CV-1 in Origin with SV40 genes, derived from the kidney of the African green monkey). Colocalization of actin and connexin-43 was significantly increased following cyclic strain in the tendon cells, but was not observed in the COS-7 cells, suggesting that COS-7 cells are not as mechanosensitive or that connexin-43 is playing a different role [246]. Additionally, inhibiting non-muscle myosin II activity to block actin contractility, and hence mechanotransduction, greatly reduced the detectable connexin-43 on actin filaments, suggesting that conexin-43 is not produced when the cell cannot contract. The colocalization of connexin-43 and actin filaments with mechanical loading, and subsequent loss of this colocalization with inhibited actin-myosin contractility indicates that gap junction production in tendon cells is mechanically regulated and partially dependent on the actin cytoskeleton.

Localization of connexin-32 and connexin-43 has been shown to vary within tendon cells as they reside in the tendon tissue: tendon cells are linked by both connexins longitudinally (e.g. within rows), but only by connexin-43 laterally (e.g. between rows) [197, 229, 230]. 3D reconstructions of sections from adult rat deep digital flexor tendons stained for connexin-32 and -43 showed that, in addition to the processes between cells, connexin-43 was prominent in the periphery of the tendon, between the epitenon and the outermost layer of tendon [197]. Connexin-32 was also prominent in the periphery, but with a more diffuse distribution than connexin-43, and with many foci between cells that appeared as plaques [197]. This reconstruction identified the precise locations of connexin-32 and -43 in tendon, and showed that the 3D structure of tendon cells is complex, with collagen bundles distributed throughout the tendon, and different localization patterns of connexin-32 and -43. The differences in positioning between these two connexins may be attributed to their different mechanoresponsive functions in Col I secretion, a hypothesis investigated by selectively blocking specific connexins in avian tendon cells. Tendon cells from chicken deep digital flexor tendons strained to 75 millistrain for 8 h/day for 3 d increased Col I secretion by 23%, compared to unstrained controls [230]. When connexin-32 and -43 were both inhibited via the biomimetic peptide gap27, Col I production was reduced compared to untreated cultures, in both strained and unstrained cells [230]. When connexin-43 was selectively inhibited via antisense oligonucleotides, Col I secretion was further increased with loading, while antisense inhibition of connexin-32 resulted in reduced Col I secretion [230]. These results suggest that mechanical signals are integrated by tendon cells to produce a coordinated response to load, and that connexin-32 may be stimulatory and connexin-43 may be inhibitory [230]. The differential actions of connexin-32 and -43, and possibly other gap junctions, could contribute to the overall cell response to mechanical loading in tendon. Excess collagen production is responsible for scarring, making targeted inhibition of stimulatory gap junction

proteins a potential clinical treatment to prevent fibrosis following tendon injury. The anabolic and catabolic cellular processes that maintain the adult tendon ECM are likely mediated in part by gap junction communication, but a deeper understanding of the relationships between mechanical signals and cell responses is needed.

Cell-Cell Junctions in Tendon Injury and Disease

Adult tendons heal poorly, and the roles of gap junctions in injury and healing have been explored. Gap junction involvement in tendinopathies may be driven in part by inflammatory cytokines. The inflammatory cytokine IL-1β is produced following tendon injury [153, 154] and directly impacts tendon cells [247, 248]. To evaluate this, cells from human flexor digitorum profundus tendons were cultured in 3D and subjected to 3.5% cyclic strain at 1 Hz for 1 h/day for up to 5 days with or without IL-1β treatment. All strained cells underwent increased apoptosis, and had upregulated gene expression of Col I, fibronectin, biglycan, TGFβ1, cyclooxygenase-2, MMP-27, and a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5), genes which are upregulated in human tendinopathies [249]. IL-1β decreased expression of all target genes to the levels of unstrained controls, with the exception of cyclooxygenase-2 and ADAMTS5, which were both increased by addition of IL-1β alone, though strain with IL-1β did not further increase their expression [249]. Cells seeded more densely made more connections and survived cyclic straining, while cells at a lower seeding density did not survive. Enhanced cell survival was lost when gap junctions were inhibited, indicating that cell survival was mediated by gap junctions [249]. Indeed, IL-1β treatment upregulated connexin-43 expression in a time- and dose-dependent manner, as early as 8 h after its addition to cell cultures. IL-1β also altered cell morphology, with treated cells forming multiple long, axon-like processes and connecting to one another [249]. Although high levels of IL-1β likely contribute to tendon pathology via upregulation of inflammatory proteins such as cyclooxygenase-2 and ADAMTS5, IL-1β may also be part of the adaptive response to loading. In particular, the ability for IL-1β to upregulate connexin-43, shown in other studies to attenuate cell death caused by strain [240, 244], may be useful for preventing or treating strain-induced tendon pathologies. However, more work is needed to determine how the progression of tendinopathies may differ between *in vivo* and *in vitro* conditions, and how connexin-43 may mediate strain-induced injury.

Fluoroquinolone antibiotics, such as ciprofloxacin, are known to cause tendinopathies and ruptures in some patients [250]. These tenotoxic side effects of ciprofloxacin may be due to altered expression of cadherins and connexins by the tendon cells. To assess this, adult human tendon cells

from the tendons of the rectus femoris, gracilis, and semitendinosus muscles were cultured for 2 d and treated with 10, 20 or 50 μg/mL ciprofloxacin. Control cells were cultured without ciprofloxacin. Ciprofloxacin administration significantly decreased expression of N-cadherin at all concentrations, and connexin-43 expression trended downwards [251]. Col I, Col III, and MMP-2 expression was unaffected by ciprofloxacin, but MMP-1 expression increased, and tissue inhibitor of MMP (TIMP)-1 decreased [251]. Expression of long lysyl hydroxylase 2 (LH2b), an enzyme involved in collagen crosslinking, trended lower, though the decrease was not statistically significant. No changes were observed in the actin, vimentin, and microtubule network between control and ciprofloxacin-treated cells [251]. The reduction in LH2b, coupled with the decrease in N-cadherin and downward trend in connexin-43, suggests that ciprofloxacin may inhibit collagen crosslinking in some patients and impact the cell-cell contact in tendon cells, possibly leading to tendon ruptures. However, the mechanisms through which N-cadherin and connexin-43 in adult tendon cells influence tissue maintenance in normal and tendinopathic tendons needs further study.

Ectopic ossification is associated with Achilles tendon injuries and repairs [60]. The exact causes are unknown, but cytokines such as bone morphogenetic protein (BMP)2 and 4, and TGFβ2 may be driving an endothelial-to-mesenchymal transition of vascular endothelial cells, which subsequently undergo osteogenic differentiation, resulting in mineralization within the tendons [252]. Smad proteins are the main signal transducers of the TGFβ pathway, and Smad2 and 3 are necessary for TGFβ2-induced tenogenesis during embryonic limb development [52, 53, 253]. Conversely, Smad7 has been shown to inhibit Smad2 signaling, and Smad7 overexpression blocks myofibroblast activation and transformation [254], inhibiting endothelial-to-mesenchymal transition. To assess the potential of Smad7 as a preventative for heterotropic ossification following tendon repair, adult rat Achilles tendons were fully transected and injected with a Smad7 overexpressing lentivirus. Histological and gene expression analysis showed that, compared to the control groups, injured tendons transduced with the Smad7 lentivirus increased production of VE-cadherin and the endothelial marker cluster-of-differentiation 31 (CD31), and decreased production of N-cadherin and vimentin, especially at 6- and 10-weeks post-surgery [252]. Histological and X-ray analysis at 10 weeks post-surgery revealed that rats treated with the Smad7 lentivirus did not show signs of heterotropic ossification, while control rats showed ossifying regions of their Achilles tendons in both X-rays and histological sections [252]. The decrease in N-cadherin in the Smad7 treated rats, coupled with the robust N-cadherin production seen in injured tendons, suggests that N-cadherin is involved in the endothelial-to-mesenchymal transition that may be underlying heterotropic ossification of injured tendons. As tenogenic differentiation of stem cells is accompanied by decreases in N-cadherin production [13], Smad7 overexpression may be creating a more tenogenic environment to promote

healing in injured tendons. Cadherins have remained underexplored as targets for regenerative therapies for tendon injuries. The extensive involvement of cadherins in tissue development, patterning, and injury response make them worthy of future investigations.

Tight Junctions in Tendon

Tendons must be able to slide over other tissues during movement to carry out their function, and both ageing and injury have been shown to result in fibrous adhesions [255]. The surface of E14 chick metatarsal and 4-week-old mouse flexor tendons was recently found to be coated in a Col IV/laminin basement membrane and contained a keratinized epithelium that was located on the outermost aspect of the tendon [256]. Immunofluorescence imaging showed that the basement membrane surrounding the tendon was composed of ZO-1 and claudin-1 tight junctions (Figure 3.3), as well as the epithelial markers keratin 1 and 10 [256]. The epithelial cells of the basement membrane had a flattened morphology and interdigitated cell processes [256]. This epithelial layer was proposed to facilitate tendon sliding, as well as prevent tendon cell migration away from the tissue. Cutting 4-week-old mouse flexor tendons and incubating them *ex vivo* for 5 d showed cells migrating from the cut ends, but not from the tendon midsubstance surrounded by the intact epithelial layer [256]. When this epithelial layer was digested by trypsin prior to 5 d of culture, cells migrated away from all the trypsin damaged surfaces, indicating that they were no longer contained by the tight junctions around the tendon [256]. Adhesions that form following injury may therefore originate from damage to this basement membrane and epithelium, a hypothesis tested in a Col IV mutant mouse line, which is not able to form intact basement membranes due to its mutated collagen [256]. Histological examination of the deep flexor tendons in the hind limbs of mutant mice showed evidence of adhesions between the tendon and the tendon synovium, and these adhesions were not present in wild-type mice [256]. Finally, when the flexor tendons of 4-week-old mice were severed and allowed to heal for 21 d, adhesions were seen between the tendon and tendon synovium and skin, though the tendon epithelium flanking the injury was intact [256]. Overall, these observations suggest that the epithelial layer surrounding tendon is crucial to preventing adhesions, and repairing damage to the epithelial layer to prevent adhesions may help mitigate the effects of injury.

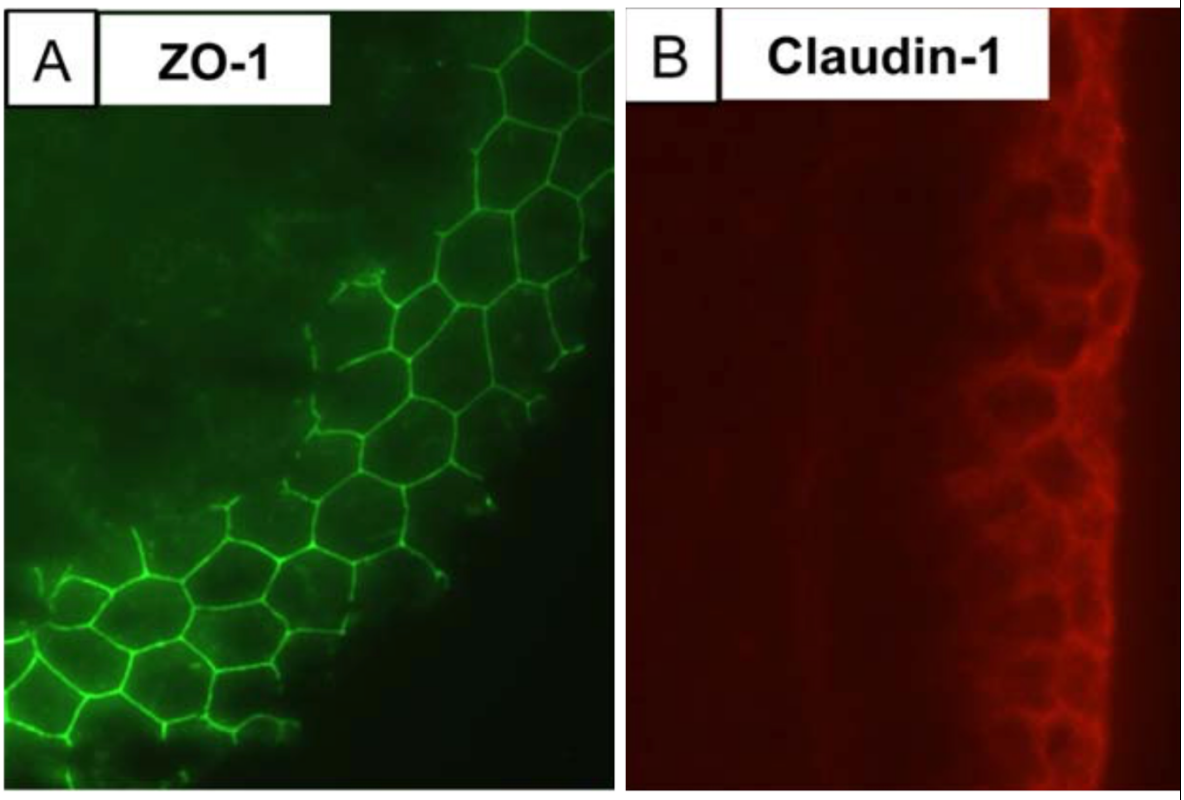


Figure 3.3 ZO-1 and claudin-1 are seen in a transverse section of E14 chick metatarsal tendon.

Immunofluorescence staining showed the presence of an epithelium containing (A) ZO-1 (green) and (B) claudin -1 (red) tight junctions on the surface of the tendon. This epithelium may prevent tendon cell migration and tendinopathic adhesion formation. Figure used with publisher's permission from Taylor et al 2011 [256].

Cell-Cell Junctions as Regulators of Musculoskeletal Tissue Formation

Regulation of Cell-Cell Junctions with Tenogenesis

Differentiation of stem cells toward the tendon lineage (tenogenesis) may be regulated by mechanical [9], biochemical [257], and combinatorial factors [49, 201]. Differentiation has been characterized by increases in expression of tendon markers such as scleraxis and tenomodulin. However, few studies have investigated how the profile of cell-cell junctions may be influenced by tenogenic differentiation. The growth factor TGFβ2 has been identified in embryonic tendon development as an important tenogenic factor [31, 47] and has been explored in tenogenesis of stem cells [48, 49, 258]. *In vitro,* N-cadherin and cadherin-11 protein production were significantly decreased in C3H10T1/2 mouse MSCs treated with TGFβ2 for 3, 7, 14, and 21 d, while scleraxis and

tenomodulin production were significantly increased after 14 and 21 d [13]. Cell morphology also changed with TGFβ2 treatment. As early as 3 d and throughout 21 d of TGFβ2 treatment, MSCs appeared fibroblastic, with an elongated actin cytoskeleton and a high degree of local actin alignment, compared to untreated controls [13]. The decrease of N-cadherin to almost undetectable levels, as well as the sustained decrease of cadherin-11, and simultaneous increase in tenogenic markers scleraxis and tenomodulin, suggests that suppression of these cadherins may occur during tenogenic induction. In the same study, connexin-43 increased significantly following 3 d of TGFβ2 treatment, but did not differ significantly from untreated controls at later timepoints [13]. Additional studies are needed to understand if N-cadherin, cadherin-11 and connexin-43 are regulators of the tenogenic stem cell response to TGFβ2. To better understand how cadherins and connexins may regulate tendon development and tenogenesis, we look to other musculoskeletal tissues of mesenchymal lineage. For example, chondrogenic differentiation is associated with changes in N-cadherin levels [259, 260], and cadherin-11 plays a role in osteogenesis [261]. Therefore, we briefly survey the cadherins and connexins involved in chondrogenesis and osteogenesis.

Cadherin Regulation of Chondrogenesis and Osteogenesis

In MSCs, N-cadherin and cadherin-11 are regulators of differentiation toward cartilage and bone [262]. The timing of N-cadherin expression appears especially critical for chondrogenesis. N-cadherin was initially shown to have a specific spatiotemporal expression pattern during limb bud chondrogenesis in chick [260]. Shell-less embryos injected during the cell condensation stage at HH22-24 with N-cadherin blocking antibodies failed to undergo chondrogenesis, and had other gross developmental and pattern deformities, likely due to widespread disruption of cell adhesion [260]. The same study also treated *in vitro* micromass cultures of chick limb mesenchymal cells with N-cadherin blocking antibodies. As in the *in vivo* experiments, cells treated with N-cadherin blocking antibodies did not undergo aggregation and condensation, and chondrogenesis was inhibited [260]. As N-cadherin expression is highest during the cellular condensation phase, blocking N-cadherin during cellular condensation is especially detrimental to chondrogenesis.

The *in vivo* increase in N-cadherin during cellular condensation and chondrogenesis may be driven by BMP2, a growth factor and member of the TGFβ signaling family [263]. BMP2-induced chondrogenesis of mouse MSCs resulted in a significant upregulation of N-cadherin after 24 h and 5 d in culture [263]. Blocking N-cadherin interactions using a N-cadherin mimicking peptide inhibited chondrogenesis, and overall loss of chondrogenesis was greater with higher concentrations of the

peptide [263]. Finally, mouse MSCs transfected to overexpress N-cadherin augmented the effect of BMP2 when N-cadherin expression was doubled, but BMP2-induced chondrogenesis was inhibited when N-cadherin expression was quadrupled [263]. Cells transfected with a mutant N-cadherin failed to undergo BMP2-induced chondrogenesis. Collectively, these results suggest that the timing of N-cadherin expression is critical to chondrogenesis, and while high levels are needed initially, N-cadherin levels must decrease in order for chondrogenesis to proceed. The initial increase and subsequent reduction of N-cadherin was further investigated using micromass cultures of embryonic chick mesenchymal cells. Cells from HH23-24 were transfected with mutant N-cadherin or wild-type N-cadherin, and in all transfected cells a transient increase in N-cadherin expression was observed for the first 2 d of culture, followed by a decrease at 3 d [259], mirroring the expression pattern documented in mouse MSCs chondrogenesis [263]. Overexpression of N-cadherin resulted in enhanced cell condensation and chondrogenesis after 2 and 3 d of culture, while micromass cultures transfected with mutant N-cadherin showed suppressed cell condensation and cartilaginous matrix generation, with reduced Col II and proteoglycan production [259].

Potential intracellular regulators of N-cadherin, such as Rho guanosine triphosphate (RhoGTP)-ases, specifically the kinase Rac1, have also been explored in chondrogenesis [264]. Inhibiting Rac1 in micromass cultures of E11.5 mouse mesenchymal limb bud cells led to reduced cell numbers, size and organization of cellular condensations, and decreased expression of N-cadherin. Overexpression of Rac1 in cultured chondrogenic ATDC5 cells resulted in increased expression of the cartilage markers Sox9, 5, and 6, collagen II, and aggrecan [264]. In micromass cultures, genetic ablation of Rac1 led to reduced expression of chondrogenic markers, including Sox9 [264], and Rac1 overexpression resulted in increased N-cadherin expression [264]. Based on these findings, it is possible that Rac1 affects the N-cadherin expression needed for cellular condensation during chondrogenesis.

TGFβ signaling may also modulate chondrogenesis via N-cadherin. TGFβ1 initiated and maintained chondrogenesis of human mesenchymal progenitor cells in addition to a corresponding initial upregulation and subsequent downregulation of N-cadherin [265]. Wnt7, a target of β-catenin, had an expression pattern that mirrored N-cadherin, with a significant increase after 1 d of TGFβ1 treatment, and a return to control levels at 3 d [265]. This suggests Wnt7 may regulate N-cadherin during TGFβ1-mediated chondrogenesis. Alongside TGFβ1, TGFβ3 is also a chondrogenic growth factor [65]. TGFβ3-induced chondrogenesis in human MSCs was accompanied by increases in N-cadherin [266]. Chondrogenic differentiation was further enhanced when cells were prevented from spreading and flattening (flattened cells differentiated into smooth muscle), suggesting cell-shape

may be mediated by cadherin cell-cell adhesions, and may affect subsequent differentiation [266]. Finally, cadherin-7 has been shown to be necessary for cartilage condensation in HH22-23 chick limb mesenchymal cells [267]. Knockdown of cadherin-7 resulted in impaired cell migration and failed precursor condensation, while overexpression of β-catenin also resulted in inhibition of cadherin-7 and suppression of cell migration [267]. Thus, in addition to N-cadherin, cadherin-7 may control cellular condensation during chondrogenesis.

N-cadherin and cadherin-11 are also involved in osteogenic differentiation and modulation of bone growth. At 6 months of age, N-cadherin $^{+/-}$ and cadherin-11 $^{-/-}$ double-knockout mice had severe phenotypic deficiencies, including smaller body mass and reduced trabecular bone mass, bone strength, and bone formation rate, and smaller diaphyses, compared to either single-knockout or wild-type mice [268]. Knocking out both N-cadherin and cadherin-11 resulted in decreased β-catenin in both the nucleus and the cytoplasm and cell membrane, leading to an overall downregulation of β-catenin signaling when cell adhesion was disrupted [268]. N-cadherin promoted osteogenic induction of human MSCs that were cultured in hydrogels functionalized with N-cadherin mimetic peptides [269], though a different study found that N-cadherin mimetic peptides enhanced chondrogenesis of human MSCs [270], highlighting N-cadherin's multifaceted roles during differentiation and development of bone and cartilage. Mature osteoblasts also express N-cadherin [271], but prolonged N-cadherin overexpression limited osteogenic differentiation of mouse MSCs by downregulating β-catenin and extracellular signal regulated kinase (ERK)1/2 signaling [272]. Additionally, ectopic bone formation in nude mice was prevented by N-cadherin overexpression [272], suggesting that N-cadherin levels are tightly regulated during mesenchymal cell condensation to ensure correct tissue differentiation, and offering a potential explanation for the drop in N-cadherin levels seen following cell condensation [260, 263]. Cadherin-11 also appears to be osteogenic during development, with reduced osteoblast differentiation and bone density observed in cadherin-11 knockout mice [261]. However, just as N-cadherin is needed for cell condensation during chondrogenesis, cadherin-11 may also be needed for cell condensation during tendon development [42, 44].

In addition to their chondrogenic and osteogenic roles, N-cadherin and cadherin-11 may be involved in the organization and maintenance of the synovial lining, the thin membrane between the joint cavity and the fibrous joint capsule that facilitates low-friction movement at the joint, and becomes inflamed during osteoarthritis [273]. Fibroblast-like synoviocytes from cadherin-11-null and wild-type mice were cultured and stained to determine the presence of cadherins. Cell-cell contacts in the wild type synoviocytes contained cadherin-11, while cadherin-11-null cells still made cell-cell contacts that contained β-catenin [274]. Wild-type cells expressed both N-cadherin and cadherin-11,

while cadherin-11-null cells expressed only N-cadherin and the mutated form of cadherin-11, though interestingly, cadherin-11-null cells expressed higher levels of N-cadherin than wild-type cells [274]. Furthermore, immunoprecipitation using β-catenin, which binds the cytoplasmic tail of cadherins, indicated that the two cadherins are not part of the same molecular complex in synoviocytes [274]. As more information emerges about the role of cadherins in the synovium, novel cadherin contributions to tendon formation may become evident.

Connexin Regulation of Chondrogenesis and Osteogenesis

In addition to cadherins, connexins are involved in the early differentiation and patterning of other musculoskeletal tissues. Connexin-43 has been shown to be necessary for chondrogenesis and osteogenesis, and its participation in both of these processes has been summarized in a recent review [275]. While less is known about the role of connexins compared to cadherins in cartilage differentiation, adult articular chondrocytes express both connexin-43 and -45 *in vitro* [276]. In one study, connexin-43 was shown to be involved in GDF-5-induced chondrogenesis [277]. Chick limb MSCs expressing mutated GDF-5 and treated with human GDF-5 had increased Col II expression in 1 d cultures and increased Alcian blue staining (indicative of glycosaminoglycan production and chondrogenic differentiation) in 3 d cultures compared to wild-type control cells, but the enhanced chondrogenesis was lost when gap junction communication was chemically blocked by oleamide [277]. Overexpressed GDF-5 functioned independently of N-cadherin, as cells with mutated N-cadherin still underwent GDF-5-induced chondrogenesis, while connexin-43 protein levels remained constant between cells expressing mutated GDF-5 and controls [277]. As GDF-5 and connexin-43 are also both present in embryonic tendon [232], it is possible that tendon and cartilage share similar developmental mechanisms. The potential involvement of connexin-43 in chondrogenesis also comes from a study that blocked gap junction communication in bone marrow-derived mesenchymal stromal cells and human chondrocytes with 18-α glycyrrhetinic acid, and observed a decrease in Col II and extracellular adenosine triphosphate (ATP) [278]. When cells were cultured farther away from each other in an engineered tissue scaffold, differentiation was not impacted, but blocking connexins again led to decreases in chondrogenic markers, suggesting functional gap junctions rather than general cell-cell contacts were needed for chondrogenesis [278].

During osteogenesis, the expression of connexin-43 has been shown to parallel osteoblastic differentiation, and inhibiting connexin-43 reduces osteoblast differentiation in human fetal osteoblastic cells [279]. In human osteoblasts, connexin-43 may regulate alkaline phosphatase

activity, as well as osteocalcin and osteopontin expression, all of which contribute to osteoblastic differentiation [279]. In contrast, 2-month-old mice with a conditional knockout of connexin-43 had increased periosteal bone formation *in vivo*, and increased periosteal and endocortical bone mass in response to axial compressive load at lower strains, indicating an overall increased sensitivity to mechanical load compared to their wild-type littermates [280]. These contradictory results are likely due to the differential effects of inhibiting connexin-43 at various developmental stages, and in specific tissues rather than globally. Complete loss of connexin-43 earlier in development leads to disruption of osteoblastic differentiation, while targeted disruptions at later stages, such as those achieved in osteoblasts on the endocortical surface, osteocytes, and periosteal cells by a conditional knockout [280], do not cause severe phenotypic deficiencies. While the causal mechanism of connexin-43-mediated disruption of osteogenesis remains unknown, the c-terminal domain of connexin-43 was recently found to bind several signaling proteins, including β-catenin [281]. Deletion of the c-terminal domain of connexin-43 in a mouse model resulted in disruption of osteoblast proliferation, differentiation, and collagen processing and organization [281], suggesting connexin-43-mediated intracellular signaling may be needed for bone formation.

Collectively, there is a need to better understand how chondrogenesis and osteogenesis are mediated by cadherins and gap junctions. Not only will a more thorough understanding of these differentiation processes point to potential tenogenic mechanisms, but aberrant differentiation of tendon cells into bone or cartilage is frequently observed in human tendinopathies [60]. Ectopic ossification or chondrogenesis within tendons implies the processes are related, and communication deficiencies or incorrect cell signaling may induce aberrant differentiation and lead to disease.

Cell-Cell Junctions in Musculoskeletal System Pathologies

Cadherins in Musculoskeletal and Skin Diseases

Cadherin-11 has been shown to participate in the inflammatory mechanisms of rheumatoid arthritis in the synovium [282]. Although cadherin-11 is required for synovial lining formation [273], it also contributed to inflammation and mediated cartilage degradation, possibly via recruitment of inflammatory molecules to the synovium [282]. Another study found that IL-17, an inflammatory cytokine involved in rheumatoid arthritis, increased cadherin-11 expression in the knee joints of mice with induced arthritis [283]. The same study found increased cadherin-11 expression in the synovium of mice with arthritis, as well as in mice with deficient IL-1 receptors, and human patients with rheumatoid arthritis. Cultured fibroblast-like synoviocytes also had significantly increased cadherin-

11 when treated with IL-17, but increases were blocked in cells treated with nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) inhibitors to suppress the inflammatory response [283]. Blocking cadherin-11 during rheumatoid arthritis may attenuate some of the symptoms, but future studies must determine other side effects of disrupting cadherin-mediated cell adhesion in the synovium. Nevertheless, cadherins may be promising targets for preventing or reversing inflammation within joints and are worth exploring in tendon and other musculoskeletal tissues.

Cadherin-11 is also implicated in pathologies of the mouth and skin. Human periodontal ligament cells express cadherin-11, but expression was suppressed by mechanical stress, as cultured cells weighted under glass coverslips from 0 to 2 g/cm^2 for up to 24 hours showed increased cadherin-11 suppression with higher stress and loading time [284]. Col I and β-catenin expression were also suppressed by loading, and reducing cadherin-11 expression with β-catenin knockdown led to rounding of periodontal ligament cells and an overall change in collagen matrix deposition [284]. Cadherin-11 was also found on the surface of fibroblasts and macrophages isolated from the skin of systemic sclerosis patients [285, 286]. Blood samples from patients with widespread skin symptoms of systemic sclerosis showed significantly increased cadherin-11 expression, compared to samples from patients with limited systemic sclerosis or other connective tissue diseases [285]. Furthermore, logistic regression analysis showed a significant correlation of increased cadherin-11 expression with extensive skin symptoms of systemic sclerosis [285]. Together, these findings illustrate that cadherin-11 may play a role in some musculoskeletal pathologies.

Connexins in Musculoskeletal and Skin Diseases

Connexins are involved in many human pathologies, and the mechanisms by which connexins contribute to various disease states have been summarized in a few recent reviews [275, 287], though it was noted that fundamental knowledge of connexin signaling in cartilage, tendon, and muscle is lacking [275]. However, connexin-43 has emerged as a potential mediator of synovium pathologies. Elevated connexin-43 expression was seen in the synovial lining of patients with osteoarthritis. Connexin-43 expression was 50% higher and MMP-1 production was increased with osteoarthritis, possibly in response to elevated IL-1β [288]. IL-1β was also detected in diseased synovia of human osteoarthritis patients [288], and IL-1β was shown to significantly increase connexin-43 production and localization to the cell membrane in adult rabbit synovial fibroblasts [289]. In a different study, human chondrocytes from the articular cartilage of the femoral head and knee of patients with osteoarthritis also had elevated expression of connexin-43 and -45, and

increased gap junction plaques [276]. Together, there is evidence of connexin-43 involvement in inflammation and osteoarthritis in synovial lining and cartilage. However, future studies are needed to identify the contributions of connexin-43 and other connexins, such as connexin-32, to tendon and other musculoskeletal pathologies.

Conclusions and Future Directions

Tendon cells in immature and mature tendons possess an array of cell-cell junction proteins, including cadherins and connexins. It is likely that additional cell-cell junction proteins are present and have yet to be explored. Improving our understanding of how the currently identified cell-cell junctions (e.g., cadherin-11, N-cadherin, connexin-32 and -43) and potential downstream signaling pathways (e.g., β-catenin) (Figure 3.4) regulate tenogenesis may result in novel strategies to direct engineered tendon formation and differentiation. A potential approach is designing engineered tissue scaffolds that are functionalized with peptides that mimic specific cell-cell junctions, similar to what has been demonstrated with N-cadherin mimicking peptides for chondrogenesis [270] and osteogenesis [269]. Tendon tissue engineering strategies that exploit the natural ability of the cells to form tissues through cadherin and connexin mediated self-assembly, such as scaffold-free techniques that found cadherin-11 in the early stages of fiber formation [44], could also be guided by a more complete understanding of the cell-cell adhesions found in developing tendon. A different approach may be to use novel biomaterials to control spatiotemporal siRNA application to stem cells to regulate cell-cell junction levels and ultimately tendon formation [290]. Future engineered tissue constructs with the appropriate types and ratios of cadherins and connexins may also acquire self-organizational capacity as the cell-cell interactions mimic *in vivo* development. Constructs that can mimic the developmental processes more closely can offer new insights into cell-cell junction-associated signaling pathways that remain underexplored in tendon development (e.g., Wnt/β-catenin). Furthermore, β-catenin may be particularly relevant in tenogenesis as it is needed for recruitment of vinculin and actin to mechanically stressed cadherin complexes [291], and β- and γ-catenin are critical to the formation of adherens junctions in *Drosophila,* but are unexplored in the developing tendons of mammals [292].

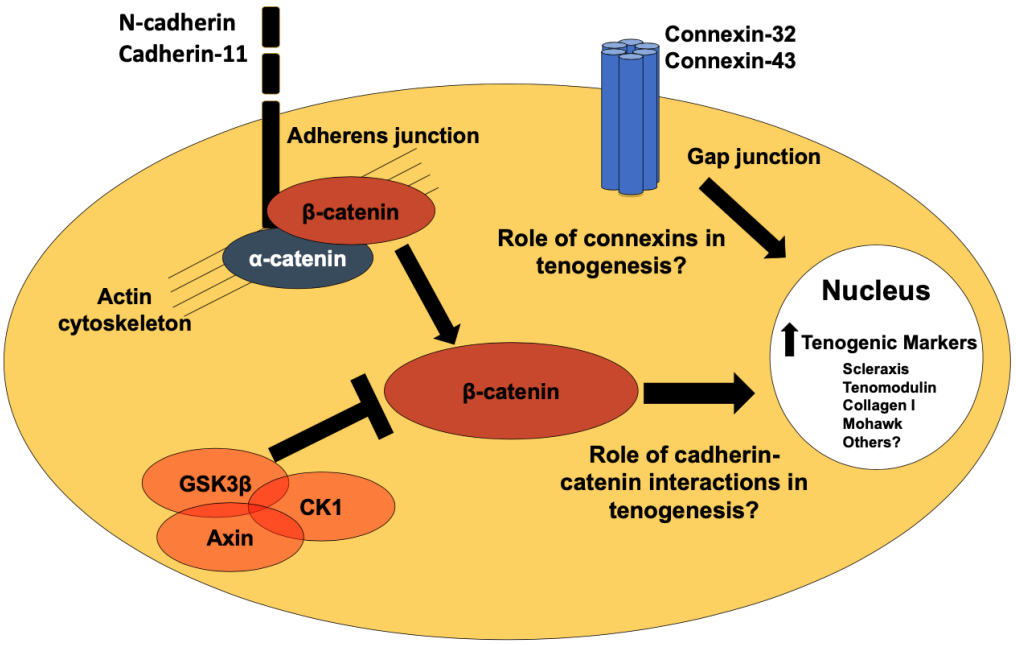


Figure 3.4 Potential cell-cell junction and cell signaling regulators of tenogenesis.

N-cadherin and cadherin-11 are found in embryonic and adult tendon cells [204,236], and are regulated in tenogenically differentiating stem cells [13]. Cadherin-β-catenin coupling and β-catenin signaling may regulate tendon and stem cell behavior [225, 226]. Connexin-32 and connexin-43 are present in embryonic, postnatal, and adult tendon cells [204, 230]. Future studies are needed to understand what the roles these cell-cell junction proteins and β-catenin are playing in tenogenesis.

Other regulators of cell-cell junctions in tendon may also emerge. The tumor suppressor protein p53, which is mutated in several cancers that also involve cadherin mutations [293], was found elevated in partially torn human supraspinatus tendons, compared to intact reference subscapularis tendons [294]. Injured tendons displayed increased apoptosis and proliferation compared to intact controls [294], and it is possible that p53 affects cadherins or other cell-cell junctions in tendons following injury, though its potential involvement has yet to be explored. In addition to p53, NF-κB was found to be elevated in tendinopathy and tendon injury, and presents a possible target for reducing inflammation in tendinopathies [295, 296]. This upregulation of NF-κB during tendinopathy may potentially affect cell-cell junction proteins, though any relationship between NF-κB and cell-cell junctions in tendon has yet to be investigated. Overall, a comprehensive understanding of potential regulators of cell-cell junction proteins in tendon will provide new targets for clinical treatment of tendinopathies.

Our current understanding of the roles of cell-cell junctions in bone and cartilage highlights the diverse contributions of cadherins and connexins to the development of musculoskeletal tissues. In comparison, the regulation of tendon formation by cell-cell junctions is poorly understood. Given that bone and cartilage form concurrently with tendon during limb development, and their development is spatiotemporally regulated by cell-cell junctions, it is possible cell-cell junctions are also critical regulators of tenogenic differentiation. Ultimately, improved understanding of the roles of cell-cell junctions in tendon differentiation, development, and maintenance has implications for advancing tissue engineering approaches and regenerative tendon healing. Additionally, cell-cell junctions are involved in pathologies of the musculoskeletal system and deserve further investigation in tendon.

Chapter 4: A 3D Printed Bioreactor for Investigation Mechanobiology and Soft Tissue Mechanics

*Raveling AR, *Theodossiou SK, Schiele NR. A 3D Printed Bioreactor for Investigating Mechanobiology and Soft Tissue Mechanics. MethodsX 5(2018) 924-932. *Denotes shared lead authorship.

Abstract

Mechanical loading is an important cue for directing stem cell fate and engineered tissue formation *in vitro*. Stem cells cultured on 2-dimensional (D) substrates and in 3D scaffolds have been shown to differentiate toward bone, tendon, cartilage, ligament, and skeletal muscle lineages depending on their exposure to mechanical stimuli. To apply this mechanical stimulus *in vitro*, mechanical bioreactors are needed. However, current bioreactor systems are challenged by their high cost, limited ability for customization, and lack of force measurement capabilities. We demonstrate the use of 3-dimensional printing (3DP) technology to design and fabricate a low-cost custom bioreactor system that can be used to apply controlled mechanical stimuli to cells in culture and measure the mechanical properties of small soft tissues. The results of our *in vitro* studies and mechanical evaluations show that 3DP technology is feasible as a platform for developing a low-cost, customizable, and multifunctional mechanical bioreactor system.

Introduction

Mechanobiology and tissue engineering approaches require a mechanical bioreactor system that can apply user-defined cyclic strains to cells and tissues *in vitro* and mechanically evaluate the developing tissues. The objective of this study was to design, build, and evaluate a low-cost, customizable, and multifunctional mechanical bioreactor system. To achieve this, we focused on developing a tensile bioreactor with potential applications for soft tissues such as tendons, ligaments, or skin. Here, we utilized 3-dimensional printing (3DP) to build a low-cost culture chamber for maintaining cells and engineered tissues in culture medium and custom grips for mounting 3D engineered tissue constructs and soft tissues. Additionally, we developed custom software to control three actuators and monitor load cells independently to conduct high-throughput loading experiments and evaluate the mechanical properties of developing tissues. Our results show that 3DP is a

promising technology that can be used to fabricate a multifunctional, low-cost, mechanical bioreactor system.

Methods and Materials

Mechanical bioreactor design

The bioreactor system design described in this study was modeled after a system developed by Kluge et al. [88] and used by colleagues [88, 90], but has several significant modifications. Here, the system was designed to be vertically orientated to reduce the potential for off-axis forces. This vertical orientation also eliminated the need for rubber gaskets associated with the moving actuator connectors, which reduced the potential for friction in the system and errors in force measurements. In addition, the bottom grip (i.e., the static grip) was fully integrated into the culture chamber to reduce the number of moving parts. The culture chambers, soft tissue grips, and actuator arm were designed in a computer-aided design (CAD) software package (SolidWorks Corp., Waltham, MA) (Figure 4.1). The rectangular culture chambers were 71 x 128 x 29 mm with inner chamber dimensions of 51 x 98 x 26 mm. The grips have a custom waveform pattern that secures a wide range of soft tissues and minimizes slipping during tensile loading. Two custom soft tissue grips were developed, one for mechanical evaluations and the other for use with soft 3D cell-seeded scaffolds *in vitro*. For mechanical testing, through-holes in the fixed grip and actuator arm grip allowed for tissues to be mounted and secured with stainless steel cap screws and nuts (Figure 4.1 A, B, C). For *in vitro* culture, the grips were designed to have snap-hooks at either end in place of through-holes. Corresponding snap-hook grooves were developed for the actuator arm and static culture chamber grip. This modification eliminated the need for through-holes spanning the depth of the chamber, which reduced the potential for culture medium leakage and allowed for more rapid and sterile mounting of cell-seeded scaffolds (Figure 4.1 D, E, G). The culture chambers were equipped with mounting posts to quickly load and secure chambers into the bottom plate of an aluminum load frame using two-piece shaft collars. A through-hole for the moving actuator arm allowed the arm to move with minimal friction. A separate port in the chambers was designed for adding and exchanging cell culture medium. A syringe filter covered the cell culture medium port during *in vitro* culture, maintaining a sterile environment while allowing for gas exchange within the culture chamber. The front of the chamber was designed to be sealed with a transparent polycarbonate cover that compresses against a rubber o-ring using stainless steel wing-nuts and bolts for quick, tool-free mounting. A list of materials is found in Table 4.1.

Table 2.1 List of materials for the bioreactor system

Name	Vendor	Catalog Number	Description
Inventor Dual Extrusion 3D Printer	Flashforge USA	6970152950192	3D printer
ABS filament	Flashforge USA	n/a	ABS filament for 3D printer
Acetone	Macron Chemicals	n/a	Acetone for waterproofing 3D printed parts
Linear Actuator	Haydon Kerk	35H4N-2.33-915	Size 14, captive, stepper motor linear actuator
Stepper Motor Controller	Peter Norberg Consulting, Inc	AR-BC4E20EU	USB, four stepper motor controller
DAQ Chassis	National Instruments	781425-01	DAQ-9171, CompactDAQ Chassis
DAQ Universal Module	National Instruments	779781-01	NI 9219 Universal AI Module
Load Cells	Honeywell Sensing & Control	n/a	Model 31 Load cell
Polycarbonate	McMaster-Carr	8574K26	Clear polycarbonate sheet
Wing nuts	McMaster-Carr	94545A220	18-8 Stainless Steel Wing Nut, M4 x 0.7 mm
Hex Head Screw	McMaster-Carr	91287A053	18-8 Stainless Steel Hex Head Screw, M4 x 0.7 mm Thread, 40 mm Long
O-rings	McMaster-Carr	9262K716	Buna-N O-Ring, 2 mm Wide, 100 mm

Shaft Coupling	McMaster-Carr	61005K411	Clamp-on Rigid Shaft Coupling Type 303 Stainless Steel
Thread Adaptor	McMaster-Carr	98434A126	18-8 Stainless Steel Female Hex Thread Adapter 6-32 to M4 x 0.7 mm
Female threaded round standoff	McMaster-Carr	91125A442	18-8 Stainless Steel, 1/4" OD, 5/16" Long, 6-32 Thread Size
Shaft Collar	McMaster-Carr	9520T8	Clamping Two-Piece Shaft Collar for 14 mm Diameter, 2024 Aluminum
Quick-Disconnect wire terminals	McMaster-Carr	72625K74/ 72625K75	Fully Insulated Heat-Shrink Quick-Disconnect Terminals Male/Female, for 22-18 Wire Gauge
Hex Head Cap Screw	McMaster-Carr	93635A025	316 Stainless Steel, M3 x 0.5 mm Thread, 30 mm Long
Thin Hex nut	McMaster-Carr	93935A320	316 Stainless Steel, M3 x 0.5 mm Thread
Socket Head Cap Screw	McMaster-Carr	91292A114	18-8 Stainless Steel, M3 x 0.5 mm Thread, 12 mm Long

Fabrication:

The 3D drawings (SolidWorks) were sliced into 2D layers using Flashforge Flashprint (Flashforge USA, City of Industry, CA) software with a slice resolution of 2.5 μm. The chambers, grips, and actuator arms were printed with 1.75-mm diameter acrylonitrile butadiene styrene (ABS) plastic filament (Flashforge) using a FlashForge Inventor 3D printer. ABS is an appealing material for use with cell culture as it is chemically and biologically inert [297], and can be sterilized using ethanol [298]. The extruder nozzle was heated to 230°C and the platform was heated to 110°C. Each print had 5 shells and a print speed ranging from 50 to 70 mm/s. The culture chambers were printed with a 0.12 mm layer height and 15% infill, while the grips and actuator arms had a 0.12 mm layer height and 30% infill. The ABS plastic culture chambers were waterproofed by treating them with an acetone vapor bath. The bottom of a glass 3 L beaker was covered with acetone (Macron Chemicals, Center Valley, PA) to a height of 3 to 4 mm and heated until boiling. The culture chambers were then lowered into the beaker and covered with Parafilm M (Bermis NA, Neenah, WI) to seal in the acetone vapor. After 5 minutes, the chambers were removed from the beaker and air-dried for 12 h. The acetone vapor-treated chambers had a smooth and waterproof finish.

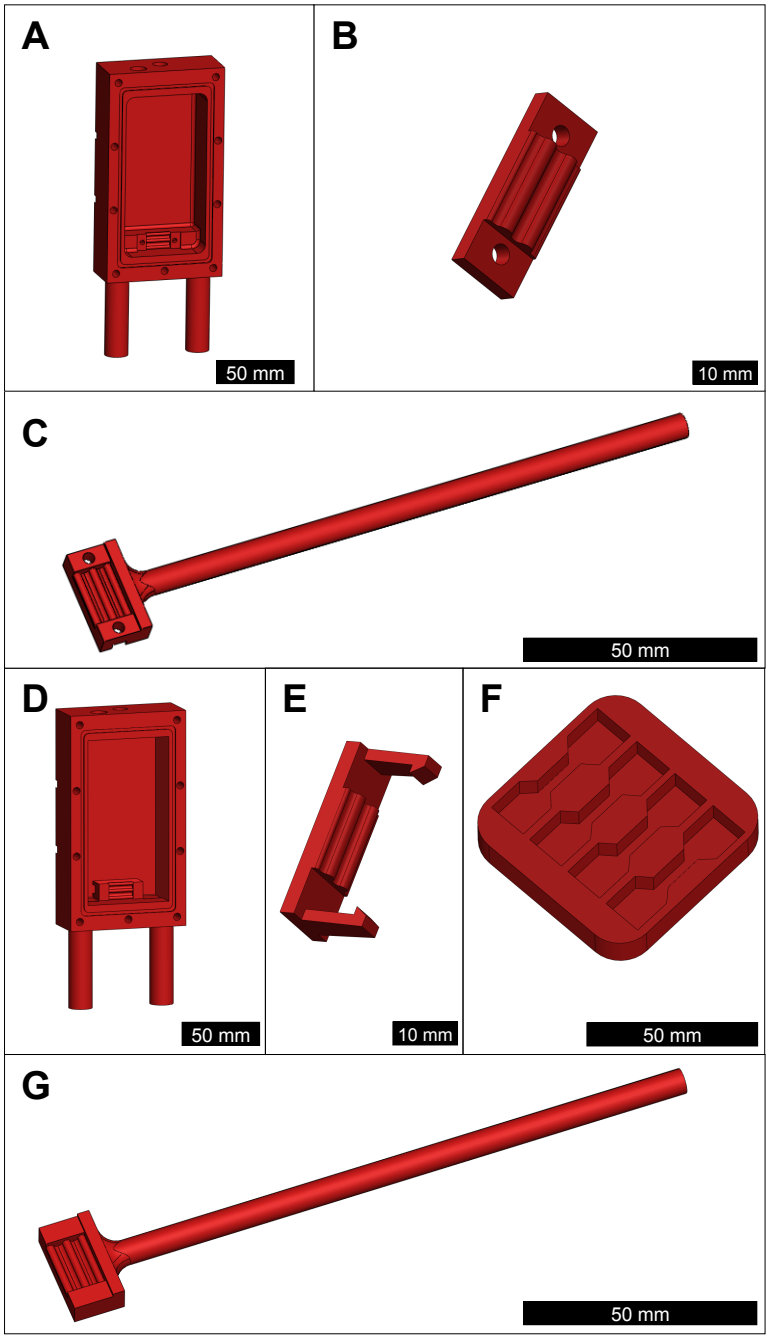


Figure 4.1 Engineering drawings of the custom mechanical bioreactor chambers, grips, and scaffold seeding wells.

A) Chamber, B) grips, and C) actuator arm designed for mechanical evaluation of soft tissues. Through-holes in the grips allow for secure mounting of tissues using stainless steel cap screws and nuts to prevent slipping during a pull-to-failure. D) Cell culture chamber, E) snap hook grip, and G) actuator arm for sterile cell culture. Through-holes are eliminated, and the snap hook system successfully secures the cell-seeded scaffolds. F) Custom wells for seeding scaffolds with cells.

Data acquisition and control

Three size 14 stepper motor linear actuators (Haydon Kerk, Waterbury CT) were mounted to the top-plate of an aluminum frame (Figure 4.2). Stainless steel mounting hardware was used to connect the load cells and actuator arms to each of the linear actuators. Heat-shrink quick-disconnect wire terminals were used to connect the actuator wiring and allow the entire bioreactor system to be easily inserted into a standard cell culture incubator through the sampling port. A 1000 g (9.81 N) capacity load cell (Model 31, Honeywell Sensing and Controls, Columbus, OH) was attached to each linear actuator. The load cells collect force data through a National Instruments (NI) data acquisition device (DAQ) (NI, Austin, TX). Calibration of the load cells was conducted using 18 different calibrated masses. Three consecutive load cell readings were taken for each calibrated mass and a calibration curve was generated. Two additional load cells with 150 g and 500 g capacities (Honeywell) were calibrated using the same procedure, but were not used in the validation experiments described below. However, the load cells can be easily interchanged in the system as needed for different tissues and force capacities. A stepper motor controller board (Peter Norberg, Ferguson, MO) and custom LabView™ programs control the movement of the actuators. To calibrate the actuators, digital images of the actuator grips were taken following actuator movements to 15 different displacement locations. ImageJ (NIH, Bethesda, MD) was used to measure the grip-to-grip displacement from 3 images taken at each location, moving the motor back to a predetermined zero position between captures. Using these calibrated load cells, actuators, and custom LabView™ controls [88], precise cyclic tensile strains can be applied and force and displacement data can be collected. The cyclic program (LabView™) provides user control over strain magnitude, strain rate, number of repetitions (cycles), frequency, and dwell time between stretches. A separate ramp control program can perform pull-to-failure tests to measure tensile mechanical properties of soft tissues and provides user control over strain magnitude, strain rate, and data collection rate.

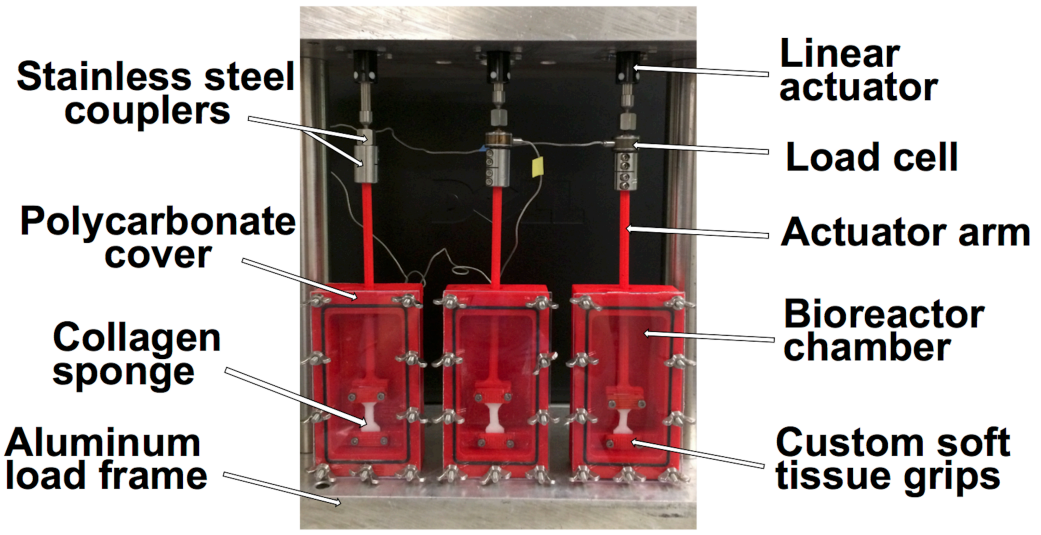


Figure 4.2 3D printed cell culture chambers mounted into the bioreactor system.

Stainless steel couplers attach the actuator arms to the linear actuators and load cells. Clear polycarbonate covers seal the chambers. Custom soft tissue grips secure the scaffolds and prevent slipping during loading. The entire system fits inside a standard cell culture incubator.

Results

In vitro bioreactor validation – dynamic mechanical stimulation of stem cells in 3D scaffolds

To evaluate the bioreactor for *in vitro* cell culture, 3D collagen type I sponges (DSM Biomaterials, Exton, PA) were prepared using a protocol previously described [299]. The collagen sponges were cut into dumbbell-shaped tensile specimens (12 mm gauge length and 4 mm width), sterilized overnight on a rocker in 70% ethanol, washed in sterile phosphate buffered saline (PBS) 6 times for 30 minutes each, and placed into custom 3D printed culture wells for cell seeding (Figure 4.1 F). Murine mesenchymal stem cells (MSCs) (C3H10T1/2, ATCC, Manassas, VA), a model MSC used in prior tendon tissue engineering studies of cyclically loaded cells [91, 201], were cultured in standard growth medium (Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS), and 1% Penicillin/Streptomycin) until 70% confluent, and used between passage 3 and 9. MSCs were trypsinized, and then $1x10^6$ cells were seeded into each collagen sponge, and incubated for 24 h in the 3D printed culture wells for initial cell attachment. The MSC-seeded collagen sponges (MSC-constructs) were mounted into the bioreactor culture chambers (Figure 4.1 D, E, G). The chambers were initially sterilized by soaking them overnight in a bath of 70% ethanol. The culture

chambers were filled with 40 mL of fresh cell culture medium to ensure the MSC-constructs were fully submerged, even when stretched. MSC-constructs were preloaded to 0.02 N to remove the slack, and the grip-to-grip length of each MSC-construct was measured using digital calipers. Independent displacement control of each actuator ensured that each MSC-construct was cyclically loaded to a peak tensile strain of 10% at a strain rate of 1%/s for 720 cycles/day (0.05 Hz) for three days (n=3), despite the slight differences in construct lengths after the initial preload was applied. MSC-constructs mounted in the culture chambers and statically loaded (e.g., 0 cycles) were used as controls (n=3). On day 4, the MSC-constructs were fixed in 10% formalin, stained with FITC-phalloidin (Life Technologies) and 4',6-diamidino-2-phenylindole (DAPI) to observe the actin cytoskeleton and cell nuclei, respectively, and then imaged on a spinning-disk confocal microscope (Nikon/Andor, Melville, NY). The staining showed that MSCs were present in both the cyclically loaded and static control groups. Furthermore, cyclic tensile strain appeared to increase actin cytoskeleton network formation, intercellular connections, and proliferation by the MSCs when compared to the static controls (Figure 4.3), which is consistent with other studies [88, 236, 300, 301]. These results demonstrate this bioreactor is useful for applying mechanical stimuli to cells in culture.

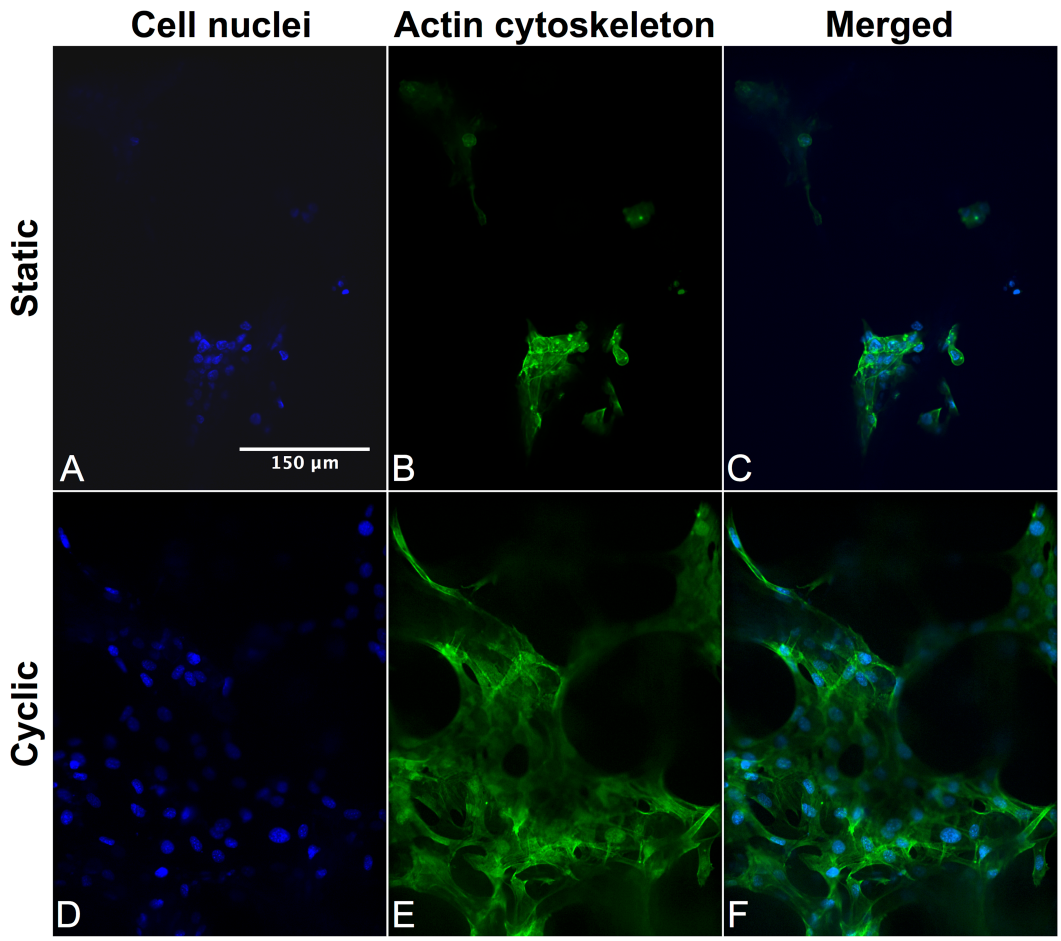


Figure 4.3 Representative images of MSCs cultured under static and cyclic tension.

Representative images (20x magnification) of cell nuclei (A, D) and actin cytoskeleton (B, E) of MSCs seeded into collagen sponges and loaded in tension statically (A, B, C) or cyclically (D, E, F) in the bioreactor for 3 days. The merged images of (C, F) of the cell nuclei (blue) and actin cytoskeleton (green) show that cyclic loading in the bioreactor appeared to increase cell proliferation and actin network formation.

Mechanical validation – tensile load frame

To evaluate the mechanical bioreactor as a small-scale tensile load frame, the mechanical properties of mouse tail tendon fascicles (MTTFs) and collagen sponges (DSM Biomaterials) were measured. MTTFs were isolated from the tails of 2.5-month old wild-type mice (n=5) with mixed C57BL/6, C3H, 129, and FVB genetic backgrounds that had been used for another University of Idaho IACUC approved study. The MTTFs were removed from the tails in PBS, secured in the mechanical testing grips (Figure 4.1 A, B, C) with sandpaper to limit slipping, mounted in the bioreactor, and submerged in PBS. Cross-sectional areas and initial lengths were measured from

calibrated digital images using ImageJ (NIH, Bethesda, MD). The MTTFs were preloaded to 0.02 N to remove the slack, and preconditioned for 10 cycles to 5% strain at a strain rate of 1%/s. After preconditioning, the MTTFs were pulled in tension to failure at a strain rate of 1%/s while recording the force and displacement. Results are reported as average ± standard deviation. The custom grips secured the MTTFs (diameter of 216 ± 67 μm) without slipping. The fascicles had a maximum (max) force of 0.59 ± 0.34 N and corresponding displacement of 1.3 ± 0.3 mm, linear-region stiffness of 0.7 ± 0.4 N/mm, max stress of 15.7 ± 4 MPa and corresponding strain of 9.2 ± 2.4%, and linear-region elastic modulus of 266 ± 72 MPa (Table 4.2). The structural and material properties of MTTFs measured in this study are within the expected range [302, 303]. Collagen sponges were also mechanically evaluated using the same mechanical testing protocol. However, the collagen sponges (n=3) were first cut into dumbbell-shaped specimens with a consistent gauge length of 12 mm and width of 4 mm, as described above for cell culture. The collagen sponges had a max force of 0.24 ± 0.03 N and corresponding displacement of 4.1 ± 0.4 mm, linear-region stiffness of 0.09 ± 0.007 N/mm, max stress of 19 ± 2 kPa and strain of 20.6 ± 2.1%, and linear-region elastic modulus of 143 ± 7 kPa (Table 4.2). As expected, the collagen sponges are dramatically softer and weaker than the MTTFs, and are consistent with a prior study that reported a tensile elastic modulus of bovine collagen sponges of approximately 50 kPa, and an ultimate stress of 12 kPa [304]. Typical force-displacement curves for the fascicles and collagen sponges are shown in Figure 4.4. Overall, we show that our bioreactor is useful as a small-scale tensile load frame.

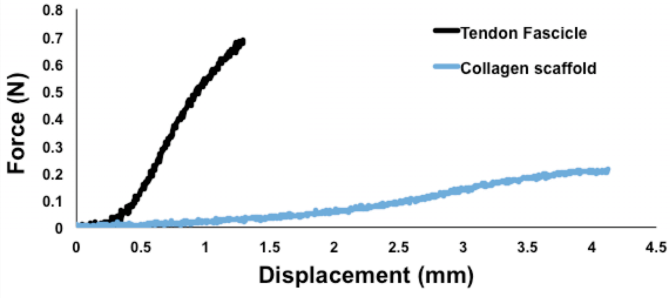


Figure 4.4 Representative force-displacement curves for a tendon fascicle and collagen scaffold.

Table 4.3 Mechanical properties of MTTFs and collagen sponges evaluated using the bioreactor system (mean ± standard deviation).

Material	Max force (N)	Displacement at max force (mm)	Stiffness (N/mm)	Max stress (MPa)	Strain at max stress (%)	Elastic modulus (MPa)
Tendon fascicle	0.59±0.34	1.3±0.3	0.7±0.4	15.7±4	9.2±2.4	266±72
Collagen sponge	0.24±0.03	4.1±0.4	0.09±0.007	0.019±0.002	20.6±2.1	0.143±0.007

Discussion

3DP is rapid, easily customizable, and lower cost in comparison to machined parts. Each chamber assembly (including the actuator arm and grips) costs approximately $4.90 to print. The 3D printed components are reusable and can be sterilized with 70% ethanol between uses, further reducing the cost. As the bioreactor system is currently configured, it can evaluate the tensile mechanical properties of small-scale soft tissues that have maximum failure loads of less than 10 N (currently limited by the maximum force capacity of the load cell). While this limitation could be addressed through use of higher capacity load cells, the linear actuators and ABS plastic actuator arms and grips further limit the maximum force capacity. The size 14 linear actuators used here have a maximum force capacity of 222 N. Given that ABS has a reported Young's Modulus in tension of about 1,600 MPa and tensile yield stress of 39 MPa [305], it is unlikely that the ABS actuator arm noticeably deforms under the small loads applied during *in vitro* culture or mechanical evaluations. Based on the cross-sectional area of the actuator arm (6-mm diameter) and 30% in-fill used during 3DP, we estimate the strain in the actuator arm at the maximum load cell capacity (10 N) to be approximately 0.0066% and 300 N would be required to reach the yield point. However, for mechanically evaluating larger tissues, a traditional materials testing load frame (e.g., an Instron) would be more appropriate.

In conclusion, we demonstrated a method for the design and fabrication of a functional, low-cost, and highly customizable 3D printed mechanical bioreactor that is useful for applications in mechanobiology and tissue engineering. Our system was able to evaluate the mechanical properties of small soft tissues (tail tendon fascicles) and engineered tissue scaffolds. Additionally, our bioreactor was successfully used to mechanically stimulate stem cells in culture for 3 days, demonstrating its value for *in vitro* cell culture. Future studies using this bioreactor system will focus on longer-term cell culture and evaluating the influence of mechanical stimuli on engineered tissue formation.

Chapter 5: TGFβ2-induced tenogenesis impacts cadherin and connexin cell-cell junction proteins in mesenchymal stem cells.

Theodossiou SK, Tokle J, Schiele NR. TGFβ2-induced tenogenesis impacts cadherin and connexin cell-cell junction proteins in mesenchymal stem cells. *Biochemical and Biophysical Research Communications* 508:3 (2018) 889-893.

Abstract

Tenogenic differentiation of stem cells is needed for tendon tissue engineering approaches. A current challenge is the limited information on the cellular-level changes during tenogenic induction. Tendon cells in embryonic and adult tendons possess an array of cell-cell junction proteins that include cadherins and connexins, but how these proteins are impacted by tenogenic differentiation is unknown. Our objective was to explore how tenogenic induction of mesenchymal stem cells (MSCs) using the transforming growth factor (TGF)β2 impacted protein markers of tendon differentiation and protein levels of N-cadherin, cadherin-11 and connexin-43. MSCs were treated with TGFβ2 for 21 days. At 3 days, TGFβ2-treated MSCs developed a fibroblastic morphology and significantly decreased levels of N-cadherin protein, which were maintained through 21 days. Similar decreases in protein levels were found for cadherin-11. Connexin-43 protein levels significantly increased at 3 days, but then decreased below control levels, though not significantly. Protein levels of scleraxis and tenomodulin were significantly increased at day 14 and 21, respectively. Taken together, our results indicate that TGFβ2 is an inducer of tendon marker proteins (scleraxis and tenomodulin) in MSCs and that tenogenesis alters the protein levels of N-cadherin, cadherin-11 and connexin-43. These findings suggest a role for connexin-43 early in tenogenesis, and show that early-onset and sustained decreases in N-cadherin and cadherin-11 may be novel markers of tenogenesis in MSCs.

Introduction

Tendons are musculoskeletal tissues that transfer forces from muscle to bone, and are essential to locomotion and movement. Tendons are frequently injured and have poor healing ability [306, 307]. Loss of function and re-rupture are common following tendon injury, and motivate regenerative tissue engineering strategies using stem cells. Mesenchymal stem cells (MSCs) have promise for tissue engineering based on their potential to differentiate into several musculoskeletal tissue lineages [308], including tendon [4-6, 9, 49, 201]. However, limited understanding of tenogenic differentiation (tenogenesis) is a challenge for using stem cells in tendon tissue engineering

approaches. Tenogenesis appears to be influenced by several biochemical [6] and mechanical [9] factors, and their combined effects [49, 201], but their impacts on MSC behavior are not fully understood.

Mature and developing tendons contain cells that are linked via cadherins and connexins (cell-cell junction proteins). Cadherins (cad) can complex with each other, bind to the intracellular actin cytoskeleton, sense forces, and trigger downstream signaling cascades [199]. Adult and embryonic tendon cells have been found to possess N-cad and cad-11 [42, 236]. Cad-11 may be necessary for regulation of cell condensation, shape, and alignment in the developing embryonic tendon [42]. Connexins (i.e., gap junctions) allow the intercellular exchange of ions and small molecules by forming direct channels between the cytoplasm of adjacent cells. In tendon, connexins (cxn) are mechanotransducers capable of altering cellular activity in response to loading [230, 242]. Cxn-43 has been found in adult [197, 309] and embryonic tendons [231], and limb buds [232]. Cells in mature tendon appear to communicate via cxn-43 and cxn-32, which link cells in a 3-dimensional network that can collectively respond to mechanical stimuli [197]. Taken together, developing and mature tendon cells possess N-cad, cad-11 and cxn-43 cell-cell junction proteins, but there is limited information on how these proteins are altered by tenogenic induction in MSCs.

The three isoforms of transforming growth factor (TGF)β, 1, 2, and 3, all affect musculoskeletal tissues, but have distinct effects on differentiation and cell behavior. TGFβ1 is involved in extracellular matrix remodeling and collagen secretion during tendon healing and scar formation [310]. TGFβ1 [265, 311] and TGFβ3 are inducers of chondrogenesis and cartilage formation [312-314]. TGFβ2 appears to be tenogenic: it upregulated tenogenic gene expression in MSCs [49, 53], was found in embryonic tendon [47], maintained tenogenic commitment of embryonic tendon cells [31], and induced tenogenic markers *in vivo* [315]. Thus, TGFβ2 is an especially promising tenogenic growth factor.

To evaluate tenogenic differentiation, a few markers have been identified. Scleraxis (Scx) is a transcription factor upregulated during early embryonic tendon formation and is necessary for tenogenic commitment of tendon progenitor cells [28]. Tenomodulin (Tnmd) is thought to be a late stage tendon marker, is highly expressed in adult tendons, and is regulated by Scx [32, 33]. Tenascin-C (TNC) is a glycoprotein present in developing and adult tendons [41]. Alongside these protein markers, cell morphology serves as additional evidence of tenogenic differentiation. *In vivo*, tenogenically differentiating embryonic tendon progenitor cells possess a highly organized actin cytoskeleton network [37], similar to adult tendon cells [236]. At present, it is not known if

biochemical factors such as TGFβ2 can initiate changes to actin organization that lead to a more tendon-like appearance in MSCs.

The objective of this study was to investigate how TGFβ2 affects cell-cell junctions and tenogenic marker protein production, as well as actin cytoskeleton organization in MSCs. We hypothesized that tenogenesis of MSCs results in altered levels of cell-cell junction proteins. Therefore, we examined proteins levels of N-cad, cad-11, and cxn-43, as well as tendon marker proteins Scx, Tnmd, and TNC throughout 21 days of TGFβ2 treatment. We demonstrated pronounced morphological changes in TGFβ2-treated MSCs, as well as significant changes in cell-cell junction protein levels as early as day 3, and significant increases in tenogenic marker proteins at day 14 and 21. Our results indicate that TGFβ2-induced tenogenesis impacts the cell-cell junctions produced by MSCs.

Methods and Materials

Cell Culture and TGFβ2 Supplementation

Murine MSCs (C3H10T1/2, ATCC, Manassas, VA), a model MSC used in prior studies investigating tenogenesis [31, 91], were cultured in standard growth medium (Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS), and 1% Penicillin/Streptomycin) until 70% confluent, and used between passage 3 and 9. MSCs were trypsinized, and 5000 cells/cm^2 were seeded into each well of a 12-well plate and incubated for 24 h to allow for initial cell attachment. Cells were washed with warmed phosphate buffered saline (PBS) (Gibco, Grand Island, NY), the medium was switched to low-serum medium (DMEM, 1% FBS, 1% Penicillin/Streptomycin), and allowed to equilibrate for 24 h. Cells were rinsed with warm PBS and cultured for 3, 7, 14, and 21 days (d) in low-serum medium (controls) or low-serum medium supplemented with 50 ng/mL recombinant human TGFβ2 (PeproTech, Rocky Hill, NJ). The medium was changed every third day. Experiments were repeated a minimum of 3 times.

Western Blot Analysis

Cells were collected for western blot (WB) analysis in RIPA cell lysis buffer and HALT protease inhibitor (Invitrogen, Carlsbad, CA). Sodium dodecyl sulfate (SDS) was added at a 1:1 ratio and samples were sonicated, heated to 100^o C for 5 minutes, and loaded into Novex Wedgewell 4-20% Tris Glycine Mini Gels (Invitrogen). To normalize total protein content (due to increased cell proliferation with TGFβ2 treatment), 20 μL of control and 10 μL of treated cell lysate were loaded per

lane. Cell lysate collected from each well of the 12-plate was run in its own lane (3 wells of each condition were run per individual experiment). Samples probed for TNC required 4-12% Tris Glycine gels (Invitrogen) due to the large protein size, and were run in duplicate, though all 3 wells of each condition were tested for each experiment and are included in the densitometry data. Following electrophoresis, gels were transferred to nitrocellulose membranes (Invitrogen), blocked in 5% milk in tris buffered saline (Boston Bioproducts, Ashland, MA) with 0.1% Tween20 (TBST) (Acros Organics, Morris Plains, NJ), and incubated overnight at 4^{o} C on an orbital shaker with appropriate primary antibodies in 5% bovine serum albumin (BSA) in TBST. Primary antibodies raised in rabbit were purchased for N-cad, cxn-43, β-actin (Abcam, Cambridge MA), cad-11 (Cell Signaling Technologies, Danvers, MA), Tnmd, Scx, and TNC (Invitrogen), and used at concentrations of 1:1000 to 1:10,000. Blots were washed 3x for 5 min in TBST and incubated for 1 h at room temperature with goat anti-rabbit HRP-linked secondary antibody (Invitrogen). Blots were then washed in TBST, developed using ECL chemiluminescence reagents (Invitrogen), imaged (Syngene, Frederick, MD), and analyzed via band densitometry in ImageJ (NIH, Bethesda, MD), with all intensities normalized to their respective β-actin bands.

Fluorescence Staining and Confocal Microscopy

To observe changes in cell morphology, cells were cultured and supplemented with TGFβ2 as described above, but on glass coverslips. At 3, 7, 14, and 21d the medium was removed, cells were rinsed with PBS, and fixed in 10% formalin overnight at 4^{o} C. Cells were washed with PBS, permeabilized with 0.1% Triton-X (Acros Organics), and stained with FITC-phalloidin and 4,6-Diamidino-2-phenylindole (DAPI) (Life Tech., Waltham, MA) to observe the actin cytoskeleton and cell nuclei, respectively. Coverslips were mounted on slides and imaged on a spinning-disk confocal microscope (Nikon/Andor, Melville, NY).

Statistical Analysis

Proteins were initially normalized to their respective β-actin bands. Since not all timepoints and experiments could be run on the same gel, TGFβ2-treated groups were normalized to their respective controls at each day, and analyzed using 2-tailed, unpaired t-tests (Prism 8, GraphPad, LaJolla, CA). Significance was set at $p<0.05$. Results are reported as mean ± standard deviation.

Results

TGFβ2 induced a fibroblastic morphology in MSCs

MSC morphology appeared fibroblastic when treated with TGFβ2, compared to controls (Figure 5.1). As early as 3d after TGFβ2 treatment, the actin cytoskeleton appeared more elongated and the MSCs were less circular (Fig. 5.1 A, B). This trend continued at 7d, accompanied by increased cell proliferation (Fig. 5.1 C, D). At 14 and 21d, control cells continued to proliferate and showed some level of localized actin filament alignment, but largely resembled earlier timepoint controls (Fig. 5.1 E, G). TGFβ2-treated cells appeared to have a high degree of local actin cytoskeleton alignment and elongation, along with a visible increase in cell number at 14 and 21d (Fig. 5.1 F, H), compared to controls.

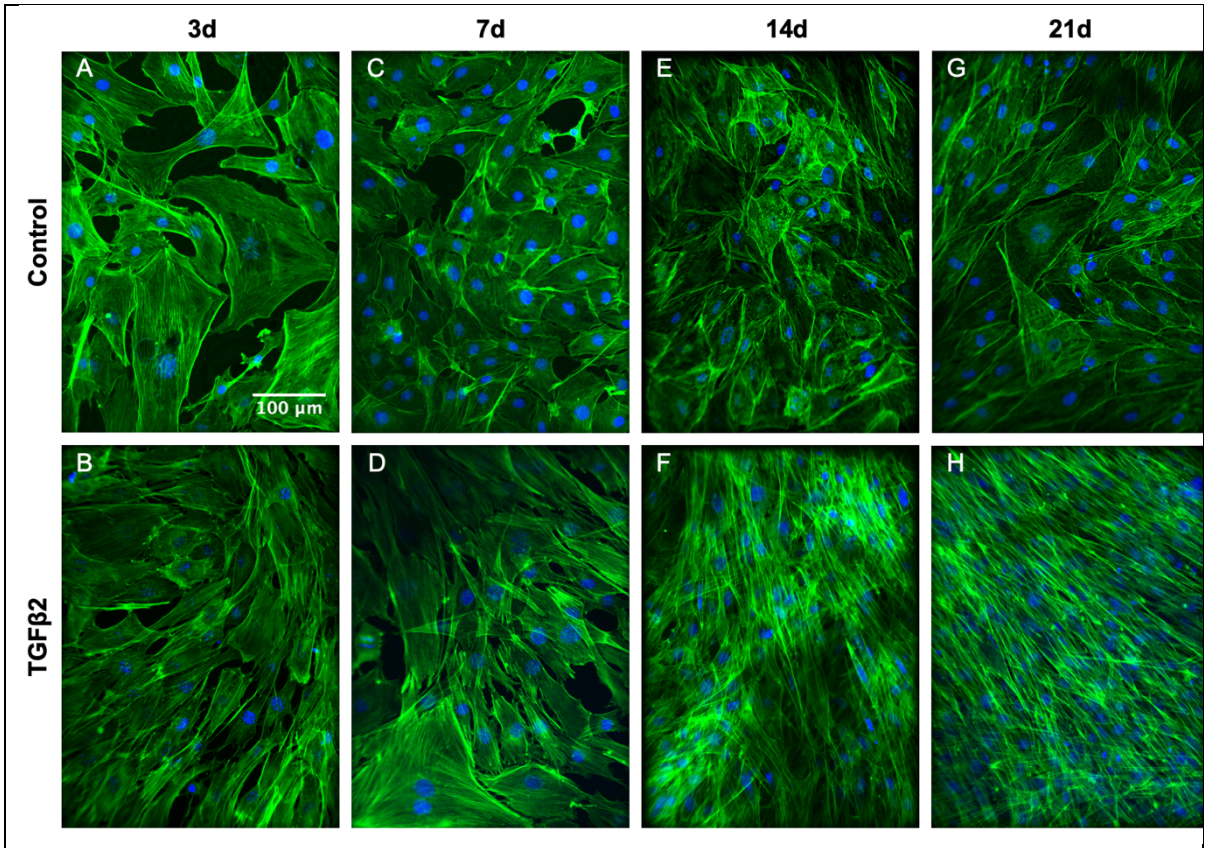


Figure 5.1 Representative images (20x) of TGFβ2-treated MSCs.

Actin cytoskeleton (green) and cell nuclei (blue) are shown in MSCs at 3, 7, 14, and 21d (A-H). TGFβ2-treated MSCs (B, D, F, H) had increased proliferation, and appeared more fibroblastic and elongated, compared to controls (A, C, E, G).

TGFβ2 induced tenogenic marker proteins and altered cell-cell junction levels in MSCs

TGFβ2 treatment significantly increased levels of tenogenic marker proteins Scx and Tnmd, compared to untreated controls (Fig. 5.2 C, D). In TGFβ2-treated MSCs, Scx trended higher at 3 ($p=0.08$) and 7d ($p=0.1$), and was significantly increased at 14d ($p<0.0001$) and 21d ($p=0.001$), compared to controls. From 3 to 14d, Tnmd trended higher ($p=0.07$-0.09) and was significantly higher at 21d ($p=0.0004$). TNC was not detected until 14 and 21d, and showed similar levels in control and treatment groups (Fig. 5.2 E).

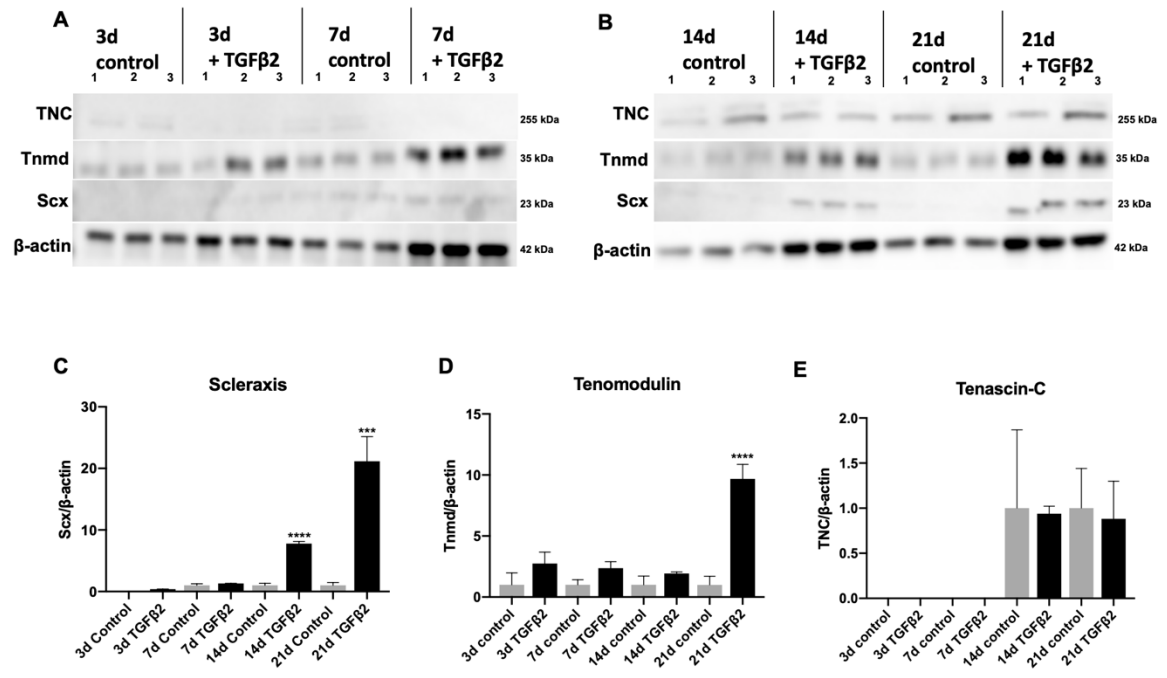


Figure 5.2 Representative WB images of tendon marker proteins produced by MSCs.

WB showed increases in Scx, Tnmd, and TNC following 3d, 7d (A), 14d, and 21d (B) of TGFβ2 treatment. Quantified band densitometry showed significant increases in Scx (C) and Tnmd (D), while TNC (E) levels were similar to controls. All bands were normalized to β-actin and to their respective timepoint controls. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$. Mean ± standard deviation.

Significant changes were also observed in the levels of cell-cell junction proteins with TGFβ2 treatment (Fig. 5.3). In TGFβ2-treated MSCs, N-cad protein levels decreased significantly at 3 ($p=0.0006$), 7 ($p=0.009$), 14 ($p=0.01$), and 21d ($p=0.0008$), compared to controls (Fig. 5.3 C). Similarly, cad-11 protein levels (Fig. 5.3 D) were significantly decreased at 3 ($p=0.02$), 7 ($p=0.0003$), 14 ($p=0.0007$), and 21d ($p=0.03$). Cxn-43 protein levels increased significantly at day 3 (Fig. 5.3 E, $p=0.0039$), and then trended lower at all other timepoints ($p=0.1$-0.3), compared to untreated controls.

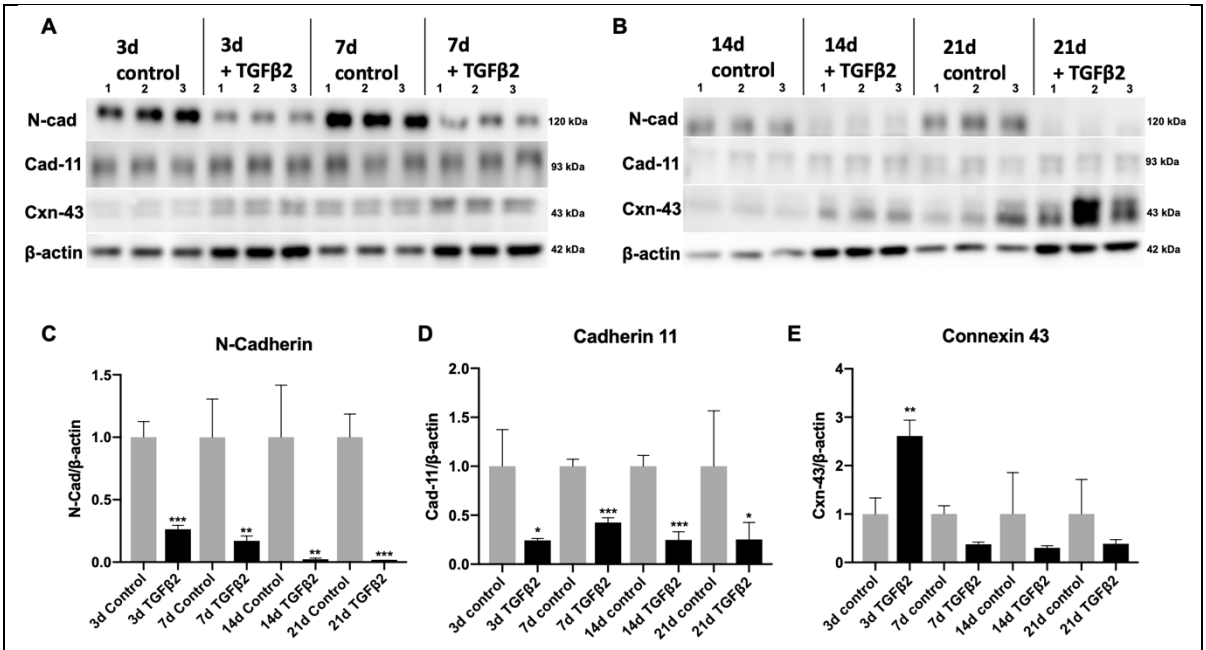


Figure 5.3 Representative WB images of cell-cell junction proteins produced by MSCs.

WB showed decreases in N-cad and cad-11, and changes in cxn-43 following 3d, 7d (A), 14d, and 21d (B) of TGFβ2 treatment. Quantified band densitometry showed significant decreases in N-cad (C) and cad-11 (D), while cxn-43 increased at 3d (E). All bands were normalized to β-actin and to their respective timepoint controls. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$. Mean ± standard deviation.

Discussion

Cell-cell junction proteins modulate cell migration, differentiation, adhesion, and communication [199], but their role in tenogenesis has not been identified. We demonstrated that tenogenic induction in MSCs by TGFβ2 resulted in significant changes in the levels of cell-cell junction proteins (Fig. 5.3). Tenogenic induction was determined by elevated protein levels of Scx and Tnmd (Fig. 5.2) and development of fibroblastic morphology (Fig. 5.1). As cell-cell junction proteins are involved in differentiation and formation of other musculoskeletal tissues [261, 266], the decreases in N-cad and cad-11, and initial increase in cxn-43, are noteworthy. Our results suggest that these cell-cell junctions may play a role in tenogenesis.

Defining the exact set of marker proteins to determine when MSCs are tenogenically differentiated is a challenge in itself. Of the various tendon markers, Scx and Tnmd have been repeatedly linked to tenogenic differentiation [28, 30, 31, 167]. To our knowledge, this is the first study to demonstrate Scx and Tnmd protein production in TGFβ2-treated MSCs over 21d (Fig. 5.2 A-D). Significant increases in Scx and Tnmd protein levels, coupled with the development of fibroblastic morphology (Fig. 5.1), suggest that TGFβ2 effectively induced tenogenic differentiation

in MSCs. Our findings in TGFβ2-treated MSCs support prior studies that found Scx upregulated early in tenogenesis [31]. Tnmd is a late stage marker of tenogenesis [30], and consistent with this, we found Tnmd to increase at day 21. Tnmd appeared to increase only after the initial increase in Scx at day 14. Another potential tendon marker, TNC, was undetected at 3d and 7d and produced at similar levels at 14d and 21d by control and treated MSCs (Fig. 5.2 E). In prior studies, TNC was found in adult tendon and increased in response to injury [316, 317], but it is not known to be a specific early stage tendon marker. Based on TNC, it is possible that the MSCs are not terminally differentiated by 21d, or that TNC is not a specific tenogenic marker. The observed accompanying changes in N-cad, cad-11 and cxn-43 protein levels may provide a novel, additional set of marker proteins to indicate tenogenic induction in MSCs.

Cadherins have been found to regulate chondrogenesis and osteogenesis. N-cad has been shown to direct chondrogenesis in the developing limb bud and in MSCs [259, 260, 265, 266, 270]. N-cad is required for early mesenchymal cell condensation and N-cad levels peak within the first 24 hours of chondrogenic induction [260], but decrease significantly by day 3 of development in the chick limb [259]. TGFβ1 has been shown to drive chondrogenesis in human mesenchymal progenitor cells by modulating N-cad to influence cell condensation [265]. TGFβ1 supplementation led to an initial increase in N-cad, followed by a decrease in N-cad, as cells committed to a chondrogenic fate, while N-cad disruption led to severe disruptions in cartilage formation [265]. Our results show that TGFβ2 treatment decreased N-cad protein in MSCs, but without the initial increase seen in prior work with TGFβ1. This newly identified distinction may be unique to tenogenesis or to the impact of TGFβ2 treatment, or related to both. However, when comparing tenogenesis to chondrogenesis, our findings suggest a similarity of decreased N-cad at later stages of differentiation [265]. The combinatorial effects of N-cad and cad-11 have been found in osteogenic differentiation and modulation of bone growth, as evidenced by the severe phenotypic deficiencies of double knockout mice lacking these cadherins [268]. Independently, N-cad appears to promote osteogenic induction of human MSCs [269], and N-cad is expressed in mature osteoblasts [271]. However, N-cad overexpression limits further osteogenesis [272]. Cad-11 also appears to play an osteogenic role during development, with reduced osteoblast differentiation and bone density observed in cad-11 knockout mice [261]. However, cad-11 may also be needed for cell condensation in tendon development [42, 44]. Up until now, how cad-11 changes during tenogenesis was unknown. Our results suggest cad-11 production is lowered with tenogenesis, a reasonable observation given its osteogenic capabilities, but cad-11 does not decrease to the nearly undetectable levels of N-cad, which supports prior evidence that cad-11 is involved in tendon formation [42]. Taken together, the dramatic and rapid (by 3d) decrease in N-cad following TGFβ2 treatment is a novel finding. The

persistence of almost undetectable levels of N-cad in TGFβ2-treated MSCs during 21d of culture suggests that N-cad levels decrease during tenogenesis, possibly distinguishing this process from differentiation toward alternative lineages, such as bone. Based on our findings, it is possible that N-cad and cad-11 are involved in tenogenic induction, but their role in regulating tenogenic markers will need to be identified in future studies.

Significant changes were also observed in cxn-43 protein levels. Cxn-43 was significantly higher in TGFβ2-treated MSCs at 3d, and then trended lower at later timepoints, compared to controls (Fig. 5.3 E). Communication via cxn-43 has been proposed as a differentiation-guiding mechanism of cartilage and tendon during embryonic limb development [232]. In adult tendons, cxn-43 was found localized to where cell processes meet as well as between cell bodies [197]. Cxn-32 was also found in adult tendon, but only between cell bodies. Both cxn-43 and cxn-32 have been implicated as mechanotransducers in tendon cells and may mediate stimulatory or inhibitory effects on collagen production in response to tensile loading [230, 242]. Future studies will aim to identify the effects of tenogenic induction on additional gap junction proteins (e.g., cxn-32) and cxn-43 by combining mechanical stimulation of cells with TGFβ2 supplementation. Mechanical stimuli are also thought to regulate tenogenesis [5], but the mechanotransductive signaling pathways are still not fully understood. It is possible that the TGFβ2 pathway in coordination with cadherins and connexins may play a role in mechanotransduction, and will be investigated in future studies.

In conclusion, our results highlight the impact of TGFβ2 on tenogenesis and identify novel changes in N-cad, cad-11 and cxn-43. A limited understanding of the many factors involved in tenogenesis and tendon development has been a challenge for stem cell-based tissue engineering approaches. The levels of these cell-cell junction proteins may be used as additional markers to assess tenogenic induction of MSCs. Future work to understand how cell-cell junctions mediate the response of stem cells to biochemical and mechanical factors is needed to direct tenogenesis and inform tendon tissue engineering strategies.

Chapter 6: Onset of Neonatal Locomotor Behavior and the Mechanical Development of Achilles and Tail Tendons

Theodossiou SK, Bozeman A, Burgett N, Brumley MR, Swann H, Raveling AR, Becker JJ, Schiele NR. Onset of neonatal locomotor behavior and the mechanical development of Achilles and tail tendons. *Journal of Biomechanics*. 2019 November; 96:109354.

Abstract

Tendon tissue engineering approaches are challenged by a limited understanding of the role mechanical loading plays in normal tendon development. We propose that the increased loading that developing postnatal tendons experience with the onset of locomotor behavior impacts tendon formation. The objective of this study was to assess the onset of spontaneous weight-bearing locomotion in postnatal day (P) 1, 5, and 10 rats, and characterize the relationship between locomotion and the mechanical development of weight-bearing and non-weight-bearing tendons. Movement was video recorded and scored to determine non-weight-bearing, partial weight-bearing, and full weight-bearing locomotor behavior at P1, P5, and P10. Achilles tendons, as weight-bearing tendons, and tail tendons, as non-weight-bearing tendons, were mechanically evaluated. We observed a significant increase in locomotor behavior in P10 rats, compared to P1 and P5. We also found corresponding significant differences in the maximum force, stiffness, displacement at maximum force, and cross-sectional area in Achilles tendons, as a function of postnatal age. However, the maximum stress, strain at maximum stress, and elastic modulus remained constant. Tail tendons of P10 rats had significantly higher maximum force, maximum stress, elastic modulus, and stiffness compared to P5. Our results suggest that the onset of locomotor behavior may be providing the mechanical cues regulating postnatal tendon growth, and their mechanical development may proceed differently in weight-bearing and non-weight-bearing tendons. Further analysis of how this loading affects developing tendons *in vivo* may inform future engineering approaches aiming to apply such mechanical cues to regulate engineered tendon formation *in vitro*.

Introduction

Tendons transfer mechanical forces from muscle to bone, and are critical for movement and locomotion. Frequent injury and poor healing are key motivators for tendon tissue engineering. To advance tendon tissue engineering, there is a need to better understand the processes that regulate normal tendon development. Tendon development can be characterized by describing the mechanical properties, but there is limited information available on how the mechanical properties of tendon progress during typical development. Structure-function relationships in developing and mature tendon have been reviewed [318, 319], though few studies have characterized early postnatal tendons. Previous studies demonstrated that the mechanical properties of developing tendon increase throughout embryonic [9, 40] and postnatal growth [39, 320]. In chick models, significant increases were observed in the ultimate tensile stress of extensor tendons [83] and elastic modulus of calcaneal tendons [40] between embryonic developmental stages (e.g., Hamburger and Hamilton (HH) stages) HH 42 and 43, and HH38 and 43, respectively. In developing mice, linear region stiffness and elastic modulus of Achilles tendons (ATs) increased with age from postnatal day (P) 4 to 28 [39]. In humans, children aged 8-10 years and adults aged over 19 years had higher AT stiffness and elastic modulus, compared to children aged 5-7 [321]. Collectively, these findings illustrate that the mechanical properties of tendon change throughout embryonic and postnatal development. However, many of the mechanical and biochemical factors that regulate tendon formation during development remain unknown.

Unlike ATs, tail tendons (TTs) are regarded as force-transmitting, but primarily non-weight-bearing tendons, whose main function in rats and most mice is to position the tail [322, 323]. In mutant embryonic mice null for scleraxis, a transcription factor and regulator of tenogenesis [28], development differed between force-transmitting (forelimb flexors, long trunk, tail) and muscle-anchoring (short-range anchoring, intercostal) tendons, suggesting distinctions in the developmental processes of various tendons [30]. Although TTs are considered non-weight-bearing, they were also impacted by scleraxis loss-of-function, much like the force-transmitting limb tendons [30]. While mechanical properties of adult mouse [66, 302, 324] and rat TTs have been evaluated [325, 326], it is unknown how the mechanical development of TTs differs from weight-bearing tendons, such as the ATs.

Tissue engineering studies show that mechanical stimulation regulates tendon formation *in vitro* [4, 5, 44, 45, 84, 91, 93-95, 201-203, 327]. Mechanical loading of cells in scaffolds enhanced collagen fibril quantity and diameter, elastic modulus, and ultimate tensile stress [84], and collagen type I and III gene expression [93, 94]. Mechanically stimulated stem cell-seeded scaffolds increased

maximum force, linear region stiffness, maximum stress, and elastic modulus of rabbit patellar tendon defects, compared to static controls [95]. These results suggest that mechanical loading impacts cell behavior and directs functional tendon tissue formation *in vitro*, but there is limited information on how mechanical stimulation impacts developing neonatal tendons *in vivo*.

Embryonic movement is a source of prenatal mechanical stimulation that may contribute to the correct development of musculoskeletal tissues [9, 11, 101, 328-331]. Tendon development in chick, mouse, and human is disrupted when embryonic movement is restricted or absent [11, 12, 332-334]. Muscle-less mice develop early-stage condensations of tendon progenitor cells that are subsequently lost by embryonic day 13.5, and this loss may be attributed to a lack of mechanical stimulation from the developing muscles [11]. While movement in the embryo is important for tissue formation, the effects of mechanical stimulation on postnatal tendon development are not as well understood, partly because changes in postnatal movement patterns and tissue formation have not been characterized.

Throughout postnatal development, the mechanical loads that tendons experience are likely to increase as locomotion and weight-bearing behaviors increase. However, it remains unknown how the development of weight-bearing movement and locomotion in the neonate is associated with the functional development of tendons. Existing research has focused mainly on adult tendon; one study found that daily running uphill significantly increased the elastic modulus and maximum stress of the AT [116]. Tendons may also adapt to increased mechanical loading in adolescent athletes with increased stiffness [335]. Restriction of motion negatively impacts adult tendon mechanical properties in humans [336], and in normal [337] and healing [338] rats. Based on these studies, we propose that developmental changes in locomotion and weight-bearing increase loading of the musculoskeletal system, and contribute to the increased mechanical properties of developing postnatal tendons. In this study, we explored the relationship between the mechanical properties of neonatal rat tendons and their locomotor behavior. Developing rats are an ideal model system because gradual and significant changes occur in spontaneous posture and locomotion during the first two postnatal weeks. Movement patterns shift from limited loading of the hindlimbs (i.e., crawling) at birth to expression of weight-bearing quadrupedal walking by P10 [339]. To date, there is limited information on the development of locomotor behavior in rats before P10.

We aimed to characterize the relationship between changes in spontaneous locomotor behavior and mechanical properties of tendon. We hypothesized that developmental changes in locomotion and weight-bearing influence the mechanical properties of tendon in postnatal rats. To test this hypothesis, we measured the structural (maximum force, displacement at maximum force,

stiffness, cross-sectional area) and material (maximum stress, strain at maximum stress, elastic modulus) properties of ATs and TTs, and the onset of weight-bearing locomotion as a function of age in neonatal rats at P1, P5, and P10. Evaluating weight-bearing and non-weight-bearing tendons allowed us to compare changes in structural and material properties of tissue subjected to different degrees of *in vivo* mechanical stimulation at the onset of locomotion.

Methods and Materials

Evaluation of weight-bearing locomotion

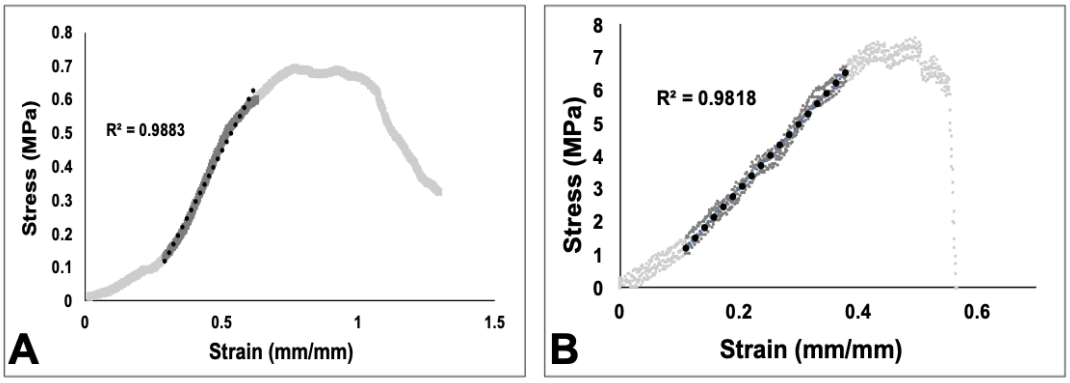
Subjects were offspring from Sprague-Dawley rats maintained in accordance with NIH guidelines (National Research Council, 2011), and the institutional animal care and use committee. To evaluate locomotion, rat pups were tested at P1, P5, or P10 (n=8 subjects per age). Subjects were removed from the home cage with the dam and individually tested in a clear, 8-in by 8-in Plexiglas box (i.e. open-field) to examine spontaneous locomotion. The open-field box was placed inside a temperature- and humidity-controlled incubator to maintain conditions during testing (35°C at P1, 33°C at P5, 30°C at P10). All behavior was video recorded for 20-minutes from a lateral and dorsal camera view, and stored on DVD. Following behavioral testing, subjects were euthanized via CO_2 inhalation, the hindlimbs and tails were removed, packaged in saline-soaked gauze, and stored at -80° C.

Scoring of spontaneous locomotor behavior was conducted during video-playback using Datavyu (Version 1.3.4; Datavyu Team, 2014). Locomotor behaviors scored included non-weight-bearing hindlimb activity (i.e., hindlimb kicking, pivoting, crawling with inactive hindlimbs), partial weight-bearing of the hindlimbs (i.e., partial rearing and hindlimb-active crawling), and full weight-bearing hindlimb activity (i.e., walking, standing, and full rearing). One person scored all of the videos. Intra- and interrater reliability with a standard file was >90%. Differences in durations of each behavior category with developmental age were analyzed with one-way analysis of variance (ANOVA) and Tukey's post-hoc test. Significance was set at $p<0.05$.

Evaluation of mechanical properties

To evaluate tendon mechanical properties, one AT per animal from P1 (n=4), P5 (n=5-7), and P10 (n=6-11) Sprague-Dawley rats was thawed and dissected in saline. Ranges in n-numbers (P5 and P10 ATs) are due to some missing images preventing calculation of cross-sectional area and subsequent

determination of material properties, though structural properties were still obtained. After the skin was removed, the muscles and fascia were teased away from the tibia and the fibula, exposing the AT. The tendon was isolated from the limb while maintaining the myotendinous junction at the gastrocnemius muscle and the insertion at the calcaneus. The myotendinous junction and calcaneus bone were mounted facing anteriorly into our custom small-scale tensile load frame with cyanoacrylate [89]. A 500 g capacity load cell (Honeywell, Columbus, OH) measured force, and custom LabVIEW software (National Instruments, Austin, TX) recorded force and displacement data. Front and side view images of each tendon were obtained using a digital camera (Thorcam DCC1645C, Thorlabs, Inc, Newton, NJ). Tendon length and width were measured using ImageJ (NIH, Bethesda, MD). ATs are wider mediolaterally than anteroposteriorly, so consistent with previous studies [340], ATs were assumed to have an elliptical cross-section. Cross-sectional area was calculated using the narrowest front and side view width of the ATs, whose longitudinal location coincided in both views. ATs were preloaded to 0.05 N, preconditioned with 10 cycles of loading to 1% strain, and pulled to failure at 0.1 mm/s. ATs and TTs were kept hydrated with saline throughout testing. Maximum stress, strain at maximum stress, linear elastic modulus, and linear stiffness were calculated from the force-displacement curves and cross-sectional areas. The linear region was determined from the slope of a line fit to curves that had a $R^2 > 0.90$ ($R^2 = 0.976 \pm 0.023$, supplementary figure S6.1). Differences with developmental age were evaluated using a one-way ANOVA and Tukey's post-hoc test.



Supplementary Figure 6.1 Linear regions of postnatal rat Achilles and tail tendon stress-strain curves.

A) Representative stress-strain curve of a P10 Achilles tendon with the linear region selected (dark gray) and the modulus fit showing (dotted line). B) Representative stress-strain curve of a P10 tail tendon with the linear region selected (dark gray) and the modulus fit showing (dotted line). R^2 values are shown for the trendlines.

TTs from P5 (n=7) and P10 (n=8) rats were dissected in saline using methods previously described [325]. Briefly, whole TTs were grasped at the proximal end of the skinned tail and gently removed. TTs were secured with cyanoacrylate into cardboard c-clamps to hold the tissues during mounting in the load frame. The c-clamps were cut following mounting. TTs were measured using ImageJ as described for ATs, but TTs were assumed to have a circular cross-section based on previous studies [325, 341] and using the narrowest diameter (measured three times and averaged) from the side view of the mounted TTs. A preload of 0.01 N for P5 tendons and 0.05 N for P10 TTs was applied to remove the slack. TTs were not preconditioned due to their fragility. TTs were pulled to failure at 0.1 mm/s and force was measured using a 150 g capacity load cell (Honeywell). TTs were evaluated with unpaired, two-tailed t-tests. Significance was set at $p<0.05$.

Results

Weight-bearing locomotor behavior increases with age

Spontaneous locomotion increased from P1 to P10 (Figure 6.1 A-D). Durations of locomotion significantly increased from P1 to P10 ($p=0.002$) (Figure 6.1 A). P10 rats showed significantly more non-weight-bearing hindlimb activity compared to P1s ($p=0.003$) and P5s ($p=0.005$) (Figure 6.1B). Specifically, P10 rats showed significantly more pivoting than P1s ($p=0.001$) and P5s ($p=0.001$). Differences in hindlimb kicks and crawling (without hindlimbs) across ages were not significant. Partial weight-bearing hindlimb activity increased significantly ($p<0.001$) during the first 10 postnatal days, with P10s exhibiting significantly more hindlimb-active crawling than P1s ($p=0.001$) and P5s ($p=0.003$) (Figure 6.1 C). Only P10 showed partial rearing. For full weight-bearing hindlimb locomotion, the effect of age was not significant ($p=0.140$); however, only P10 pups showed any full weight-bearing behavior (Figure 6.1 D). There were no significant differences in any category of weight-bearing locomotion between P1 and P5.

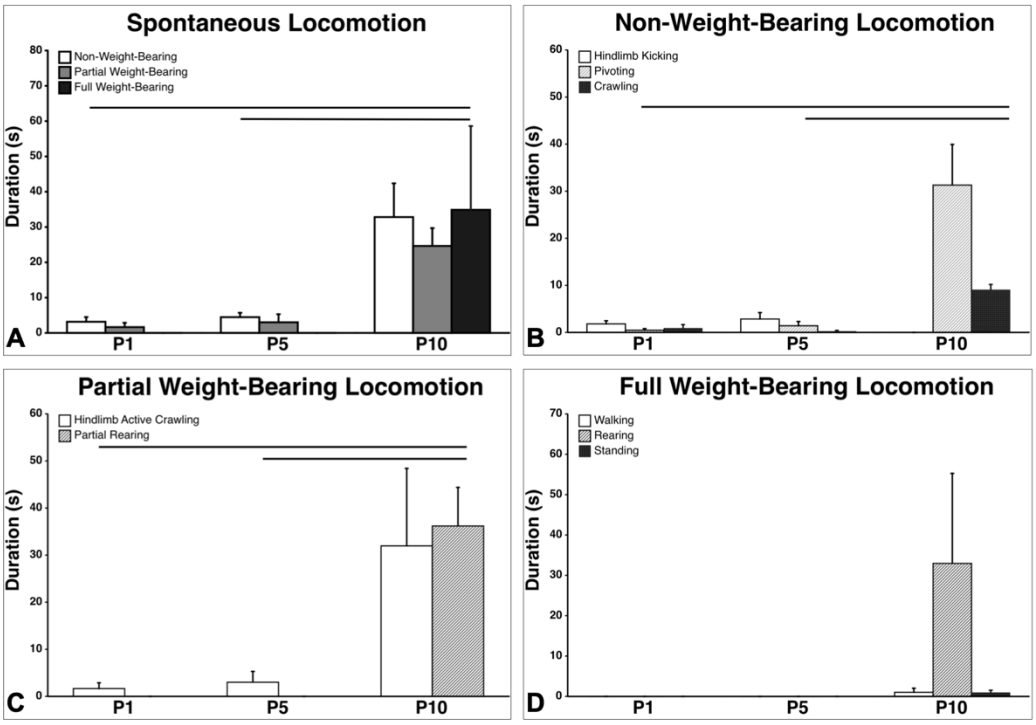


Figure 6.1 Spontaneous locomotion in postnatal rats, when tested in an open field.

A) Durations of non, partial, and full weight-bearing spontaneous locomotion across developmental age. B) Categories of non-weight-bearing locomotion (hindlimb kicking, pivoting, and crawling with inactive hindlimbs). C) Categories of partial weight-bearing locomotion (hindlimb active crawling and partial rearing). D) Categories of full weight-bearing locomotion (walking, full rearing, and standing). Only P10 rats showed any full weight-bearing behavior. Lines denote significant differences between groups. Bars show mean durations; vertical lines depict SEM.

Achilles tendon mechanical properties

Average mechanical properties of rat tendons and gauge lengths are listed in Table 6.1. All samples included in the data analysis failed at the midsubstance. Representative force-displacement and stress-strain curves of P1, P5, and P10 ATs showed changes with age (Figure 6.2 A, 6.3 A). The maximum force, displacement at maximum force, stiffness, and cross-sectional area of ATs increased as a function of age (Figure 6.2 B, C, D, E). Maximum force for P10 ATs was significantly higher compared to P1 ($p=0.0001$) and P5 ($p=0.0004$) (Figure 6.2 B). Displacement at maximum force was significantly higher for P10 ATs compared to P1 ($p=0.0032$) and P5 ($p=0.0046$) (Figure 6.2 C). Stiffness was significantly higher for P10 ATs compared to P1 ($p=0.03$) (Figure 6.2 D). Cross-sectional area of P10 ATs was significantly larger than P1 ($p=0.0023$) and P5 ($p=0.0016$) ATs (Figure 6.2 E). Elastic modulus of P10 ATs trended lower than P5 ($p=0.06$), while maximum stress and strain at maximum stress remained consistent between age groups (Figure 6.3 B, C, D).

Table 6.1 Mechanical properties of P1, P5, and P10 rat ATs, and P5 and P10 rat TTs (mean +/- standard deviation)

Tendon	Max force (N)	Displacement at max force (mm)	Stiffness (N/mm)	Max stress (MPa)	Strain at max stress (mm/mm)	Elastic modulus (MPa)	Cross-sectional area (mm^2)	Mechanical testing sample gauge length (mm)
P1 AT	0.31±0.16	0.63±0.22	0.68±0.43	0.56±0.34	0.47±0.21	1.64±1.14	0.61±0.31	0.89±0.46
P5 AT	0.91±0.27	0.92±0.34	1.66±0.78	1.04±0.030	0.50±0.18	2.64±1.02	0.66±0.62	1.55±0.29
P10 AT	2.65±1.03	2.04±0.82	2.18±1.15	0.94±0.66	0.68±0.28	1.21±0.60	3.50±1.45	3.17±1.40
P5 TT	0.30±0.18	1.59±0.74	0.26±0.16	1.15±0.55	0.61±0.29	2.66±1.57	0.27±0.12	2.89±1.23
P10 TT	0.66±0.17	1.69±0.58	0.50±0.21	3.23±2.33	0.40±0.19	10.33±6.48	0.31±0.15	4.76±1.63

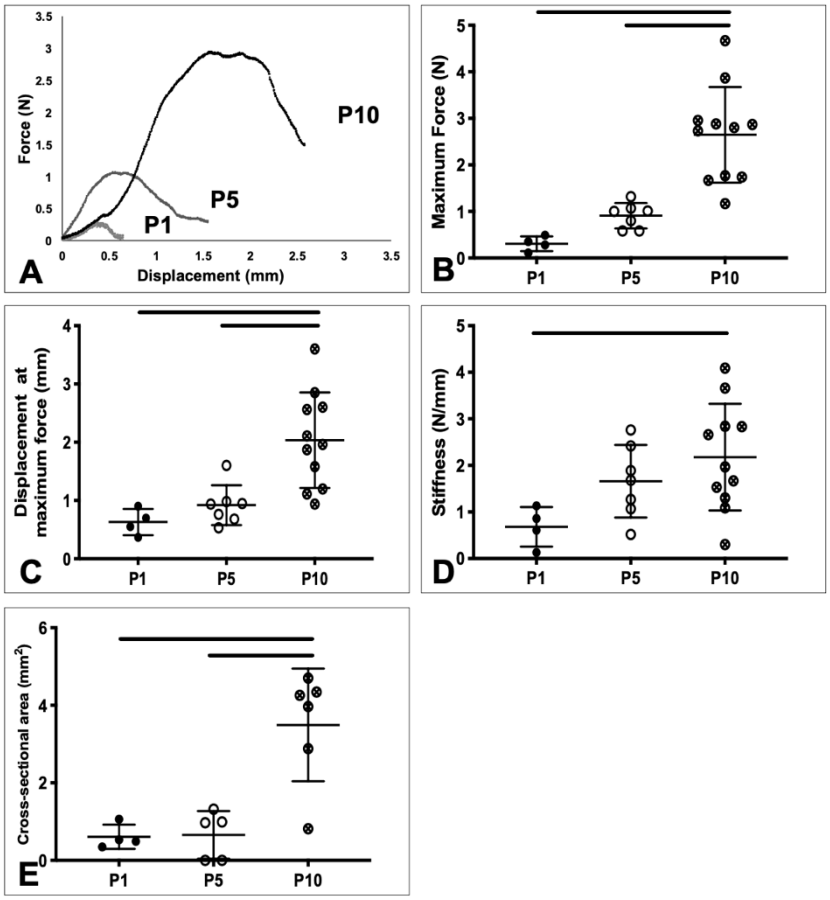


Figure 6.2 Postnatal rat Achilles tendon structural properties.

A) Representative force-displacement curves of P1, P5, and P10 Achilles tendons. B) Maximum force, C) displacement at maximum force, D) stiffness, and E) cross-sectional area. P10 Achilles tendons had significantly increased structural properties, compared to P1 and P5. Lines denote significant differences between groups. Bars represent mean ± standard deviation.

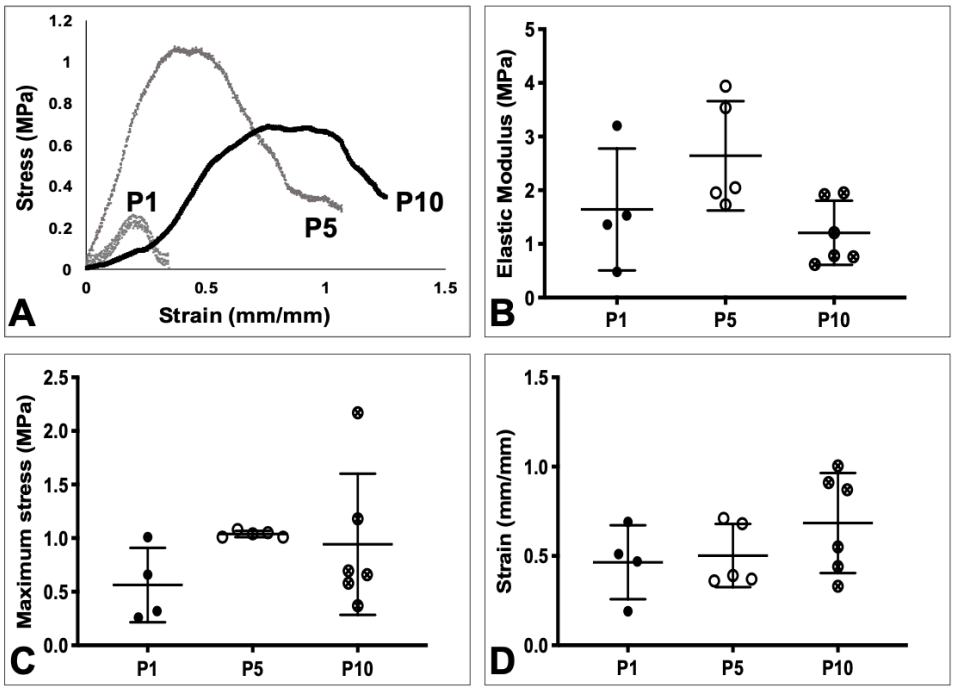


Figure 6.3 Postnatal rat Achilles tendon material properties.

A) Representative stress-strain curves of P1, P5, and P10 Achilles tendons. B) Elastic modulus, C) maximum stress, and D) strain at maximum stress. P10 Achilles tendon material properties remained relatively constant between groups. Bars represent mean ± standard deviation.

Tail tendon mechanical properties

Representative force-displacement and stress-strain curves of TTs showed increases from P5 to P10 (Figure 6.4 A, 6.5 A). The maximum force ($p=0.0015$), stiffness ($p=0.027$), elastic modulus ($p=0.009$), and maximum stress ($p=0.039$) of P10 TTs were significantly higher than the corresponding properties in P5 TTs (Figure 6.4 B, 6.4 D, Figure 6.5 B, C). No significant differences were observed for displacement at maximum force, cross-sectional area, and strain at maximum stress between age groups (Figure 6.4 C, E, Figure 6.5 D).

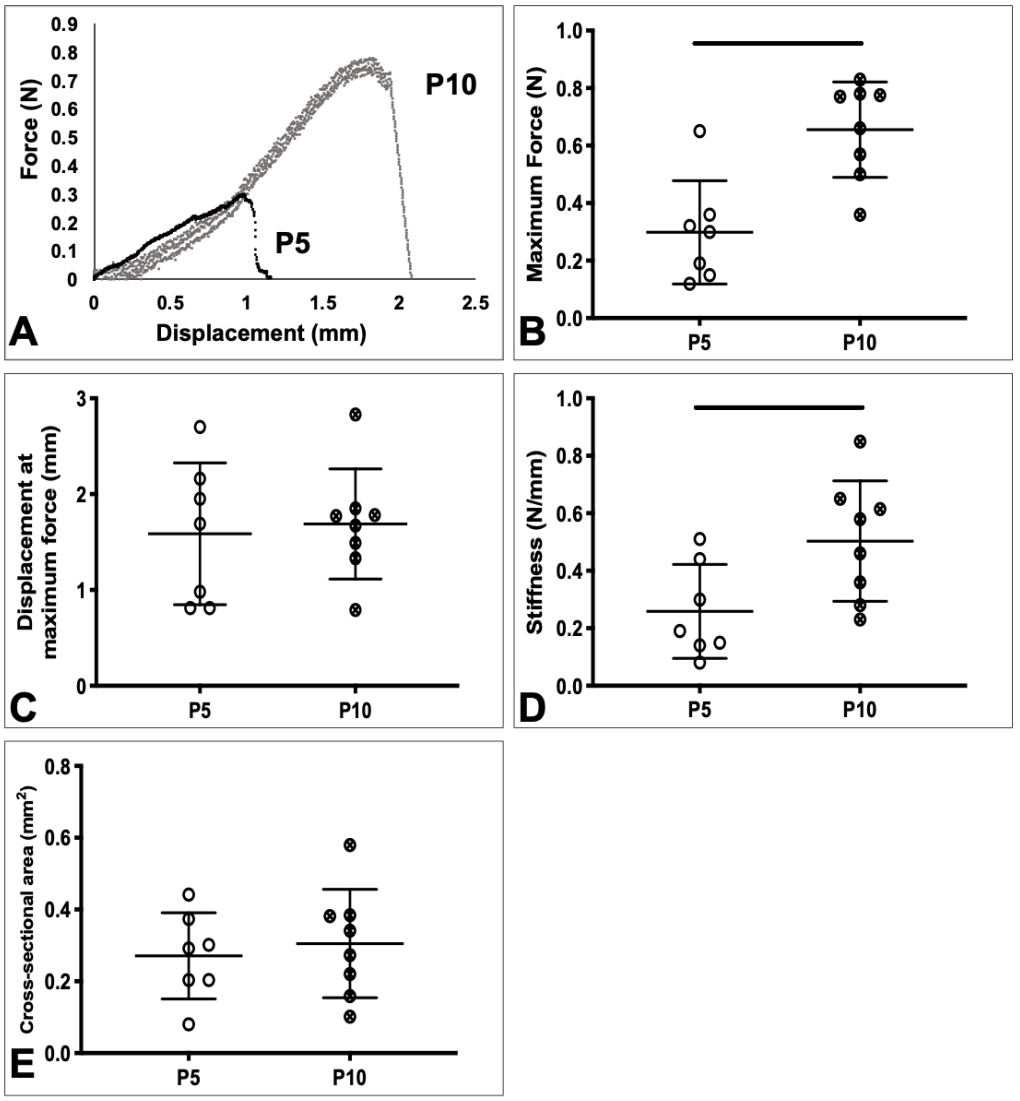


Figure 6.4 Postnatal rat tail tendon structural properties.

A) Representative force-displacement curves of P5 and P10 tail tendons. B) Maximum force, C) displacement at maximum force, D) stiffness, and E) cross-sectional area. P10 tails tendons had significantly increased structural properties, compared to P5 tail tendons. Lines denote significant differences between groups. Bars represent mean ± standard deviation.

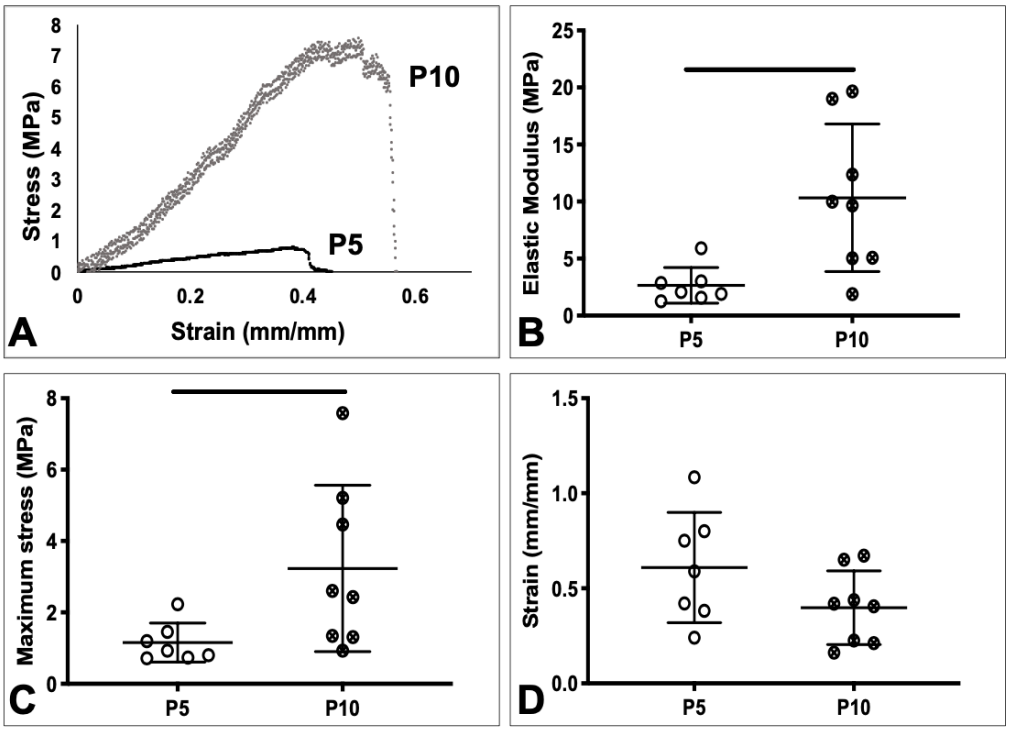


Figure 6.5 Postnatal rat tail tendon material properties.

A) Representative stress-strain curves of P5 and P10 tail tendons. B) Elastic modulus, C) maximum stress, and D) strain at maximum stress. P10 tails tendons had significantly increased material properties, compared to P5 tail tendons. Lines denote significant differences between groups. Bars represent mean ± standard deviation.

Discussion

An attractive approach for tendon tissue engineering involves mimicking native tissue development to produce functional constructs. A challenge has been limited information on how the mechanical function of tendon forms during development, and the role mechanical loading plays in tendon formation. Several developmental processes, including the onset of locomotion, may regulate tendon formation. In this study, we examined how the onset of weight-bearing locomotion coincides with changes in the structural and material properties of weight-bearing ATs and non-weight-bearing TTs. We found increases in hindlimb weight-bearing locomotion behavior beginning between P1 and P10. At the same time, structural properties (maximum force, displacement at maximum force, stiffness, and cross-sectional area) of ATs increased significantly at P10, compared to P1 and P5, whereas material properties (maximum stress, strain at maximum stress, and elastic modulus) did not. Structural and material properties of TTs increased between P5 and P10. These findings suggest that increased locomotor behavior coincides with neonatal AT growth and maintained material properties,

and that the developmental mechanisms of the non-weight-bearing TTs may be unique from the weight-bearing ATs.

Increased locomotion initiating between P1 and P10 may provide the mechanical loading needed for regulating tendon growth. Narrowing down a specific time point during which important developmental changes might arise is novel information that can aid in the search for potential biomechanical cues to direct tenogenesis *in vitro*, or to stimulate repair *in vivo*. Previous characterization of age-related locomotor patterns during postnatal development showed quadrupedal walking is not consistent in rats until P10, and gradually increases in duration throughout the second postnatal week [339]. The current finding that full weight-bearing locomotion was higher in P10s than at earlier ages supports this, suggesting that the developmental range in which significant mechanical stimulation of hindlimb tendons begins may be quite small, and initiated by mechanical cues from this new movement pattern. Future work should examine the correlation between AT mechanical properties and weight-bearing locomotion in rats during the second and third postnatal weeks (i.e., time points with increased weight-bearing locomotion), to investigate dose effects related to mechanical loading. Experimental manipulation of weight-bearing locomotion during the postnatal period would further elucidate the coordination between locomotor behavior and musculoskeletal development.

Changes in structural and material properties of neonatal tendon during the days immediately following birth are less understood. To our knowledge, this is the first study assessing the mechanical properties of rat ATs and TTs at these early postnatal ages. In rat ATs, we found significant increases in structural properties, specifically maximum force and displacement at maximum force, while no significant differences were found in the material properties (maximum stress, strain at maximum stress, and elastic modulus). Our AT results are consistent with another study that found no significant differences in the material properties of normally developing mouse ATs from P7 to P10 [39]. The significant increase in AT cross-sectional area at P10, compared to both P1 and P5 (Figure 6.2 E), could explain why material properties were unchanged. It is possible that increased mechanical stimuli from initiation of weight-bearing behavior lead to lateral tissue expansion, increasing cross-sectional area. The differences we observed in AT dimensions and structural mechanical properties coincide temporally with changes in the collagen of rat ATs [342]. At P4, extracellular matrix in the ATs became denser, and long parallel fibers with defined crimping were visible by P7, though at the nanoscale, collagen fibril diameter did not appear to increase until P14 [342]. Similarly, collagen fibril diameter in postnatal mouse AT was significantly increased at P21, compared to P4, 7, and 10 [39]. Together, these findings indicate that neonatal ATs are still immature

and the collagen structure is dynamic and developing. Weight-bearing loading may induce rapid lateral expansion in neonatal ATs and hence increase the dimension-dependent structural properties by P10, but underlying material properties have not yet been significantly impacted. Collagen cross-linking influences elastic modulus [99, 343], and may be a regulator of material properties in developing ATs. In neonatal ATs, significant increases in dimensions and structural properties may couple with minimal material property changes, to ensure that tendon is responsive to mechanical stimuli before becoming fully cross-linked and less adaptable. Future studies investigating collagen fibril organization, cross-linking, diameter, and density from P1 to P10 and beyond may help to identify impacts of weight-bearing loading on the collagen structure in ATs.

In contrast to AT, both structural and material properties of TTs increased significantly from P5 to P10. Elastic modulus, stiffness, maximum stress, and maximum force were significantly higher in TTs at P10, compared to P5. Since rat TTs are non-weight-bearing, they may experience a smaller increase in mechanical loading as locomotion develops, and hence lateral tissue expansion proceeds more slowly, leading to no significant increase in cross-sectional area at P10. Significant increases in structural and material properties of TTs suggest a more rapid maturation, compared to ATs. Future studies are needed to explore the mechanisms that regulate TT compared to AT development.

Neonatal rat tendons are challenging to mechanically test, as the tissues are small and soft, requiring a small-scale load frame with low force-capacity load cells (e.g., 150 g), leading to some limitations. Tendon isolation and mounting is difficult, and measuring their small cross-sectional areas may be a source of some variability and error. To control for this, all tendons were evaluated in the same way. Cross-sectional areas were measured multiple times and averaged, which reduces error [341]. Neonatal AT is particularly challenging to evaluate. The AT is anterior to and overlaid by the plantaris tendon, and composed of three different bundles [344]. To avoid damaging the tissue, the plantaris tendon was not removed and the whole AT complex was used. Neonatal AT is also relatively short, compared to its width, and may be prone to gripping artifacts. The tibialis anterior (TA), another limb tendon, has an increased length to width ratio, and has been evaluated at P2 [345]. TA tendons may be evaluated in future studies. With regard to the TTs, we could only mechanically evaluate P5 and P10 groups. P1 TTs were too small and fragile to reliably isolate from the tails and mount into our load frame.

Finally, we did not control for sex during this study. At more mature ages, differences have been detected in AT mechanical properties between male and female rodents [346, 347]. At younger ages (4 weeks), body mass, collagen content, elastic modulus, and maximum stress have been shown

to be similar between male and female, and no significant differences were found in TTs [347]. Sex differences of the neonates likely did not affect our results, though future studies will control for sex.

Taken together, increased locomotor behavior coincides with AT growth and maintained material properties, and development of non-weight-bearing TTs may be unique from the weight-bearing ATs. Future studies will manipulate the mechanical environment during development to better understand the impacts of locomotion-associated loading on tendon formation. Future investigations will also explore the mechanisms driven by this interaction, and how the developmental process may be different in weight-bearing and non-weight-bearing tendons. Overall, we found a parallel increase in locomotor behavior and functional tendon formation, narrowing down the postnatal time period during which locomotion may affect tendon development. New information on the postnatal developmental timeline of rat tendon and the relationship between locomotion and tendon mechanical properties will provide insights into how these processes occur during normal development of the entire organism. These findings have implications for directing functional tendon formation in engineered tissues.

Chapter 7: Neonatal Spinal Cord Transection Decreases Hindlimb Weight-Bearing and Affects Mechanical Development of Neonatal Rat Achilles and Tail Tendons.

Theodossiou SK, Pancheri NM, Bozeman AL, Martes AC, Bozeman AL, Brumley MR, Raveling AR, Courtright JM, Schiele NR.

Submitted to *ASME Journal of Biomechanical Engineering.*

Abstract

Tendon tissue engineering approaches are challenged by a limited understanding of the role mechanical loading plays in normal tendon development. Mechanical loading resulting from the development of weight-bearing locomotor behavior may be required for proper tendon formation. This study assessed development of the mechanical properties of energy-storing and positional tendons, and concomitant changes in weight-bearing locomotion, in neonatal rats subjected to a low thoracic spinal cord transection or a sham surgery at postnatal day (P)1. On P10, spontaneous locomotion was video recorded and scored to determine non-weight-bearing, partial weight-bearing, and full weight-bearing hindlimb movement. Achilles tendons (ATs), as energy-storing tendons, and tail tendons (TTs), as positional tendons, were mechanically evaluated. Non- and partial weight-bearing hindlimb activity decreased in spinal-transected rats compared to sham controls. No spinal-transected rats showed full weight-bearing locomotion. ATs from spinal-transected rats had increased elastic modulus, while cross-sectional area trended lower compared to sham rats. TTs from spinal-transected rats had higher stiffness, while the cross-sectional area trended higher. Collagen structure of ATs and TTs did not appear impacted by surgery condition, and no significant differences were detected in the collagen crimp pattern. Our findings suggest that mechanical loading from weight-bearing locomotor activity during development regulates neonatal AT lateral expansion and maintains tendon compliance, and that TTs may be differentially regulated. The onset and gradual increase of weight-bearing movement may provide the mechanical cues directing functional postnatal tendon formation.

Introduction

Tendons are collagenous tissues that transfer mechanical forces from muscle to bone to enable movement and locomotion. Tendons are frequently injured and heal poorly [3], making them an active area of investigation for tissue engineering and regenerative medicine. A number of cellular [34, 52,

53, 348-351], biochemical [52, 53, 57, 58, 226, 348, 349, 352], and mechanical [4, 5, 9, 84, 91, 93-95, 200-203, 327, 353] cues have been found to influence tenogenesis (e.g., tenogenic differentiation and tendon formation) *in vivo* and *in vitro,* but there is a need to further understand how mechanical loading regulates functional tendon formation.

Recent studies have characterized the mechanical properties of tendon throughout embryonic, neonatal, and postnatal development to better understand their formation. Tendon mechanical properties increase throughout development in embryonic chick [9, 40, 83], postnatal mouse [39] and rat [233], and humans [321], but the factors driving these changes remain largely unknown. Mechanical loading is involved in regulating tenogenic differentiation of stem cells, and it impacts collagen production *in vivo* and *in vitro* [5, 91, 167, 354]. Mechanical loading from embryonic movement or the onset of postnatal locomotion may be necessary for functional tendon development. *In vivo*, mechanical loading originates from contractions of the adjacent, concurrently forming skeletal muscles [11], and increases as the embryo or neonate begins to move [12, 102, 106, 233]. Chick embryos subjected to both rigid and flaccid paralysis at embryonic day (E)5.5, 6.5, and 7.5 had downregulated expression of the tenogenic markers, scleraxis and tenomodulin, compared to non-paralyzed controls [52]. E15 chick embryos subjected to rigid paralysis for 48 hours (h) had significantly reduced calcaneal tendon elastic modulus by E17, along with reduced activity of lysyl oxidase, an enzyme that mediates the formation of collagen crosslinks [12]. Conversely, induced hypermotility led to increased elastic modulus in E17 chick calcaneal tendons [12]. In Pax3 knockout mice ($Pax3^{Spd/Spd}$), which lack skeletal muscle, tendon progenitor cells were identifiable before E12.5, but were lost without the mechanical stimulation from the developing muscles [11]. Both acute (e.g., transection) and gradual (e.g., botulinum toxin (Botox) injection) loss of mechanical loading led to apoptotic tendon cell death and loss of scleraxis expression in the Achilles tendons of adult mice [167]. In a different study, Botox unloading led to increased stiffness and collagen deposition in adult rat Achilles tendons [355]. Taken together, these studies highlight the importance of mechanical loading for embryonic tendon development and adult tendon maintenance. However, few studies have assessed the role of mechanical loading during postnatal development.

Postnatal growth in rodents and humans is associated with the development of full weight-bearing locomotor behavior [233, 339], suggesting that the developing tendons are exposed to increasingly high levels of mechanical loading postnatally, especially compared to embryonic ages. Though there are limited studies on how this locomotor development impacts postnatal tendon formation, mechanical loading has been explored in the postnatal maturation of the enthesis (the tendon-to-bone attachment). Mice receiving a Botox injection in their supraspinatus muscle at birth had

decreased muscle volume and bone mineralization, and delayed fibrocartilage development in the enthesis at postnatal day (P)21, 28, and 56, compared to controls that received saline injections [102]. In another study, enthesis development was disrupted in mice that received daily Botox injections in their supraspinatus from birth (to paralyze the surrounding muscles), compared to non-injected controls at P28 and P56 [101]. Maximum force and stiffness of the supraspinatus attachment decreased following 4 and 8 weeks of Botox unloading, compared to controls, while tendon cross-sectional area, tensile strength, elastic modulus, collagen fibril alignment, and toughness were also decreased [101]. Furthermore, cellular formation of the enthesis is disrupted with Botox unloading in postnatal mice. Unloading significantly increased the numbers of Hedgehog (Hh)-responsive cells within the enthesis of P7 to P56 animals, resulting in decreased fibrocartilage mineralization and disrupted enthesis formation [106]. Results of these studies suggest that enthesis and tendon development require the mechanical loading associated with normal movement.

We recently identified that increases in neonatal rat tendon mechanical properties coincide with the increased locomotor behavior that occurs during the first 10 postnatal days [233]. Interestingly, the observed increases in mechanical properties differed between the energy-storing, weight-bearing Achilles tendons (AT) and positional, non-weight-bearing tail tendons (TT), possibly due to the different mechanical loads these tendons experience as locomotion develops. Structural (maximum force, displacement at maximum force, stiffness, and cross-sectional area) and material (maximum stress, strain, elastic modulus) properties increased at different rates in the different tendon types. Energy-storing ATs displayed significant increases in their structural properties during the first 10 postnatal days, while positional TTs had increased structural and material properties between P5 and P10. Specifically, in ATs, maximum force, displacement at maximum force, and cross-sectional area increased at P10, compared to P5 and P1, whereas in TTs, maximum force, maximum stress, elastic modulus, and stiffness increased from P5 to P10. These changes coincided with a significant increase in weight-bearing locomotion in P10 rats, compared to P1 and P5 [233]. Overall, our previous results suggest that increased mechanical loading from the postnatal development of weight-bearing locomotor behavior may differentially impact functionally distinct tendons.

Collectively, prior studies indicate that increased mechanical loading, possibly from the developing locomotor behavior, may be contributing to the changes observed in the postnatal tendons, and that tendons may respond to this loading differently based on their function. Disruption in the development of locomotor behavior may lead to changes in tendon mechanical properties, but how the onset of weight-bearing locomotion may affect developing neonatal tendon mechanical properties remains unknown. We hypothesized that altering locomotion, and hence mechanical loading, would

impact neonatal tendon development. To test this hypothesis, we evaluated the collagen structure and measured the structural (maximum force, displacement at maximum force, stiffness, cross-sectional area) and material (maximum stress, strain, elastic modulus) properties of energy-storing ATs and positional TTs in P10 rats, following a neonatal spinal cord transection or sham surgery at P1. Prior research has shown that a neonatal spinal transection significantly reduces, but does not eliminate, hindlimb locomotor behavior in rats during early postnatal development [356]. Thus, we also assessed changes in full, partial, and non-weight-bearing hindlimb movement during spontaneous open-field locomotion on P10. Disrupting locomotor development in neonates and assessing the impact on postnatal tendon mechanical properties provides novel insights into how the mechanical function of tendon emerges during development.

Methods and Materials

Animals and spinal cord surgery

Subjects were female offspring of Sprague-Dawley rats acquired from Simonson laboratories. Adult animals were socially housed and mated. Pregnant females were individually housed and closely monitored during the week they were scheduled to deliver. On P1 (postnatal day 1; ~24 h after birth), litters were culled to 8 pups. At that time, all pups remaining in the litter received a low thoracic spinal cord transection, or a sham surgery. All pups within a litter received the same surgical treatment. On P10, one female pup from a litter was randomly selected for behavioral testing. The animal colony room was maintained in accordance with the NIH, Institutes on Laboratory Animal Resources, and Idaho State University, Institutional Animal Care and Use Committee guidelines. Animals were provided with *ad libitum* access to food and water and the room was kept on a 12h light:dark cycle.

To begin surgery on P1, rat pups were voided, weighed, and anesthetized via hypothermia. A small incision was made to expose the lower thoracic and lumbar spine, and a partial laminectomy was performed between T8 and T10. For animals in the transection group, the spinal cord was completely cut at one spinal level (between T8-T10) and a collagen matrix was injected into the injury site. Rat pups in the sham surgery group underwent all procedures except the transection cut and collagen insertion. In both groups, animals received internal and external sutures and subcutaneous injections of 0.04 mg/kg of buprenorphine (50 μl) for analgesia and 9% saline to maintain internal fluid balance. Pups were placed in an infant incubator to recover before being returned to the home cage with the dam. The total time of separation from the dam was ~40 min. Pups remained with the dam in their home cage until behavioral testing occurred on P10, and were monitored regularly to ensure recovery.

Evaluation of weight-bearing locomotion

On P10, a total of 16 female rat subjects (8 spinal-transected + 8 sham) were tested in an open field for measurement of spontaneous locomotion. Individual rat pups were placed in an 8" x 8" x 8" Plexiglas box that was located inside a 30 °C infant incubator to maintain temperature. After a 30-min acclimation period, spontaneous locomotion was recorded for 20 min from a lateral camera angle. Following testing, subjects were euthanized via CO_2 inhalation, and hindlimbs and tails were dissected and stored at -80 °C.

Locomotion was assessed from video records using the scoring program Datavyu (version 1.3.4; Datavyu Team, New York, NY). The duration of hindlimb locomotor behavior was evaluated and categorized as full weight-bearing, partial weight-bearing, or non-weight-bearing [29]. Full weight-bearing behaviors include standing and walking (plantar contact with all 4 paws and belly off the ground) or full rearing (standing on hindlimbs with both forelimbs off the ground and head above the abdomen). Partial weight-bearing behaviors include hindlimb-active crawling (hindlimbs used to facilitate movement and belly in contact with the ground) and partial rearing (one forelimb off the ground and head above the abdomen). Non-weight-bearing behaviors include crawling (hindlimbs inactive), pivoting (torso and head moving while hindlimbs remain anchored), and hindlimb kicking. The categorization of these behaviors depended on the degree to which the hindlimbs supported the weight of the animal. The duration of each of these behaviors was scored and summed over the entire 20-min video. Intra- and inter-rater reliability was >90%. Unpaired t-tests were used to assess differences in duration of non-, partial, and full weight-bearing locomotion between sham and spinal-transected rat subjects on P10. The independent variable was surgical condition, and the dependent variables were the durations of the weight-bearing behaviors listed above. Significance was set at $p < 0.05$.

Evaluation of tendon mechanical properties

Mechanical evaluation was conducted as previously described [233]. Briefly, one AT per animal from P10 (n=11-14 sham, 9-11 spinal) female rats was isolated, mounted and secured with cyanoacrylate in the grips of our small-scale tensile load frame [89]. A 500 g capacity load cell (Honeywell, Columbus, OH) was used to measure force, and a custom LabVIEW program (National Instruments, Austin, TX) recorded force and displacement data. A digital camera captured front and side view images of each tendon (Thorcam DCC1645C, Thorlabs Inc., Newton, NJ). Tendon length and width were measured using ImageJ (NIH, Bethesda, MD) to calculate gauge length and cross-

sectional area, respectively. ATs were assumed to have an elliptical cross-section [340], which was calculated using the narrowest front and side view width of the AT, whose longitudinal location coincided in both views. Ranges in n-numbers (P10 ATs) are due to obtaining only structural properties for some samples due to missing images. ATs were preloaded to 0.05 N, preconditioned with 10 cycles of loading to 5% strain, and pulled to failure at 0.1 mm/s. ATs and TTs were kept hydrated with saline throughout testing. The force-displacement and cross-sectional area data were used to calculate maximum force, displacement at maximum force, stiffness, maximum stress, strain, and elastic modulus. The linear region, for calculation of linear elastic modulus and stiffness, was determined from the slope of a line fit to curves that had a $R^2 > 0.90$ (average $R^2 = 0.961 \pm 0.017$). To assess differences in toe-region elastic moduli and toe-to-linear region transition strain between sham and spinal tendons, a bilinear fit ($R^2>0.90$) was applied to the stress-strain curve [39, 357]. Differences in mechanical properties between sham and spinal conditions were evaluated using unpaired, two-tailed t-tests, with significance set at $p < 0.05$.

Similarly, TTs from P10 (n=10 sham, n=10 spinal) female rats were dissected in saline using methods previously described [233, 325]. Briefly, whole TTs were grasped at the proximal end of the tail and gently separated from the rest of the tail. TTs were secured with cyanoacrylate into cardboard c-clamps to hold the tissues during mounting in the load frame. C-clamps were cut following mounting to allow tensile loading of the TTs. TTs were measured using ImageJ as described for ATs, but were assumed to have a circular cross-section based on previous studies [325, 341], using the narrowest diameter (measured 3 times and averaged) from the side view image of the mounted TTs. A preload of 0.05 N was applied to remove the slack. TTs were not preconditioned due to their fragility. TTs were pulled to failure at 0.1 mm/s and force was measured using a 150 g capacity load cell (Honeywell). TT mechanical properties between sham and spinal conditions were evaluated with unpaired, two-tailed t-tests. Significance was set at $p < 0.05$.

Imaging and evaluation of collagen structure

To visualize collagen structure, P10 ATs (n=4 sham, n=3 spinal) and TTs (n=5 sham and spinal) were imaged via second harmonic generation (SHG) [358] on an Olympus FluoView 1000 Multiphoton Confocal Microscope (Olympus, Tokyo, Japan). One TT and one AT, left or right, from each animal was dissected as described for mechanical testing, and fixed overnight at 4^{o}C in 10% formalin. Following fixation, and to image the AT, the myotendinous junction and calcaneous bone were trimmed off. Three images were taken for each tendon (left, right, and center position). Distance between crests of the crimp pattern was measured using ImageJ (NIH) in 5 locations per image, and the average measurement was the crimp distance for that image. The average crimp distance of an

image set was used to determine the average crimp distance of the whole tendon. Crimp distance between sham and spinal conditions was evaluated with unpaired, two-tailed t-tests. Significance was set at $p < 0.05$.

Results

Weight-bearing locomotor behavior changes following spinal transection

Overall durations of spontaneous non-, partial, and full weight-bearing locomotion during the open-field test for spinal and sham subjects are shown in Figure 7.1A. Spinal-transected rats showed significantly less non-weight-bearing ($p = 0.017$) and partial weight-bearing ($p = 0.004$) locomotion, compared to sham subjects. No spinal subjects engaged in full weight-bearing behavior, while 6 of the 8 sham subjects did. The duration of full weight-bearing locomotion trended higher ($p = 0.055$) in sham rats (Figure 1), though differences were not significant.

Durations of non-weight-bearing locomotor activities are seen in Figure 7.1B. There were no significant differences in duration of hindlimb kicks ($p = 0.456$) or crawling ($p = 0.296$) between sham and spinal subjects. However, spinal pups showed significantly less pivoting compared to shams ($p = 0.003$). Within partial weight-bearing behaviors (Figure 7.1C), spinal pups showed significantly less hindlimb-active crawling ($p = 0.035$), and less partial rearing ($p = 0.022$) than shams. Within the full weight-bearing category (Figure 7.1D), there were no differences in walking ($p = 0.115$), standing ($p = 0.149$), or rearing ($p = 0.141$) between sham and spinal subjects, although full weight-bearing was only shown by shams. .

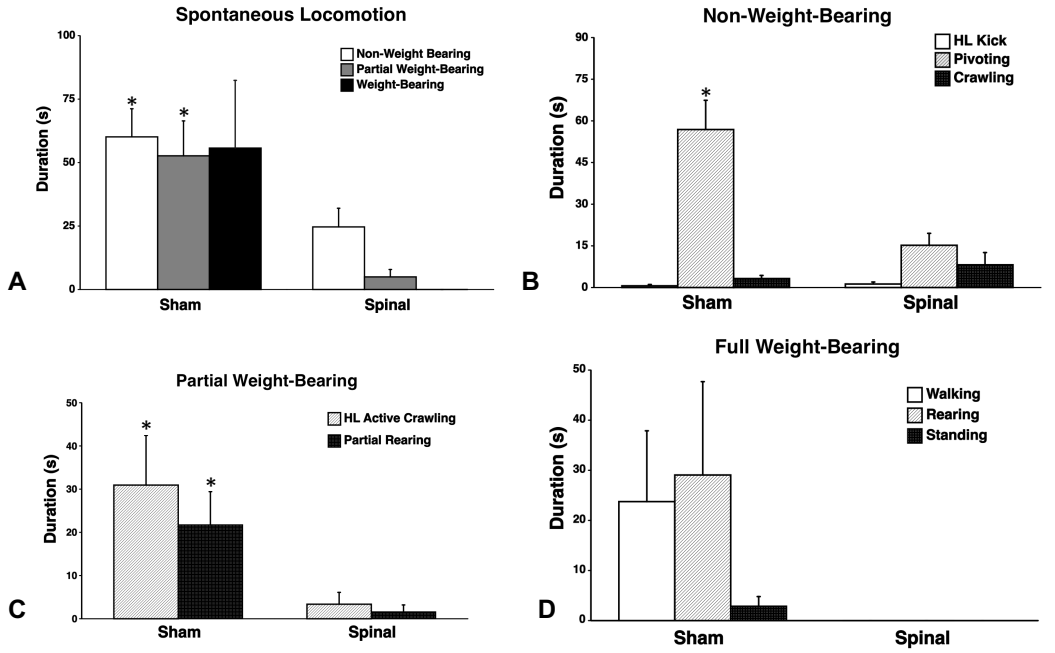


Figure 7.1 Duration of spontaneous locomotion in the open field by sham and spinal female P10 rats.

(A) Spinal rats (n=8) showed significantly less overall spontaneous non- and partial weight-bearing locomotion compared to sham rats (n=8). (B) Within the category of non-weight-bearing behavior, spinal rats showed significantly less pivoting compared to shams. (C) For partial weight-bearing, both hindlimb-active crawling and rearing were significantly reduced in spinal rats. (D) For full weight-bearing activity, only sham rats showed any behaviors in this category, including walking (6 out of 8 subjects). Bars show group mean durations; vertical lines show SEM. * $p < 0.05$.

Achilles tendon mechanical properties

Average mechanical properties of rat tendons are listed in Table 7.1. Representative force-displacement and stress-strain curves for sham and spinal P10 ATs are shown in Figure 7.2A, B. ATs from spinal-transected rats had increased linear region elastic modulus ($p = 0.042$), compared to the sham condition (Figure 7.3G), while cross-sectional area trended lower ($p = 0.05$) (Figure 7.3C). Maximum force ($p = 0.84$), displacement at maximum force ($p = 0.52$), linear region stiffness ($p = 0.19$), toe region elastic modulus ($p = 0.13$), maximum stress ($p = 0.22$), strain at maximum stress ($p = 0.62$), and transition strain ($p = 0.55$) were consistent between the sham and spinal subjects (Figure 7.3A, B, D, E, F).

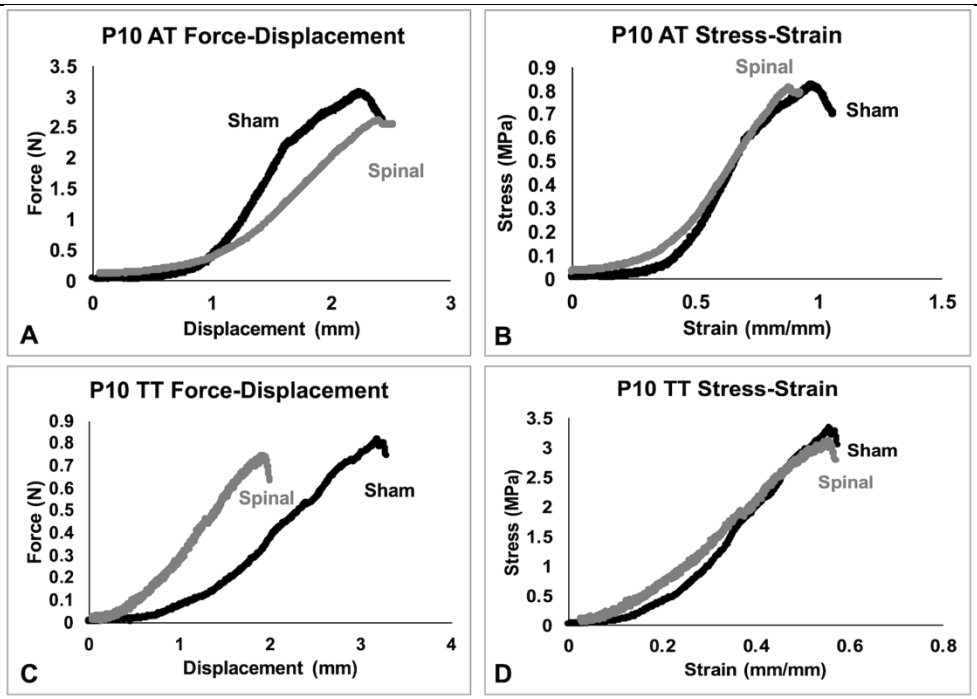


Figure 7.2 Representative structural and material property curves.

(A, B) Representative force-displacement and (C, D) stress-strain curves for ATs and TTs from sham and spinal P10 rats.

Tail tendon mechanical properties

Representative force-displacement and stress-strain curves for sham and spinal P10 TTs are shown in Figure 7.2C, D. TTs from spinal-transected rats had higher linear region stiffness ($p = 0.03$; Figure 7.4D), and their cross-sectional area trended higher ($p = 0.05$) (Figure 7.4C). Maximum force ($p = 0.73$), displacement at maximum force ($p = 0.19$), toe ($p = 0.26$) and linear region elastic modulus ($p = 0.12$), maximum stress ($p = 0.11$), strain at maximum stress ($p = 0.31$), and transition strain ($p = 0.42$) were consistent between sham and spinal subjects (Figure 7.4A, B, E, F, G, H).

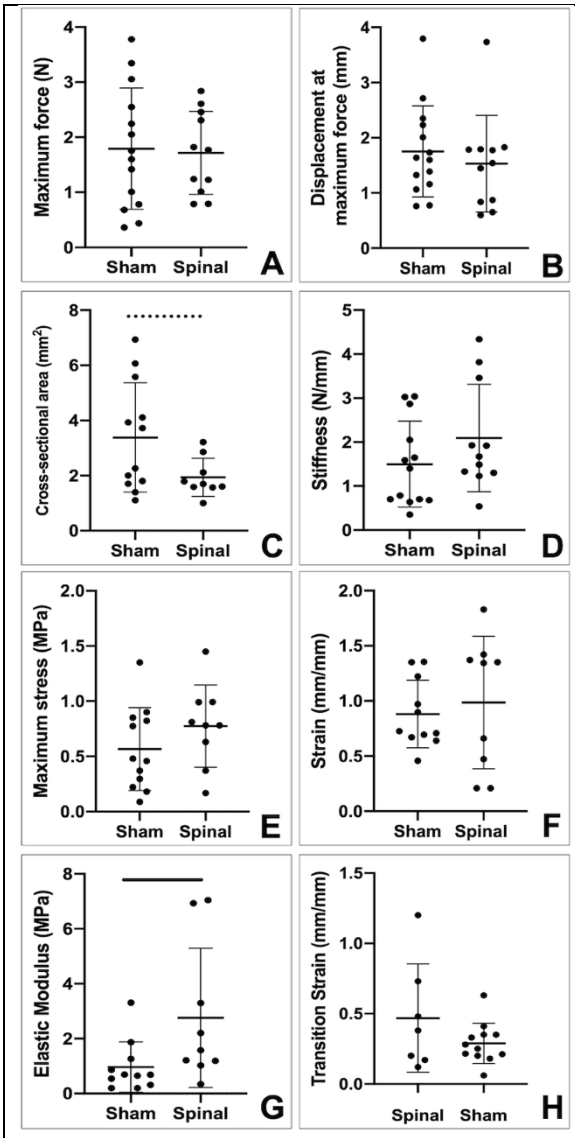


Figure 7.3 AT mechanical properties.

(A) Maximum force, (B) displacement at maximum force, (C) cross-sectional area, (D) stiffness, (E) maximum stress, (F) strain at maximum stress, (G) elastic modulus, and (H) transition strain for sham and spinal conditions. ATs from spinal transected rats had significantly higher linear region elastic modulus and cross-sectional area trended lower, compared to sham controls. Lines denote significant differences between groups. Bars represent mean ± standard deviation.

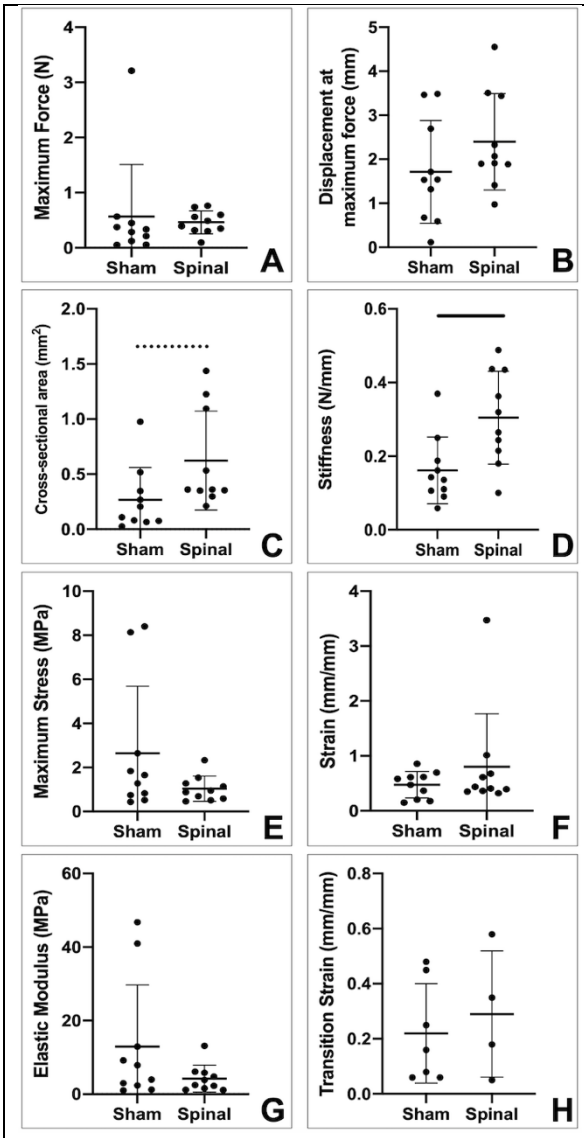


Figure 7.4 TT mechanical properties.

(A) Maximum force, (B) displacement at maximum force, (C) cross-sectional area, (D) stiffness, (E) maximum stress, (F) strain at maximum stress, (G) elastic modulus, and (H) transition strain for sham and spinal conditions. TTs from spinal transected rats had significantly higher linear region stiffness and cross-sectional area trended higher, compared to sham controls. Lines denote significant differences between groups. Bars represent mean ± standard deviation.

Collagen structure

Collagen structure of ATs and TTs did not appear to be impacted by the surgery condition (Figure 7.5A-D). Statistical analysis showed there were no significant differences in crimp distance between sham and spinal conditions in ATs ($p = 0.78$) and TTs ($p = 0.72$) (Figure 7.5E, F), or between sham ATs and TTs ($p = 0.50$) or spinal ATs and TTs ($p = 0.48$).

Table 7.1 Mechanical properties of P10 sham and spinal rat ATs and TTs (mean +/- standard deviation)

Tendon	Max force (N)	Displacement at max force (mm)	Stiffness (N/mm)	Max stress (MPa)	Strain at max stress (mm/mm)	Linear region elastic modulus (MPa)	Toe region elastic modulus (MPa)	Toe-to-linear region transition strain (mm/mm)	Cross-sectional area (mm^2)	Gauge length (mm)
P10 AT sham	1.79±1.10	1.75±0.83	1.50±0.98	0.57±0.37	0.88±0.31	0.96±0.92	0.57±0.52	0.29±0.14	3.39±1.98	2.12±0.51
P10 AT spinal	1.72±0.75	1.53±0.88	2.09±1.22	0.77±0.37	0.99±0.60	2.76±2.54	0.86±0.56	0.47±0.39	1.94±0.69	1.96±1.13
P10 TT sham	0.57±0.94	1.71±1.17	0.16±0.09	2.65±3.04	0.48±0.24	12.93±16.80	0.95±1.20	0.29±0.23	0.27±0.29	6.47±1.08
P10 TT spinal	0.46±0.21	2.40±1.10	0.30±0.13	1.04±0.57	0.81±0.96	4.22±3.63	2.30±2.87	0.22±0.18	0.59±0.49	6.04±0.99

Discussion

There is a need to enhance our understanding of the relationship between locomotor behavior and tendon development, as the limited information on the role of mechanical loading in tendon formation challenges efforts to engineer functional tendon replacements. In this study, we examined the effects of disrupting weight-bearing locomotion in early postnatal tendon development for two functionally distinct tendon types (e.g., the energy-storing AT and the positional TT). In spinal-transected rats, the amount of non- and partial weight-bearing hindlimb locomotion was lower when compared to sham controls, and no spinal-transected rats showed full weight-bearing locomotion during the study period (Figure 7.1), providing an *in vivo* system to study mechanical regulators of tendon formation. Characterization of this *in vivo* model is useful, as investigating the impacts of loading on tendon formation during postnatal development in other systems is a challenge. Tendon cells harvested postnatally for *in vitro* studies may have altered phenotypes, different characteristics depending on the postnatal isolation day, or de-differentiate when isolated from tendons that are already formed [342]. Additionally, shifts in cell behavior in response to load *in vitro* may not result in readily apparent changes in tissue function at the tendon level. Therefore, using spinal-transection surgery as a model system to disrupt locomotor development in neonates provides insights into how the mechanical function of tendon emerges during development, possibly driven by the typical increases in weight-bearing locomotor behavior.

In P10 ATs, we observed significantly increased elastic modulus and a trend toward lower cross-sectional area ($p = 0.05$) with spinal transection (Figure 7.3G, C). Previously, we found that the cross-sectional area of ATs increased as a function of age and with the onset of locomotor behavior [233]. Taken together, weight-bearing locomotor activity may regulate the lateral expansion of developing neonatal ATs. The increase in elastic modulus of P10 ATs with decreased loading is consistent with a prior study that identified increased elastic modulus in ATs with Botox unloading in 4-month old rats [355]. These findings suggest that decreased mechanical stimulation leads to smaller energy-storing tendons that are less compliant and more rigid. It is also possible that the reduced loading causes the ATs to resemble positional tendons, as positional tendons typically having higher elastic moduli [359-365]. Interestingly, SHG imaging did not identify changes in the collagen crimp distance of ATs between sham and spinal conditions (Figure 7.5A, B). Though unexpected, this finding is consistent with a prior study in embryonic chick calcaneal tendon that found neither paralysis nor hypermotility visibly affected collagen structure [12]. The changes in mechanical properties that follow alterations in the mechanical loading environment during early postnatal development may be due to cellular or biochemical factors, such as enzymatic crosslinking of the collagen fibers [99], rather than changes to the overall collagen structure.

Similarly, P10 TT mechanical properties remained mostly unaltered with spinal transection, with the exception of increased stiffness and a trend towards a larger cross-sectional area in spinal-transected rats (Figure 7.4C, D). This suggests the TTs may experience increased loading demands in the spinal condition as the animals attempt to maintain balance without normal use of their hindlimbs. Thus the TTs may increase in size and stiffness to compensate for the additional mechanical loading, similar to the AT during normal development of weight-bearing locomotion [233]. As with the ATs, SHG imaging showed no changes in the collagen crimp distance of the TTs between sham and spinal conditions (Figure 7.5C, D).

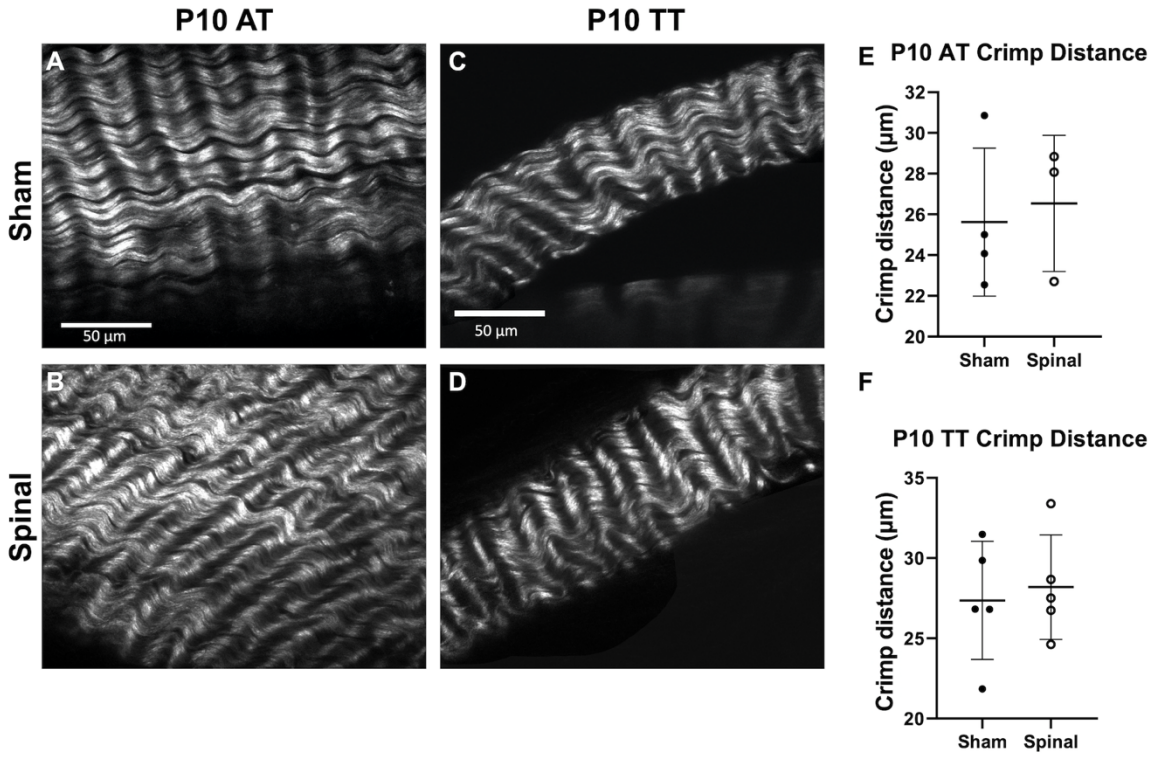


Figure 7.5 SHG images of collagen in neonatal tendons.

Representative SHG images of collagen structure in ATs of (A) sham and (B) spinal transected rats, and in TTs of (C) sham and (D) spinal transected rats. No differences were detected in (E, F) crimp distance between sham and spinal conditions in ATs or TTs, or between ATs and TTs. Scale bar = 50 µm. Bars represent mean ± standard deviation.

Taken together, the spinal surgery and reduced mechanical stimuli influenced both AT and TT postnatal development, but the impact was unique between the two tendon types. With spinal surgery, elastic modulus and cross-sectional area were altered in ATs, whereas stiffness increased in

TTs. One possible explanation for the differential impact on ATs compared to TTs is the physiological function of each tendon. ATs are weight-bearing, energy storing tendons that must withstand large mechanical loads during daily activity. In contrast, TTs are generally regarded as non-weight-bearing, positional tendons, whose main function in rodents is tail positioning [322, 323]. The increase in stiffness of TTs from spinal subjects may be due to the rats using their tail to supplement their impaired locomotion, and hence exposing their tails to increased loads, compared to normal development. Additionally, neonatal ATs may mature more slowly than TTs [233], and thus be more plastic and susceptible to manipulations of the loading environment at P10. Other studies demonstrated that neonatal ATs regenerate following a transection and subsequent unloading [157]. While we did not induce a tendon injury, an unidentified regenerative mechanism in neonates may be preventing more pronounced changes in mechanical properties following tendon unloading due to the spinal injury-induced decrease in movement. As weight-bearing tendons that may require exposure to mechanical stimulation to develop mechanical function, ATs may depend on the onset of normal locomotion, and hence normal loading. Our previous findings suggest that increases in hindlimb weight-bearing locomotion behavior and tendon mechanical properties are coordinated during postnatal development, and first occur between P5 and P10 [233]. Since mechanical loading impacts the expression of tenogenic markers, such as scleraxis [91], and the production of the later stage tenogenic marker tenomodulin [74, 366], postnatal weight-bearing locomotor behavior may regulate both cellular maturation and functional tendon development, but future studies are needed to determine how ATs and TTs are differentially regulated at the cellular level.

Mechanical loading has been shown to influence mechanical properties of cell-seeded scaffolds *in vitro* [4, 5, 9, 84, 91, 93-95, 200, 202, 203, 327]. Tendon cells respond to mechanical stimulation by upregulating genes for collagen (Col) I and III [93, 94], synthesizing collagen fibrils and aligning them along the axis of tension [84], and remodeling their extracellular matrix (ECM) [46]. During embryonic and postnatal development, tendons initially display high cellularity and relatively low collagen content [36, 37, 39-41]. As they mature, they contain 60-85% collagen by dry weight, 95% of which is Col I, and are relatively hypocellular [196]. Cells in mature tendon are sensitive to mechanical loading and respond by remodeling their ECM or secreting inflammatory cytokines [125-127, 154], both of which ultimately lead to alterations in the tissue mechanical properties. Unloading also impacts healing in mature tendons. In adult rats, immobilization following a supraspinatus tendon injury led to upregulation of chondrogenic genes [142], and immobilization after an Achilles tendon transection led to increased laxity of healed tendons, compared to controls [144]. Collectively, these results illustrate that cells and tendons respond to their mechanical environment to direct tendon formation and maintenance via upregulation of tenogenic genes and

secretion of biochemicals, but mechanically regulated cellular mechanisms and the possibility for unique developmental pathways in functionally distinct postnatal tendons deserve further study.

This study has some limitations. The neonatal spinal transection model used here does not lead to elimination of hindlimb movement. During the early neonatal period, rats that have undergone a mid- or low-thoracic spinal cord transection show less myoclonic twitching [367] and spontaneous locomotion in the hindlimbs [356]. However, when the transection occurs early in postnatal life (i.e., before P14 in rats [368]), spinal-injured animals still go on to develop hindlimb locomotor behavior, albeit not as coordinated as in normal controls [369], and remain able to adapt their locomotor behavior to sensory stimulation [370]. Findings from the current study of decreased locomotor activity in spinal-transected female P10 rats are consistent with a recent study showing similar decreases in spinal-transected male P10 rats, compared to shams [356]. Thus, because the neonatal rat transection procedure leads to alterations (i.e., decreases) in weight-bearing locomotion, and not paralysis, it provides a useful model to examine activity-based developmental processes such as tendon development. In future studies it will be important to extend the age range out to older animals, as P10/P11 is the typical age that quadrupedal walking is regularly shown in rats [339]. Thus, examining tendon properties at a later time point from surgery will likely reveal more drastic and activity-dependent effects between sham and spinal-transected animals. Two-tailed unpaired t-tests showed that P10 ATs and TTs from rats subjected to the sham surgery did not differ significantly in their mechanical properties from P10 ATs and TTs from rats allowed to develop normal locomotor behavior, as reported in our prior study (data not shown) [233]. Thus, the sham surgery, on its own, did not appear to alter mechanical properties of developing tendons. Additionally, there are inherent challenges to testing neonatal tissues (small size, fragility, damage during dissection, and slipping during testing). To minimize errors, any data with apparent damage during tissue isolation or slipping during testing were excluded from the analysis. Finally, although sex differences in tendons have been detected at later ages [347, 371], at ages below 4 weeks, body mass, collagen content, elastic modulus, maximum stress [347], and early locomotor development [339] do not differ between male and female mice. Therefore, we do not believe it is likely that using only female rats affected our results.

In this study, we demonstrated that disrupting the development of normal locomotion of postnatal rats via spinal transection at P1 results in unique changes to AT and TT mechanical properties, without disrupting the collagen structure. Future investigations will identify the cellular mechanisms affected by manipulating the postnatal mechanical environment, with particular focus on how these mechanisms may be impacted differently in the energy-storing and weight-bearing (e.g.

AT) and the positional and non-weight-bearing (e.g. TTs) tendons. These results provide new insights into how mechanical stimuli, particularly the onset of weight-bearing locomotion during neonatal development, impact tendon formation.

Chapter 8: TGFβ2-Induced Tenogenesis of Mesenchymal Stem Cells Proceeds in the Absence of Smad3 Signaling and Activates PI3K/Akt/mTOR/P70S6K Signaling

Theodossiou SK, Murray JB, Hold LA, Courtright JM, Carper AM, Schiele NR.

Submitted to *Stem Cell Research and Therapy.*

Abstract

Tissue engineered and regenerative approaches for treating tendon injuries are challenged by the limited information on the cellular signaling pathways driving tenogenic differentiation of stem cells. Members of the transforming growth factor (TGF)β family play a role in tenogenesis, which may proceed via Smad-mediated signaling. However, recent evidence suggests some aspects of tenogenesis may be independent of Smad signaling, and other pathways potentially involved in tenogenesis are understudied. Here, we examined the role of Akt/mTORC1/P70S6K signaling in TGFβ2-induced tenogenesis of mesenchymal stem cells (MSCs), and evaluated TGFβ2-induced tenogenic differentiation when Smad3 is inhibited. Mouse MSCs were treated with TGFβ2 to induce tenogenesis, and Akt or Smad3 signaling were chemically inhibited using the Akt inhibitor, MK-2206, or the Smad3 inhibitor, SIS3. Effects of TGFβ2 alone and in combination with these inhibitors on the activation of Akt signaling and its downstream targets mTOR and P70S6K were quantified using western blot analysis, and cell morphology was assessed using confocal microscopy. Levels of the tendon marker protein, tenomodulin, were also assessed. TGFβ2 alone activated Akt signaling during tenogenic induction. Inhibiting Akt prevented increases in tenomodulin, and attenuated tenogenic morphology of the MSCs in response to TGFβ2. Inhibiting Smad3 did not prevent tenogenesis, but appeared to accelerate it. MSCs treated with both TGFβ2 and SIS3 produced significantly higher levels of tenomodulin at 7 d and morphology appeared tenogenic, with localized cell alignment and elongation. Finally, inhibiting Smad3 did not appear to impact Akt signaling, suggesting that Akt may allow TGFβ2-induced tenogenesis to proceed independently of Smad3 signaling. These findings show that Akt signaling plays a role in TGFβ2-induced tenogenesis, and that tenogenesis of MSCs can be initiated by TGFβ2 independent of Smad3. These finding provide new insights into the signaling pathways that regulate tenogenic induction in stem cells.

Introduction

Tendons, the collagenous musculoskeletal tissues that connect muscle to bone to enable movement, are frequently injured and heal poorly, leading to long-term loss of function [1]. Tendon injuries are common in both the general population and athletes, and limited clinical treatment options combined with their increasing incidence and high costs in terms of both healthcare and productivity losses make tendinopathies a significant public health concern [1, 14, 372]. Mesenchymal stem cells (MSCs) are attractive for use in regenerative therapies to treat tendon injuries, as they are relatively easy to isolate and can differentiate into a variety of tissue lineages, including tendon [4, 5, 7, 9, 257, 373]. However, the limited understanding of the cell signaling pathways involved in tenogenesis (differentiation toward the tendon lineage) is a challenge for tissue engineering and regenerative approaches.

Currently identified tenogenic pathways include those related to transforming growth factor beta (TGFβ) signaling [374]. TGFβ has three isoforms (TGFβ1, 2, and 3), which, in canonical signaling, appear to depend on small mothers against decapentaplegic (Smad) 2/3 signaling [53, 375]. Smads are intracellular signaling proteins that regulate several cellular processes including growth, differentiation, and proliferation [55]. Previously, we and others showed that TGFβ2 is a potent inducer of tenogenesis in murine MSCs *in vitro* [13, 48, 49, 53, 55, 350]. MSCs supplemented with recombinant human TGFβ2 significantly increased production of the tendon marker proteins scleraxis and tenomodulin over 21 days (d) in culture, and underwent extensive morphology changes indicative of a transition towards tendon-like cells [13]. TGFβ2 also effectively induced tenogenesis in mouse embryonic fibroblasts grown in an *in vitro* tendon model [55]. Interestingly, the same study showed that when Smad4, the downstream effector of Smad2 and 3, was knocked out in mouse embryonic fibroblasts via Adenovirus/Cre mediated floxing, proliferation was disrupted, but the cells still produced scleraxis, indicating early tenogenesis was able to proceed [55]. Collectively, while canonical TGFβ2 signaling likely depends on Smad2/3, this recent evidence suggests that other cell signaling pathways may be active in tenogenesis that are independent of Smad.

Though pathways beyond TGFβ and Smad2/3 have yet to be extensively investigated in tenogenesis, the phosphatidylinositol 3 kinase (PI3K)/Akt/mTORC1/P70S6K pathway has been implicated in the TGFβ-mediated tendon response to injury [376]. Treatment of murine MSCs with TGFβ1, which induces a pro-inflammatory and pro-fibrogenic response in tendon [167], activated (phosphorylated) Akt [377]. Akt, a kinase with extensive downstream roles in cell survival, growth, protein synthesis, and apoptosis, is activated by the phospholipid membrane-bound PI3K in response

to several extracellular cues, including various growth factors, and potentially TGFβ signaling [378]. Once phosphorylated, Akt can indirectly activate the mammalian target of rapamycin, or mTORC1 complex. mTOR knockout mice display abnormalities in the gross anatomy of their Achilles, patellar, and tail tendons [377], suggesting mTOR is involved in tendon development and maintenance. mTOR also activates protein S6 kinase, or P70S6K, which mediates protein synthesis and cell growth [379]. As P70S6K is a downstream effector of Akt, it is possible that P70S6K may be activated during TGFβ2-induced tenogenesis. P70S6K has not been investigated in the context of tenogenic differentiation, though it may contribute to proliferation and migration of other fibroblastic cells, such as lung fibroblasts [380]. Overall, although components of the PI3K/Akt/mTORC1/P70S6K pathway have been implicated in tendon development, interactions between this pathway and the tenogenic isoform of TGFβ, TGFβ2, have not been investigated.

Existing data suggest activation of Akt by TGFβ signaling can take place independently of Smad2 and 3 [381], but Akt activation has not been investigated in response to TGFβ2 specifically. Prior studies have mainly explored exogenous TGFβ1 to enhance TGFβ signaling in experimental settings. However, TGFβ isoforms induce differentiation towards distinct musculoskeletal tissue lineages. TGFβ3 is known to be chondrogenic [374], while TGFβ1 may induce chondrogenesis [265] or fibrosis and inflammation [171], and TGFβ2 is tenogenic [13, 31, 55, 382]. Thus, there remains a need to assess interactions between TGFβ2 and Akt signaling, specifically for tenogenesis. Notably, there is conflicting evidence as to whether Akt signaling enhances or attenuates cellular responses to TGFβ [383, 384], further highlighting the need for additional studies. Though Akt signaling is a precursor to a multitude of cellular events, establishing its potential involvement in tenogenesis is beneficial due to the limited understanding of the signaling events preceding tenogenic differentiation. Non-canonical and Smad-independent interactions between TGFβ and other signaling pathways have been documented in various cell types, and summarized in a comprehensive review [379]. While TGFβ is known to interact with bone morphogenic protein (BMP), mitogen-activated protein kinase (MAPK), Wingless/Integrated (Wnt) [225], Hedgehog (Hh), and Notch signaling [379], potential crosstalk between TGFβ and the Akt/mTORC1/P70S6K pathway is of particular interest in the context of tenogenesis due to the availability of therapeutic agents that can target Akt/mTORC1/P70S6K [385].

Taken together, additional signaling pathways may be involved in TGFβ2-induced tenogenesis of stem cells, and one such pathway may be PI3K/Akt/mTORC1/P70S6K. We hypothesized that Akt/mTORC1/P70S6K would be activated during TGFβ2-induced tenogenesis,

independent of Smad3 signaling. To test this, we treated mouse MSCs with TGFβ2 to induce tenogenesis, and chemically inhibited Akt and Smad3 signaling for up to 7 d. We examined cell morphology, and activation of Akt, mTOR, and P70S6K, and evaluated levels of the tendon marker, tenomodulin. Our results showed that TGFβ2 activated Akt, and inhibiting Akt prevented TGFβ2-induced tenogenesis. Inhibiting Smad3 appeared to accelerate TGFβ2-induced tenogenesis. The tenogenic involvement of pathways other than Smad3 provides additional targets for investigations in tendon development.

Materials and Methods

Cell Culture and Tenogenic Induction

Murine MSCs (C3H10T1/2, ATCC, Manassas, VA), a model MSC used in prior studies investigating tenogenesis and tendon injury [31, 91, 377], were cultured and supplemented with TGFβ2 to induce tenogenesis as previously described [13]. Briefly, cells were expanded in standard growth medium (Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS), and 1% Penicillin/Streptomycin) until 70% confluent, and used between passage 5 and 13. MSCs were trypsinized, and seeded into each well of a 24-well plate. Cells used for 15 minute (min), 30 min, 1 hour (h) and 24 h timepoints were seeded at 25,000 cells/cm^2. Cells for 3 and 7 d timepoints were seeded at 5000 cells/cm^2. Cells were incubated for 24 h to allow for initial cell attachment, and then washed with warmed phosphate buffered saline (PBS) (Gibco, Grand Island, NY). The medium was switched to low-serum medium (DMEM, 1% FBS, 1% Penicillin/Streptomycin), and allowed to equilibrate for 24 h. Cells were rinsed with warm PBS and cultured for 15 min, 30 min, 1 h, 24 h, 3 d, 7 d, or 14 d in low-serum medium with the corresponding amount of sterile water (vehicle controls) or low-serum medium supplemented with 50 ng/mL recombinant human TGFβ2 (PeproTech, Rocky Hill, NJ). The medium was changed every third day. Experiments were repeated a minimum of 3 times.

Inhibition of Akt and Smad3

To inhibit Akt signaling, cells were seeded in 24-well plates and cultured for 15 min, 30 min, 60 min, 24 h, 3 d and 7 d in low-serum medium with water and dimethyl sulfoxide (DMSO) (vehicle controls), and low-serum medium supplemented with 50 ng/mL recombinant human TGFβ2 (PeproTech), 500 nM of the Akt inhibitor MK-2206 [386] (MedChem Express, Monmouth Junction, NJ), or both (TGFβ2+MK-2206). To inhibit Smad3 signaling, cells were cultured and seeded into 24-

well plates as described above for 15 min, 30 min, 60 min, 24 h, 3 d, and 7 d in low-serum medium with water and DMSO (vehicle controls), and low-serum medium supplemented with 50 ng/mL recombinant human TGFβ2 (PeproTech), 2 μM of the Smad3 inhibitor SIS3 [53] (Tocris, Bristol, UK), or both (TGFβ2+SIS3).

Western Blot Analysis

Cells were collected for western blot (WB) analysis in RIPA cell lysis buffer and HALT protease inhibitor (Invitrogen, Carlsbad, CA). Sodium dodecyl sulfate (SDS) was added at a 1:1 ratio and samples were sonicated, heated to 100^{o} C for 5 minutes, and loaded into Novex Wedgewell 4-20% Tris Glycine Mini Gels (Invitrogen). Lanes were loaded differentially to normalize total protein content. Cell lysate collected from each well of the 24-well plate was run in its own lane (2 to 3 wells of each condition were run, analyzed, and averaged per individual experiment). Samples probed for mTOR and phosphorylated (P)-mTOR required 4-12% Tris Glycine gels (Invitrogen) due to the large protein size. Following electrophoresis, gels were transferred to nitrocellulose membranes (Invitrogen), blocked in 5% milk in tris buffered saline (Boston Bioproducts, Ashland, MA) with 0.1% Tween20 (TBST) (Acros Organics, Morris Plains, NJ), and incubated overnight at 4^{o} C on an orbital shaker with appropriate primary antibodies in 5% bovine serum albumin (BSA) in TBST. Primary antibodies raised in rabbit were purchased for P-Smad3, β-actin (Abcam, Cambridge MA), mTOR, P-mTOR, Akt, P-Akt, P70S6K, P-P70S6K, Smad2/3 (Cell Signaling Technologies, Danvers, MA), and tenomodulin (Tnmd) (Invitrogen), and used at concentrations of 1:1000 to 1:10,000. Phosphorylation indicates activation, and increases in levels of P-Akt, P-mTOR, P-P70S6K, and P-Smad3 were used as representations of increased activation. Blots were washed 3x for 5 min in TBST and incubated for 1 h at room temperature with goat anti-rabbit HRP-linked secondary antibody (Invitrogen). Blots were then washed in TBST, developed using ECL chemiluminescence reagents (Invitrogen), imaged using a Genesis Pi6x imager (Syngene, Frederick, MD), and analyzed via band densitometry in ImageJ (NIH, Bethesda, MD), with all intensities normalized to their respective β-actin bands.

Fluorescence Staining and Confocal Microscopy

To observe changes in cell morphology, cells were cultured and supplemented with TGFβ2, MK-2206 and SIS3, as described above, but on glass coverslips. At 24 h, 3 d, and 7 d, the medium

was removed, cells were rinsed with PBS, and fixed in 10% formalin overnight at 4° C. Cells were washed with PBS, permeabilized with 0.1% Triton-X (Acros Organics), and stained with FITC-phalloidin and 4,6-Diamidino-2-phenylindole (DAPI) (Life Tech., Waltham, MA) to observe the actin cytoskeleton and cell nuclei, respectively. Coverslips were mounted on slides and imaged on a spinning-disk confocal microscope (Nikon/Andor, Melville, NY).

Statistical Analysis

Proteins were initially normalized to their respective β-actin bands. As not all timepoints and experiments could be run on the same gel, treatment groups were normalized to their respective controls at each timepoint. Phosphorylated protein content was then normalized to total Akt, P70S6K, mTOR, or Smad2/3 content, as previously described [160]. Each experimental run was averaged (minimum n = 3 independent runs, 2-3 technical replicates per run) and ratios were calculated from bands imaged on the same membrane. Resulting ratios were analyzed using 1-way analysis of variance (ANOVA) with Sidak's multiple comparison test (Prism 8, GraphPad, LaJolla, CA). Significance was set at $p < 0.05$. Results are reported as mean ± standard deviation.

Results

Akt signaling is active during TGFβ2-induced tenogenesis

TGFβ2 treatment significantly increased the ratio of P-Akt (activated) to total Akt at 30 min ($p < 0.05$; Fig. 1B) and 60 min ($p < 0.001$; Fig. 8.1C), and at 24 h ($p < 0.01$; Fig. 1D), compared to controls. TGFβ2 increased the average P-Akt/Akt ratio, though not significantly, at 15 min and 3 d (Fig. 8.1A, E). Compared to controls, TGFβ2 did not alter the ratio of P-mTOR to mTOR at any timepoint (Fig. S1A-E). TGFβ2 significantly increased the ratio of P-P70S6K to P70S6K compared to controls at 24 h ($p < 0.05$; Fig. S2D), and P-P70S6K activation trended higher at 60 min ($p = 0.08$; Fig. S2C). TGFβ2 did not impact the ratio of P-P70S6K to P70S6K at 15 and 30 min, or 3 and 7 d (Fig. S2A, B, E, F).

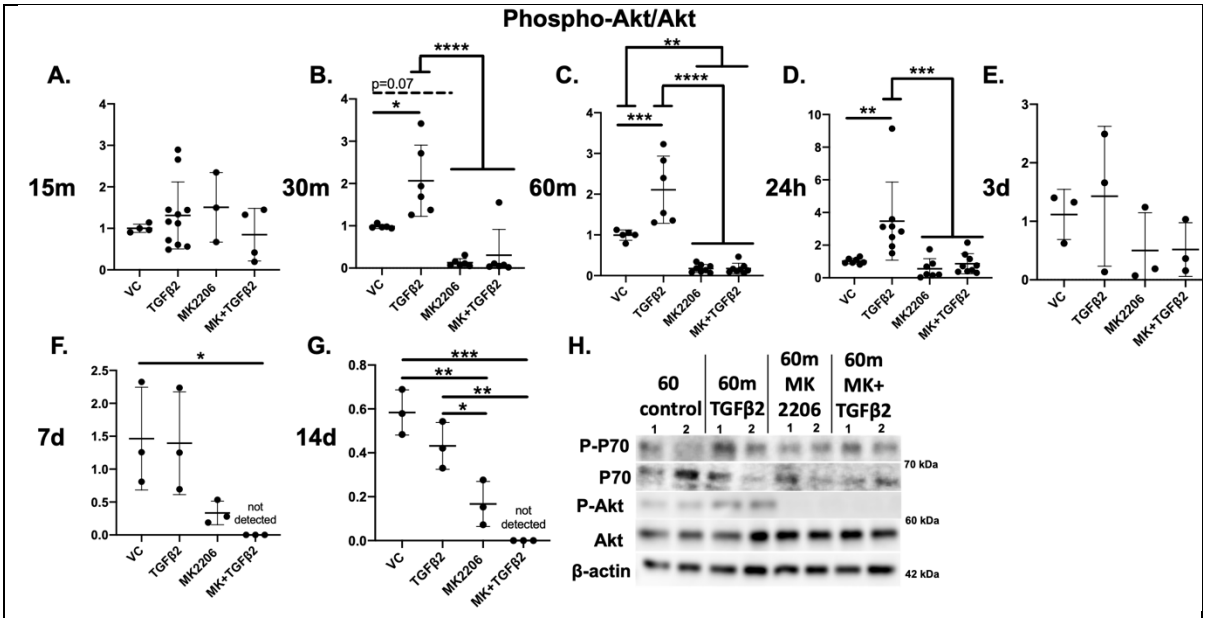


Figure 8.1 Akt is activated by TGFβ2 and inhibited with MK-2206 as a function of time.

(A-G) Quantified western blot band densitometry showing the ratio of P-Akt to Akt as a function of time. The ratio of P-Akt to Akt was significantly increased by TGFβ2 treatment at (B) 30 m, (C) 60 m, and (D) 24 h. Akt activation levels in MSCs were significantly decreased by MK-2206 at (B) 30 m, (C) 60 m, (D) 24 h, (F) 7 d and (G) 14 d. (H) Representative western blot showing MK-2206 prevents Akt activation at 60 m, while TGFβ2 increases Akt phosphorylation. P-P70S6K and P70S6K remained unaffected. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. Bars = mean ± standard deviation.

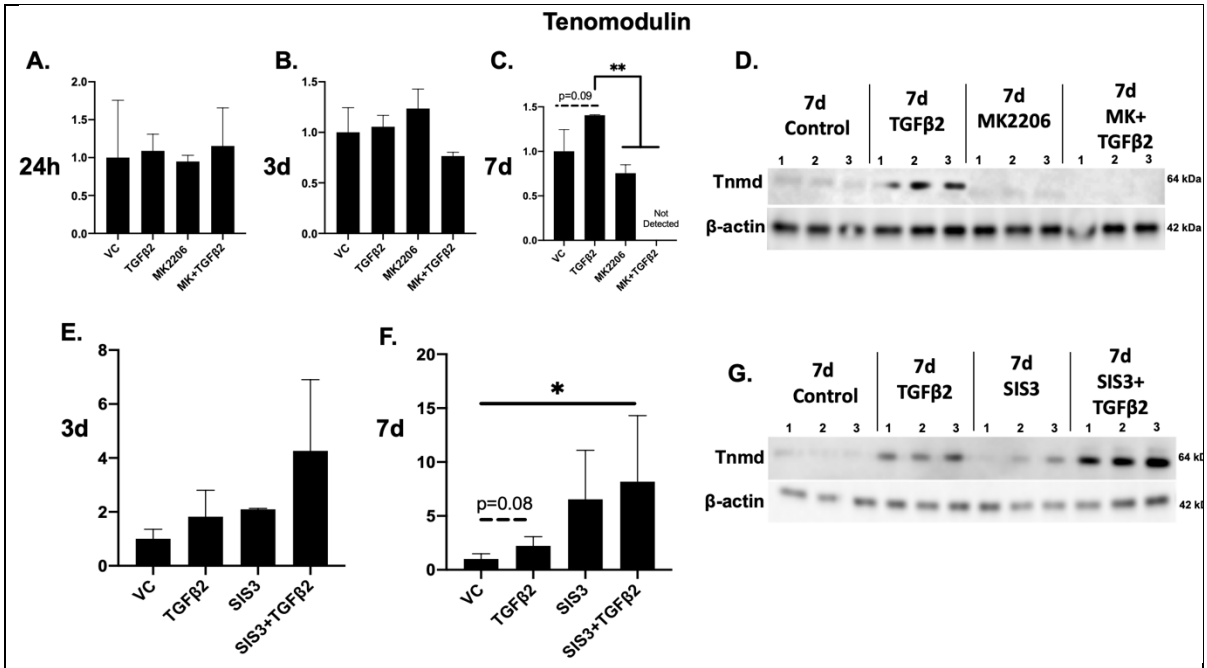


Figure 8.2 Akt inhibition prevents increases in tenomodulin, while Smad3 inhibition appears to accelerate tenomodulin production.

(A-C) Quantified western blot band densitometry showing tenomodulin is not detectable following 7 d of treatment with MK-2206+TGFβ2, indicating tenogenesis is disrupted when Akt signaling is inhibited. (D) Representative western blot showing cells treated with MK-2206+TGFβ2 do not produce tenomodulin at 7 d. (E-F) MSCs treated with SIS3+TGFβ2 produce more tenomodulin than cells treated with TGFβ2 alone at 7 d. (G) Representative western blot showing MSCs production of tenomodulin increases with 7 d of TGFβ2 and SIS3 treatment, compared to controls, and MSCs treated with TGFβ2 or SIS3 alone. * = $p < 0.05$, ** = $p < 0.01$. Bars = mean ± standard deviation.

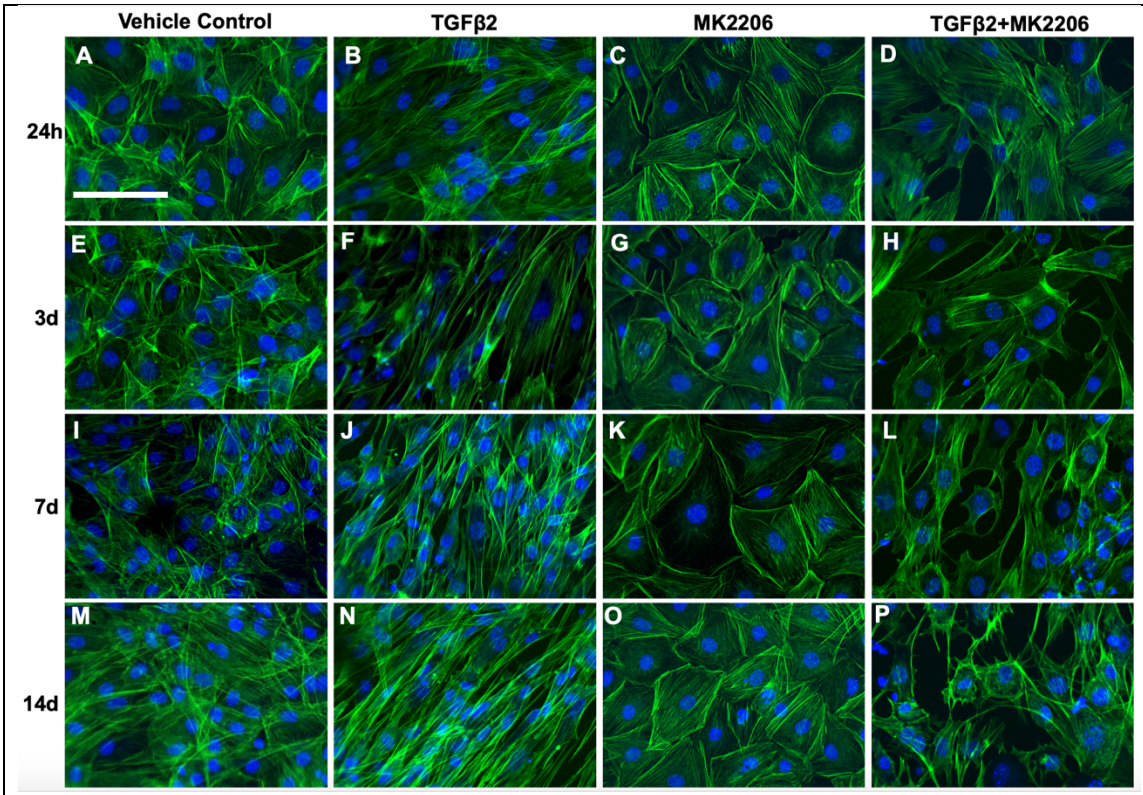


Figure 8.3 Akt inhibition impacts tenogenic cell morphology.

Representative images (20x) of MSCs treated with TGFβ2 and MK-2206. Actin cytoskeleton (green) and cell nuclei (blue) are shown in MSCs at 24 h, and 3, 7, and 14 d (A-P). (D, H, L, P) MSCs treated with both TGFβ2 and the Akt inhibitor, MK-2206, had disruptions in cell morphology and did not appear elongated or fibroblastic, compared to (B, F, J, N) cells treated with TGFβ2 only and (A, E, I, M) controls. (C, G, K, O) MK-2206 alone did not appear to drastically impact cell morphology. Scale bar = 100 μm.

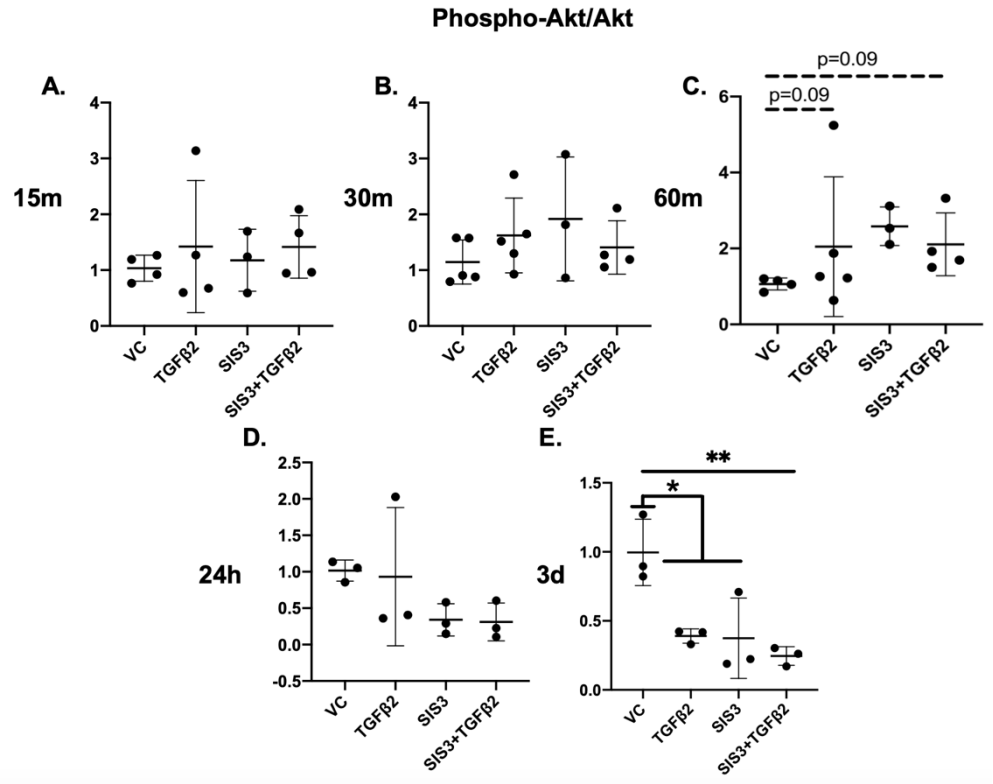


Figure 8.4 Smad3 inhibition has limited impact on Akt activation.

Quantified western blot band densitometry showing the ratio of P-Akt to Akt in MSCs treated with SIS3 to block Smad3 signaling. The ratio of P-Akt to Akt was not significantly altered by SIS3 at (A) 15 m, (B) 30 m, and (D) 24 h. (C) Akt activation trended higher in cells treated with TGFβ2 and SIS3+TGFβ2 at 60 m ($p = 0.09$). (E) Akt activation levels were significantly decreased in all conditions except vehicle controls by 3 d. * = $p < 0.05$, ** = $p < 0.01$. Bars = mean ± standard deviation.

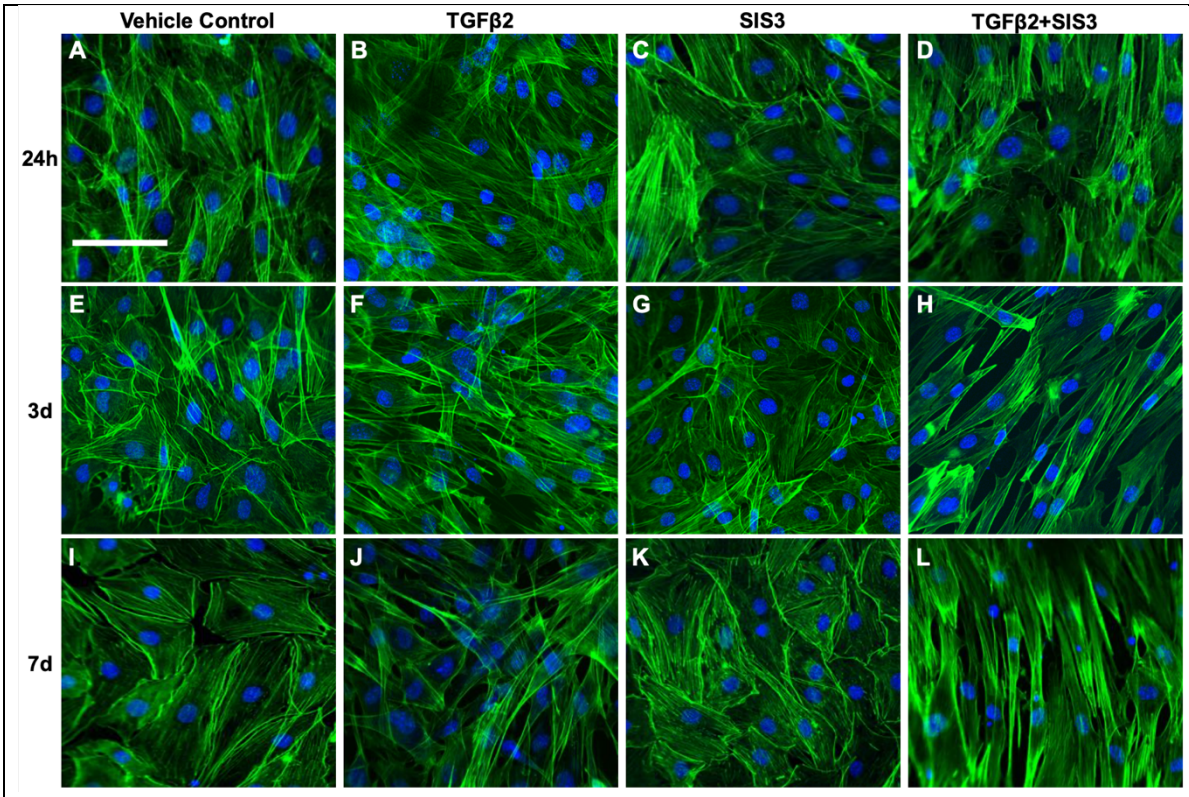


Figure 8.5 Smad3 inhibition does not alter tenogenic cell morphology.

Representative images (20x) of TGFβ2 and SIS3-treated MSCs. (A-H) Actin cytoskeleton (green) and cell nuclei (blue) are shown in MSCs at 24 h, 3 d and 7 d. (D, H, L) MSCs treated with both TGFβ2 and the Smad3 inhibitor, SIS3, appeared more fibroblastic and elongated, compared to (A, E, I) controls and (B, F, J) cells treated with TGFβ2 only. (C, G, K) SIS3 alone did not appear to impact cell morphology. Scale bar = 100 μm.

Akt inhibition prevents TGFβ2-induced tenogenesis

Cells were treated with TGFβ2, MK-2206, or MK-2206+TGFβ2. MK-2206 effectively inhibited Akt activation both on its own and when used in combination with TGFβ2. The ratio of P-Akt to Akt was significantly lower in both MK-2206 and MK-2206+TGFβ2 treated cells, compared to TGFβ2-only treated cells at 30 min ($p < 0.0001$; Fig. 8.1B), compared to all other groups at 60 min ($p < 0.01$ to $p < 0.001$; Fig. 8.1C, H), and compared to TGFβ2-only treated cells ($p < 0.001$) at 24 h (Fig. 8.1D). The P-Akt to Akt ratio remained lower in MK-2206-treated groups, and P-Akt was not detected in MK-2206+TGFβ2 treated cells at 7 d (Fig. 8.1F) and 14 d (Fig. 8.1G). MK-2206 did not significant impact the ratio of P-mTOR to mTOR at most timepoints (Fig. S1). At 24 h, mTOR activation was significantly lower in MK-2206-only treated cells, compared to TGFβ2-only cells ($p < 0.05$; Fig. S1D), and at 3 d, activation was significantly lower in MK-2206+TGFβ2 treated cells, compared to TGFβ2-

only cells ($p < 0.05$; Fig. S1E). MK-2206 did not affect the ratio of P-P70S6K to P70S6K at most timepoints, though P-P70S6K activation trended lower ($p = 0.08$) in MK-2206 and MK-2206+TGFβ2 treated cells at 60 min (Fig. S2C) and was significantly lower ($p < 0.05$) in MK-2206 treated cells compared to TGFβ2-only treated cells at 24 h (Fig. S2D). At this same timepoint, MK-2206+TGFβ2 treated cells had a significantly higher ratio of P-P70S6K to P70S6K compared to vehicle controls ($p < 0.05$; Fig. S2D), and the ratio in MK-2206+TGFβ2 treated cells trended higher ($p = 0.055$) than in MK-2206-only treated cells. At 7 d, levels of tenomodulin were undetectable in the MK-2206+TGFβ2-treated cells, indicating tenogenesis had been disrupted ($p < 0.01$; Fig. 8.2C, D). Tenogenic cell morphology was disrupted with MK-2206+TGFβ2 treatment. Cells showed reduced elongation, proliferation, and localized alignment, compared to cells treated with TGFβ2 alone, at 24 h, 3 d, and 7 d (Fig. 8.3). Finally, although cells treated with MK-2206+TGFβ2 had a significantly higher ratio of P-Smad3 to Smad2/3 at 60 min ($p < 0.05$; Fig. S3A), levels of activated Smad3 were similar to controls at 24 h (Fig. S3B).

TGFβ2-induced tenogenesis proceeds when Smad3 signaling is inhibited

Cells were treated with TGFβ2, SIS3, or SIS3+TGFβ2. Cells treated with SIS3 had decreased activation of Smad3 and a lower ratio ($p = 0.08$) of P-Smad3 to total Smad2/3 at 60 min, as expected (Fig. S3C). SIS3 did not decrease the ratio of P-Akt to Akt alone or in combination with TGFβ2 at any timepoint (Fig. 8.4), though all conditions had significantly lower Akt activation compared to controls at 3 d (Fig. 8.4E). SIS3 did not impact the ratio of P-mTOR to mTOR, except at 15 min, where SIS3+TGFβ2 treated cells had significantly lower mTOR activation compared to other timepoints ($p < 0.01$; Fig. S4A). Finally, at 15 min, SIS3 and SIS3+TGFβ2 treated cells had a significantly lower ratio of P-P70S6K to P70S6K ($p < 0.05$; Fig. S5A), compared to cells treated with TGFβ2 alone, but SIS3 did not affect this ratio at any other timepoint (Fig. S5). Inhibiting Smad3 signaling with SIS3 did not prevent TGFβ2-induced tenogenesis. The morphology of cells treated with SIS3+TGFβ2 displayed changes consistent with tenogenesis at earlier timepoints, compared to cells treated with TGFβ2 alone (Fig. 8.5). Levels of tenomodulin were also significantly higher ($p < 0.01$) at 7 d in cells treated with SIS3+TGFβ2, compared to the TGFβ2-only group (Fig. 8.2E, F, G).

Discussion

In this study, we showed that TGFβ2 activated Akt signaling during tenogenesis, that inhibiting Akt signaling prevented TGFβ2-induced tenogenesis, and that TGFβ2-induced tenogenesis proceeded independently of Smad3. Activation of mTOR and P70S6K, which are downstream in the Akt signaling pathway, also changed in response to TGFβ2, though these changes appeared muted compared to the impact of TGFβ2 on Akt activation. Inhibiting Akt and Smad3 had limited impact on the activation of these downstream effectors (mTOR and P70S6K). Finally, we showed that inhibiting Smad3 signaling during TGFβ2-induced tenogenesis appeared to accelerate morphological changes and tenomodulin production by MSCs. While previous work has shown that TGFβ signaling can proceed independently of Smad functionality [55], to our knowledge, this is the first study to investigate the activation of potential alternative cellular signaling pathways in TGFβ2-induced tenogenesis.

Our results suggest that Akt activation is a possible tenogenic pathway that may regulate the cellular response to TGFβ2-induced tenogenesis. TGFβ2-treatment alone significantly increased Akt activation early in tenogenic induction (Fig. 8.1). Additionally, Akt activation with TGFβ2 across various timepoints showed an increasing trend up until 24 h, and a decreasing trend until 14 d, where the P-Akt to Akt ratio is significantly lower compared to 24 h (Fig. S6). Although the ratio of P-Akt to Akt increased by a maximum of only ~2.5:1 in response to TGFβ2, as a kinase, Akt activates a cascade of intracellular events, and only modest increases in signaling may be needed to induce robust changes in transcription and protein synthesis [378]. While MK-2206 prevented Akt activation by TGFβ2 and diminished tenogenic markers, levels of total (non-phosphorylated) Akt, as well as levels of phosphorylated and non-phosphorylated mTOR and P70S6K, were not changed by MK-2206 addition alone, except at some later timepoints. This suggests that even low levels of P-Akt may be enough to maintain some downstream signaling, that other non-canonical pathways may be maintaining mTOR and P70S6K signaling in the absence of Akt activation, or that these downstream signaling molecules are not involved in TGFβ2-induced tenogenesis. Overall, our findings show that TGFβ2 activates Akt signaling during tenogenic induction.

Interestingly, in addition to not decreasing in response to MK-2206, mTOR activation was not significantly altered by TGFβ2 treatment and the apparent changes in Akt activation. This finding could be explained by Akt only indirectly activating mTOR in the canonical PI3K/Akt/mTORC1/P70S6K pathway, and this activation may be tissue-specific [378, 387]. The

TGFβ-induced epithelial-to-mesenchymal transition is also regulated by mTOR [388]. Since TGFβ2-induced tenogenesis in these MSCs is occurring after the epithelial-to-mesenchymal transition that occurred *in utero* prior to MSC isolation, mTOR regulation may not directly influence tenogenic differentiation. It is also possible that mTOR activation is exclusive to tendon injury [376], and not activated during tenogenic differentiation. However, mTOR knockout mice display some abnormalities in gross tendon morphology [377], suggesting mTOR is involved in tendon development. Similarly, the ratio of P-P70S6K to P70S6K was not significantly increased at early timepoints. However, unlike mTOR, P70S6K activation was significantly higher at 24 h with TGFβ2 treatment. The significant increases in P70S6K activation at later timepoints (24 h) suggest some mTOR activation, and the delayed peak in activation is logical given its position downstream in the pathway. Ultimately, both mTOR and P70S6K showed some increases (though not significant) in activation in response to TGFβ2 at earlier timepoints, indicating that the PI3K/Akt/mTORC1/P70S6K pathway remains a potential driver of TGFβ2-induced tenogenesis.

Akt may allow TGFβ2-induced tenogenesis to proceed in the absence of Smad3. While Smad3-independent activation of Akt by TGFβ signaling is not a novel finding [375, 381], Smad3-independent TGFβ2-induced tenogenesis has not been previously shown. Inhibiting Smad3 with SIS3 did not decrease activation of Akt (Fig. 8.4) or downstream effectors, mTOR (Fig. S4, S5), except at isolated, later timepoints. While Akt activation did not increase significantly in the TGFβ2+SIS3 treated cells, levels of P-Akt trended higher ($p = 0.09$) at 60 m (Fig. 4C), and it is possible that low levels of Akt activation are sufficient for tenogenesis to proceed. Unlike Akt, P-P70S6K activation showed an early, transient significant decrease in SIS3-treated cells at 15 min, but the ratio of P-P70S6K to P70S6K was otherwise similar to other groups at all timepoints (Fig. S5). The cause of this pattern is not clear, but could be due to effects of endogenous TGFβ1 on P70S6K signaling being inhibited by SIS3 [387]. Future studies are needed to determine the specific downstream effects of each TGFβ isoform within the context of Akt signaling. Furthermore, while other pathways not assessed in this study may be initiating tenogenesis in response to TGFβ2 addition, Akt is a promising candidate due to the extensive morphological changes observed with Akt inhibition (Fig. 8.3). Akt inhibition does not lead to alterations in cell morphology, except in the MK-2206+TGFβ2 groups. Similarly, SIS3 alone appears to do little to cell morphology or protein production, until combined with TGFβ2. While qualitative, the images of cell morphology showed enhanced tenogenic morphology with Smad3 inhibition, and extensive disruption of tenogenic morphology with Akt inhibition. Disrupted tenogenic morphology with Akt inhibition, along with loss of tenomodulin production, suggest that the Akt pathway is playing a role in early TGFβ2-induced tenogenesis.

The finding that TGFβ2 not only induces tenogenesis when Smad3 is inhibited, but that differentiation may be accelerated, is unexpected, but consistent with recent evidence that the downstream effector of Smad3 signaling (e.g. Smad4) may not be necessary for tenogenic differentiation [55]. However, Smad4 was shown to play a role in cell proliferation in response to TGFβ2 [55]. While cellular proliferation was not quantified in this study, images show that changes in cell morphology (elongation and localized alignment) are not accompanied by the large increases in cell number observed in prior studies of TGFβ2-only treated cells [13], suggesting Smad3 and hence downstream Smad4 signaling were effectively inhibited. Unlike TGFβ2-only and SIS3-only treated cells, most SIS3+TGFβ2-treated groups did not survive past 10 d in culture, suggesting long-term SIS3 accumulation or inhibition of Smad3 is problematic for TGFβ2-induced tenogenesis. For this reason, only timepoints up until a maximum 7 d are included in the data analysis.

It is possible that the apparent acceleration in TGFβ2-induced tenogenesis with Smad3 inhibition is due inhibiting cell signaling associated with endogenous TGFβ1. Fibroblasts and MSCs that are precursors to musculoskeletal tissues produce TGFβ1 during development [11, 31, 382, 389, 390], and TGFβ1 signaling is known to participate in the tendon response to injury and fibrosis [171, 391-393]. Multiple TGFβ isoforms may be active at any given time, but not all activate the same pathways, especially when non-canonical signaling is considered [394]. During early musculoskeletal tissue differentiation, TGFβ1 signaling may proceed exclusively via Smad, while TGFβ2 may be able to induce differentiation via alternate pathways that other TGFβ isoforms cannot activate [375]. As TGFβ1 may not be tenogenic and in other studies is chondrogenic [395, 396] or fibrogenic [171], inhibiting Smad3 may prevent the non-tenogenic effects of TGFβ1. Simultaneously, TGFβ2 signaling may proceed via an alternate pathway, thus accelerating tenogenesis in the absence of competing signals from endogenous TGFβ1. Other aspects of tendon development, such as production of matrix components like fibronectin, may require Smad4-dependent and independent TGFβ1 signaling [397], which long-term Smad3 inhibition prohibits. Furthermore, injury in a neonatal tendon is associated with Smad2/3 activation, and Smad2/3 activation meditated by TGFβ signaling through the TGFβ type I receptor ALK4/5/7 impacts regenerative healing [398]. Finally, interactions between multiple TGFβ isoforms and other downstream Smad-dependent signals, including BMPs [71-73, 379], may be necessary for continued differentiation and could be explored in future long-term studies. Taken together, Smad activation by TGFβ family members may play unique and time-dependent roles in differentiation and regeneration, but more work is needed to elucidate the impact of Smads in regulating tendon formation.

This study is not without its limitations. Chemical inhibitors were used to inhibit Akt and Smad3 signaling. Although western blotting showed activated Akt was lowered to almost undetectable levels by MK-2206, and the overall ratio of activated Smad3 to total Smad2/3 content decreased in SIS3-treated groups, it is possible that some activity persisted. Studies in animal knockouts can control for small levels of activation in proteins of interest, though other off-target effects of the deletions may confound results. For example, a mouse knockout model of almost all Akt isoforms (*Akt1* $^{+/-}$, *Akt2* $^{-/-}$, and *Akt3* $^{-/-}$) is viable, but the animals display generalized abnormalities in metabolism and body weight [399]. Not all proteins of interest were assessed for 7 days. Proteins activated earlier in the pathway (for example, P-mTOR) were only quantified until 24 h or 3 d in either the MK-2206+TGFβ2 or SIS3+TGFβ2 experiments, though any notable changes in activation would likely occur within these time frames. We also only assessed Akt activation using a pan-Akt antibody, rather than examining individual Akt isoform activation. It is possible individual Akt isoforms impact tenogenic differentiation in different ways, though Akt isoforms are generally considered redundant, with overlapping functions *in vivo* [399]. Additionally, culture medium was supplemented with 50 ng/mL exogenous TGFβ2 every 3 days based on concentrations used in previous studies [13, 52, 53]. While this concentration is shown to be tenogenic in MSCs *in vitro*, it is possible it does not represent the *in vivo* availability and concentration of TGFβ2. We also did not supplement cells with other TGFβ isoforms, such as TGFβ1, or quantify TGFβ1 production by MSCs. Though TGFβ1 is generally considered fibrogenic, it is possible that it activates alternative pathways when either Akt or Smad3 signaling is inhibited. Future studies will integrate assessment of alternative pathways, such as those associated with BMPs and other TGFβ isoforms. Future studies in adult human MSCs will be needed to assess the potential benefits of clinical interventions targeting these pathways. Despite various limitations, this study represents a valuable contribution to the understanding of cell signaling pathways involved in TGFβ2-induced tenogenesis in MSCs, and can be used to inform cellular studies of early tendon development.

Overall, our findings show that Akt signaling is activated during TGFβ2-induced tenogenesis of MSCs, and Akt activation appears to regulate tenogenic markers. Furthermore, inhibiting Smad3 signaling and adding exogenous TGFβ2 did not prevent tenogenesis. Instead, tenogenic differentiation appeared to be accelerated, with earlier increases in tenomodulin production and the appearance of tenogenic cell morphology. Collectively, our results suggest that Akt/mTORC1/P70S6K and other cell signaling pathways independent of Smad3 may be involved in tenogenesis. These pathways provide novel targets for future studies aiming to improve understanding of the cellular processes driving tenogenesis in stem cells.

Chapter 9: Conclusions and Future Directions

The field of tendon tissue engineering and regenerative medicine is rapidly emerging as a promising avenue for improved clinical outcomes for tendon injuries. As increased interest in tendon development and mechanical behavior is relatively recent, much remains to be discovered about this tissue before engineered approaches can effectively recapitulate the mechanical and biological properties of native tendon. The studies within this dissertation represent novel contributions to the field and provide potential starting points for researchers attempting to incorporate cues from normal tendon development into regenerative strategies. This conclusion chapter highlights our main findings and offers possible future directions for continuation of our research objectives.

When framing the overall goal of our research, two topics were reviewed: engineered models of tendon development and disease, and the role of cell-cell junction proteins in developing and mature tendons. Prior engineered models of tendon development have produced much of the current knowledge of the mechanical and cellular development of tendons, as well as revealed the mechanical and biochemical consequences of tendon injuries at multiple scales and severities. These models were summarized in Chapter 2. Similarly, prior work established the cell-cell junction proteins involved in tendon development and homeostasis, and the role cell-cell junction proteins play in other musculoskeletal tissues. However, much remains to be elucidated about how tendon cells communicate with each other and the surrounding matrix to develop and maintain tendon tissue. A survey of existing research on cell-cell junction proteins in tendon was summarized in Chapter 3. These two chapters highlight what is currently known about tendon development and disease, and illustrate the significant need for more information regarding the cellular, biochemical, and mechanical development of tendon. Our work addressed some of the gaps in knowledge outlined in Chapters 2 and 3, beginning with the role of mechanical loading in tendon development.

Mechanical loading is a tenogenic factor we explored in greater depth in Chapters 4, 6, and 7. To assess how mechanical loading can be applied to tenogenic differentiation of stem cells, we designed and validated a custom bioreactor system that allows for the culture of cells under well-controlled cyclic mechanical load. The design and validation of this bioreactor were summarized in Chapter 4. Open-source drawings of this system were also published, allowing other researchers to adapt the design for their needs, furthering the investigation of how mechanical stimuli influence cell behavior.

The mechanical bioreactor we designed can be used to not only mechanically stimulate stem cells in culture, but also as a small-scale tensile load frame. This custom small-scale load frame allowed us to mechanically evaluate tendons from rats as young as 1-day old. Comparing the Achilles (as representative energy-storing tendons) and tail (as representative positional tendons) tendons resulted in novel data showing the importance of mechanical loading in early postnatal tendon development. Notably, we showed that postnatal mechanical loading follows the onset of normal locomotor behavior in rats, and that when normal locomotion is disrupted, the mechanical development of tendons is impacted. Finally, we provided evidence that energy-storing (i.e. Achilles) and positional (i.e. tail) tendons may develop differently, an important finding for regenerative strategies incorporating cues from normal tendon formation. Future work should continue to explore the role of mechanical loading from the onset of normal locomotion at later postnatal ages. Similarly, future studies can build on this foundation and assess potential cellular mechanisms involved in mechanoregulation of postnatal tendon formation. Cellular mechanisms we identified in our MSC model of tenogenesis, namely Connexin-43, N-cadherin, Caherin-11, and β-catenin, would be worthwhile to explore in the developing tendons.

In addition to exploring mechanical stimuli, we also explored biochemical and cellular factors that drive tenogenic differentiation of stem cells. Our results helped to further establish TGFβ2 as an especially relevant tool for differentiating stem cells towards the tendon lineage, which enable the study of tendon development via *in vitro* cellular models. Using TGFβ2 allowed us to quantify how cell-cell junction proteins, N-cadherin, Cadherin-11 and Connexin-43, are impacted by tenogenesis. We also established a sharp decrease in N-cadherin as a novel potential marker of tenogenesis in stem cells. The results of our exploration of how TGFβ2 can be utilized to differentiate stem cells *in vitro* were described in Chapter 5. In Chapter 8, we expanded our cellular model of tenogenesis to include inhibition of Smad3, the cell signaling pathway by which TGFβ2 was thought to impact differentiation, and our results showed that other pathways may be active during tenogenesis. We identified the PI3K/Akt/mTOR/P70S6K pathway as a potential candidate through which Smad-independent tenogenesis may proceed. We further showed that inhibition of the Akt pathway disrupts tenogenic differentiation of stem cells. Identifying activation of PI3K/Akt/mTOR/P70S6K in tenogenesis provides a novel clinical target for enhancing tendon regeneration. Future studies should assess the impacts of enhanced Akt activation on tenogenesis, and further explore activation of Smad-independent signaling pathways in tenogenesis, namely PI3K/Akt/mTORC1/P70S6K, Wnt/β-catenin, and BMP signaling, using siRNA in cells, and, potentially, animal knockouts.

While not included in the chapters within this dissertation, work we have published at various conferences suggested that changes in levels and localization of β-catenin accompany TGFβ2-induced tenogenesis in stem cells. Exploring the alteration of β-catenin with tenogenesis needs to be followed-up in future studies. β-catenin is of particular interest due to its presence in embryonic and adult tendon, and wide-ranging roles in regulation of cell behavior. Preliminary data in whole tendon tissue from postnatal rats also identified significant changes in levels of both β-catenin and connexin-43, as a function of age, and differences varied based on tendon type (Achilles versus tail). Future work should focus on further elucidating the possible roles of β-catenin and connexin-43 in tenogenic differentiation of stem cells and development of whole tendons.

Collectively, the published and preliminary studies comprising this dissertation explored mechanical and cellular factors regulating tendon development and differentiation. We developed a custom system for mechanically evaluating neonatal tendons, and for applying tensile loading to cells *in vitro*. We demonstrated that tendon formation is impacted by the onset of locomotor development and the development of tendon mechanical properties may be unique between energy storing and positional tendons. Our *in vitro* data in stem cells indicated that connexin-43, N-cadherin, Cadherin-11, and the PI3K/Akt/mTORC1/P70S6K pathway are involved in tenogenesis. Ultimately, this work and future studies will reveal novel clinical targets for the treatment of tendon injuries, as well as improve our understanding of how tendons develop, and how the mechanical, cellular, and biochemical pathways that guide tendon development can be manipulated to treat and reverse tendon injury and disease.

Literature Cited

[1] S. Thomopoulos, W.C. Parks, D.B. Rifkin, K.A. Derwin, Mechanisms of tendon injury and repair, J Orthop Res 33(6) (2015) 832-839.

[2] I. Lantto, J. Heikkinen, T. Flinkkila, P. Ohtonen, J. Leppilahti, Epidemiology of Achilles tendon ruptures: Increasing incidence over a 33-year period, Scandinavian journal of medicine & science in sports 25(1) (2014) e133-8.

[3] T.W. Lin, L. Cardenas, L.J. Soslowsky, Biomechanics of tendon injury and repair, J Biomech 37(6) (2004) 865-77.

[4] S.D. Subramony, B.R. Dargis, M. Castillo, E.U. Azeloglu, M.S. Tracey, A. Su, H.H. Lu, The guidance of stem cell differentiation by substrate alignment and mechanical stimulation, Biomaterials 34(8) (2013) 1942-53.

[5] C.K. Kuo, R.S. Tuan, Mechanoactive tenogenic differentiation of human mesenchymal stem cells, Tissue Eng Part A 14(10) (2008) 1615-27.

[6] A.I. Goncalves, M.T. Rodrigues, S. Lee, A. Atala, J.J. Yoo, R.L. Reis, M.E. Gomes, Understanding the Role of Growth Factors in Modulating Stem Cell Tenogenesis, PloS one 8(12) (2013) e83734.

[7] J.P. Brown, T.V. Galassi, M. Stoppato, N.R. Schiele, C.K. Kuo, Comparative analysis of mesenchymal stem cell and embryonic tendon progenitor cell response to embryonic tendon biochemical and mechanical factors, Stem cell research & therapy 6 (2015) 89.

[8] Y.H. Li, M. Ramcharan, Z.P. Zhou, D.J. Leong, T. Akinbiyi, R.J. Majeska, H.B. Sun, The Role of Scleraxis in Fate Determination of Mesenchymal Stem Cells for Tenocyte Differentiation, Sci Rep-Uk 5 (2015) 13149.

[9] N.R. Schiele, J.E. Marturano, C.K. Kuo, Mechanical factors in embryonic tendon development: potential cues for stem cell tenogenesis, Curr Opin Biotechnol 24(5) (2013) 834-40.

[10] V. Arvind, A.H. Huang, Mechanobiology of limb musculoskeletal development, Ann. N.Y. Acad. Sci. 1409 (2018) 18-32.

[11] A.H. Huang, T.J. Riordan, B.A. Pryce, J.L. Weibel, S.S. Watson, F. Long, V. Lefebvre, B.D. Harfe, H.S. Stadler, H. Akiyama, S.F. Tufa, D.R. Keene, R. Schweitzer, Musculoskeletal integration at the wrist underlies the modular development of limb tendons, Development 142(14) (2015) 2431-2441.

[12] X.S. Pan, J. Li, E.B. Brown, C.K. Kuo, Embryo movements regulate tendon mechanical property development, Philos Trans R Soc B 373(1759) (2018).

[13] S.K. Theodossiou, J. Tokle, N.R. Schiele, TGFbeta2-induced tenogenesis impacts cadherin and connexin cell-cell junction proteins in mesenchymal stem cells, Biochem Biophys Res Commun 508(3) (2019) 889-893.
[14] I. Lantto, J. Heikkinen, T. Flinkkila, P. Ohtonen, J. Leppilahti, Epidemiology of Achilles tendon ruptures: increasing incidence over a 33-year period, Scand J Med Sci Sports 25(1) (2015) e133-8.
[15] M. Kedia, M. Williams, L. Jain, M. Barron, N. Bird, B. Blackwell, D.R. Richardson, S. Ishikawa, G.A. Murphy, The effects of conventional physical therapy and eccentric strengthening for insertional Achilles tendinopathy, Int J Sports Phys Ther 9(4) (2014) 488-497.
[16] Z.A. Glass, N.R. Schiele, C.K. Kuo, Informing tendon tissue engineering with embryonic development, J Biomech 47 (2014) 1964-1968.
[17] P.K. Nguyen, X.S. Pan, J. Li, C.K. Kuo, Roadmap of molecular, compositional, and functional markers during embryonic tendon development, Connect Tissue Res 59(5) (2018) 495-508.
[18] A.H. Huang, Coordinated development of the limb musculoskeletal system: Tendon and muscle patterning and integration with the skeleton, Dev Biol 429(2) (2017) 420-428.
[19] R. Schweitzer, E. Zelzer, T. Volk, Connecting muscles to tendons: tendons and musculoskeletal development in flies and vertebrates, Development 137(17) (2010) 2807-2817.
[20] N. Felsenthal, E. Zelzer, Mechanical regulation of musculoskeletal system development, Development 144(23) (2017) 4271-4283.
[21] N.A. Dyment, J.L. Galloway, Regenerative biology of tendon: mechanisms for renewal and repair, Current molecular biology reports 1(3) (2015) 124-131.
[22] E. Zelzer, E. Blitz, M.L. Killian, S. Thomopoulos, Tendon-to-bone attachment: from development to maturity, Birth Defects Res C Embryo Today 102(1) (2014) 101-12.
[23] S. Thomopoulos, G.M. Genin, L.M. Galatz, The development and morphogenesis of the tendon-to-bone insertion - what development can teach us about healing, J Musculoskelet Neuronal Interact 10(1) (2010) 35-45.
[24] A. Subramanian, T.F. Schilling, Tendon development and musculoskeletal assembly: emerging roles for the extracellular matrix, Development 142(24) (2015) 4191-204.
[25] A.H. Huang, H.H. Lu, R. Schweitzer, Molecular regulation of tendon cell fate during development, J Orthop Res 33(6) (2015) 800-12.
[26] G. Yang, B.B. Rothrauff, R.S. Tuan, Tendon and ligament regeneration and repair: clinical relevance and developmental paradigm, Birth Defects Res C Embryo Today 99(3) (2013) 203-22.

[27] C.F. Liu, L. Aschbacher-Smith, N.J. Barthelery, N. Dyment, D. Butler, C. Wylie, What we should know before using tissue engineering techniques to repair injured tendons: a developmental biology perspective, Tissue Eng Part B Rev 17(3) (2011) 165-76.
[28] R. Schweitzer, J.H. Chyung, L.C. Murtaugh, A.E. Brent, V. Rosen, E.N. Olson, A. Lassar, C.J. Tabin, Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments, Development 128(19) (2001) 3855-66.
[29] A.E. Brent, R. Schweitzer, C.J. Tabin, A somitic compartment of tendon progenitors, Cell 113(2) (2003) 235-48.
[30] N.D. Murchison, B.A. Price, D.A. Conner, D.R. Keene, E.N. Olson, C.J. Tabin, R. Schweitzer, Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons, Development 134(14) (2007) 2697-708.
[31] B.A. Pryce, S.S. Watson, N.D. Murchison, J.A. Staverosky, N. Dunker, R. Schweitzer, Recruitment and maintenance of tendon progenitors by TGFbeta signaling are essential for tendon formation, Development 136(8) (2009) 1351-61.
[32] D. Docheva, E.B. Hunziker, R. Fassler, O. Brandau, Tenomodulin is necessary for tenocyte proliferation and tendon maturation, Mol Cell Biol 25(2) (2005) 699-705.
[33] C. Shukunami, A. Takimoto, M. Oro, Y. Hiraki, Scleraxis positively regulates the expression of tenomodulin, a differentiation marker of tenocytes, Dev Biol 298(1) (2006) 234-47.
[34] Y. Ito, N. Toriuchi, T. Yoshitaka, H. Ueno-Kudoh, T. Sato, S. Yokoyama, K. Nishida, T. Akimoto, M. Takahashi, S. Miyaki, H. Asahara, The Mohawk homeobox gene is a critical regulator of tendon differentiation, Proc Natl Acad Sci U S A 107(23) (2010) 10538-42.
[35] M.J. Guerquin, B. Charvet, G. Nourissat, E. Havis, O. Ronsin, M.A. Bonnin, M. Ruggiu, I. Olivera-Martinez, N. Robert, Y. Lu, K.E. Kadler, T. Baumberger, L. Doursounian, F. Berenbaum, D. Duprez, Transcription factor EGR1 directs tendon differentiation and promotes tendon repair, The Journal of clinical investigation 123(8) (2013) 3564-76.
[36] D.M. Chaplin, T.K. Greenlee, Jr., The development of human digital tendons, J Anat 120(Pt 2) (1975) 253-74.
[37] N.R. Schiele, F. von Flotow, Z.L. Tochka, L.A. Hockaday, J.E. Marturano, J.J. Thibodeau, C.K. Kuo, Actin cytoskeleton contributes to the elastic modulus of embryonic tendon during early development, J Orthop Res 33(6) (2015) 874-81.

[38] R. Meller, F. Schiborra, G. Brandes, K. Knobloch, T. Tschernig, S. Hankemeier, C. Haasper, A. Schmiedl, M. Jagodzinski, C. Krettek, E. Willbold, Postnatal maturation of tendon, cruciate ligament, meniscus and articular cartilage: a histological study in sheep, Annals of anatomy = Anatomischer Anzeiger : official organ of the Anatomische Gesellschaft 191(6) (2009) 575-85.
[39] H.L. Ansorge, S. Adams, D.E. Birk, L.J. Soslowsky, Mechanical, compositional, and structural properties of the post-natal mouse Achilles tendon, Ann Biomed Eng 39(7) (2011) 1904-13.
[40] J.E. Marturano, J.D. Arena, Z.A. Schiller, I. Georgakoudi, C.K. Kuo, Characterization of mechanical and biochemical properties of developing embryonic tendon, Proc Natl Acad Sci U S A 110(16) (2013) 6370-5.
[41] C.F. Liu, L. Aschbacher-Smith, N.J. Barthelery, N. Dyment, D. Butler, C. Wylie, Spatial and temporal expression of molecular markers and cell signals during normal development of the mouse patellar tendon, Tissue Eng Part A 18(5-6) (2012) 598-608.
[42] S.H. Richardson, T. Starborg, Y. Lu, S.M. Humphries, R.S. Meadows, K.E. Kadler, Tendon development requires regulation of cell condensation and cell shape via cadherin-11-mediated cell-cell junctions, Mol Cell Biol 27(17) (2007) 6218-28.
[43] N.S. Kalson, Y. Lu, S.H. Taylor, T. Starborg, D.F. Holmes, K.E. Kadler, A structure-based extracellular matrix expansion mechanism of fibrous tissue growth, eLife 4 (2015).
[44] N.R. Schiele, R.A. Koppes, D.B. Chrisey, D.T. Corr, Engineering cellular fibers for musculoskeletal soft tissues using directed self-assembly, Tissue Eng Part A 19(9-10) (2013) 1223-32.
[45] K. Mubyana, D.T. Corr, Cyclic Uniaxial Tensile Strain Enhances the Mechanical Properties of Engineered, Scaffold-Free Tendon Fibers, Tissue Eng Part A (2018).
[46] N.S. Kalson, D.F. Holmes, Z. Kapacee, I. Otermin, Y. Lu, R.A. Ennos, E.G. Canty-Laird, K.E. Kadler, An experimental model for studying the biomechanics of embryonic tendon: Evidence that the development of mechanical properties depends on the actinomyosin machinery, Matrix Biol 29(8) (2010) 678-89.
[47] C.K. Kuo, B.C. Petersen, R.S. Tuan, Spatiotemporal protein distribution of TGF-betas, their receptors, and extracellular matrix molecules during embryonic tendon development, Dev Dyn 237(5) (2008) 1477-89.
[48] J.P. Brown, V.G. Finley, C.K. Kuo, Embryonic mechanical and soluble cues regulate tendon progenitor cell gene expression as a function of developmental stage and anatomical origin, J Biomech 47(1) (2014) 214-22.

[49] J.P. Brown, T.V. Galassi, M. Stoppato, N.R. Schiele, C.K. Kuo, Comparative analysis of mesenchymal stem cell and embryonic tendon progenitor cell response to embryonic tendon biochemical and mechanical factors, Stem cell research & therapy 6(1) (2015) 89.
[50] F. Edom-Vovard, B. Schuler, M.A. Bonnin, M.A. Teillet, D. Duprez, Fgf4 positively regulates scleraxis and tenascin expression in chick limb tendons, Dev Biol 247(2) (2002) 351-66.
[51] A.E. Brent, T. Braun, C.J. Tabin, Genetic analysis of interactions between the somitic muscle, cartilage and tendon cell lineages during mouse development, Development 132(3) (2005) 515-28.
[52] E. Havis, M.A. Bonnin, J.E. de Lima, B. Charvet, C. Milet, D. Duprez, TGFβ and FGF promote tendon progenitor fate and act downstream of muscle contraction to regulate tendon differentiation during chick limb development, Development 143 (2016) 3839-3851.
[53] E. Havis, M.A. Bonnin, I. Olivera-Martinez, N. Nazaret, M. Ruggiu, J. Weibel, C. Durand, M.J. Guerquin, C. Bonod-Bidaud, F. Ruggiero, R. Schweitzer, D. Duprez, Transcriptomic analysis of mouse limb tendon cells during development, Development 141(19) (2014) 3683-96.
[54] J. Bénazet, E. Pignatti, A. Nugent, E. Unal, F. Laurent, R. Zeller, Smad4 is required to induce digit ray primordia and to initiate the aggregation and differentiation of chondrogenic progenitors in mouse limb buds, Development 139 (2012) 4250-4260.
[55] C. Chien, B. Pryce, S.F. Tufa, D.R. Keene, A.H. Huang, Optimizing a 3D model system for molecular manipulation of tenogenesis., Connect Tissue Res 59(4) (2018) 295-308.
[56] V. Russo, A. Mauro, A. Martelli, O. Di Giacinto, L. Di Marcantonio, D. Nardinocchi, P. Berardinelli, B. Barboni, Cellular and molecular maturation in fetal and adult ovine calcaneal tendons, J Anat 226(2) (2015) 126-42.
[57] H. Liu, C. Zhang, S. Zhu, P. Lu, T. Zhu, X. Gong, Z. Zhang, J. Hu, Z. Yin, B.C. Heng, Z. Chen, H.W. Ouyang, Mohawk Promotes the Tenogenesis of Mesenchymal Stem Cells Through Activation of the TGFβ Signaling Pathway, Stem Cells 33 (2015) 443-455.
[58] K. Otabe, H. Nakahara, A. Hasegawa, T. Matsukawa, F. Ayabe, N. Onizuka, M. Inui, S. Takada, Y. Ito, I. Sekiya, T. Muneta, M. Lotz, H. Asahara, The transcription factor Mohawk controls tenogenic differentiation of bone marrow mesenchymal stem cells in vitro and in vivo, J Orthop Res 33(1) (2015) 1-8.
[59] H. Suzuki, Y. Ito, M. Shinohara, S. Yamashita, S. Ichinose, A. Kishida, T. Oyaizu, T. Kayama, R. Nakamichi, N. Koda, K. Yagishita, M.K. Lotzg, A. Okawa, H. Asahara, Gene targeting of the transcription factor Mohawk in rats causes heterotopic ossification of Achilles tendon via failed tenogenesis, PNAS 113(28) (2016) 7840-7845.

[60] E.J.O. O'Brien, C.B. Frank, N.G. Shrive, B. Hallgrimsson, D.A. Hart, Heterotropic mineralization (ossification or calcification) in tendinopathy or following surgical tendon trauma., Int J Exp Pathol 93(5) (2012) 319-331.
[61] X. Chen, Z. Yin, J.L. Chen, H.H. Liu, W.L. Shen, Z. Fang, T. Zhu, J. Ji, H.W. Ouyang, X.H. Zou, Scleraxis-overexpressed human embryonic stem cell-derived mesenchymal stem cells for tendon tissue engineering with knitted silk-collagen scaffold., Tissue Eng Part A 20 (2014) 1583-1592.
[62] C. Shukunami, A. Takimoto, Y. Nishizaki, Y. Yoshimoto, S. Tanaka, S. Miura, H. Watanabe, T. Sakuma, T. Yamamoto, G. Kondoh, Y. Hiraki, Scleraxis is a transcriptional activator that regulates the expression of Tenomodulin, a marker of mature tenocytes and ligamentocytes, Sci Rep 8(3155) (2018).
[63] E.P. Bavin, F. Atkinson, T. Barsby, D.J. Guest, Scleraxis Is Essential for Tendon Differentiation by Equine Embryonic Stem Cells and in Equine Fetal Tenocytes., Stem Cells Dev 26(6) (2017) 441-450.
[64] Z. Kapacee, C.Y. Yeung, Y. Lu, D. Crabtree, D.F. Holmes, K.E. Kadler, Synthesis of embryonic tendon-like tissue by human marrow stromal/mesenchymal stem cells requires a three-dimensional environment and transforming growth factor beta3, Matrix Biol 29(8) (2010) 668-77.
[65] L. Bian, D.Y. Zhai, E. Tous, R. Rai, R.L. Mauck, J.A. Burdick, Enhanced MSC chondrogenesis following delivery of TGF-beta3 from alginate microspheres within hyaluronic acid hydrogels in vitro and in vivo, Biomaterials 32(27) (2011) 6425-34.
[66] B. Mikic, R. Entwistle, K. Rossmeier, L. Bierwert, Effect of GDF-7 deficiency on tail tendon phenotype in mice, J Orthop Res 26(6) (2008) 834-9.
[67] A. Maloul, K. Rossmeier, B. Mikic, V. Pogue, T. Battaglia, Geometric and material contributions to whole bone structural behavior in GDF-7-deficient mice, Connect Tissue Res 47(3) (2006) 157-62.
[68] B. Mikic, L. Bierwert, D. Tsou, Achilles tendon characterization in GDF-7 deficient mice, J Orthop Res 24(4) (2006) 831-41.
[69] R.N. Wang, J. Green, Z. Wang, Y. Deng, M. Qiao, M. Peabody, Q. Zhang, J. Ye, Z. Yan, S. Denduluri, O. Idowu, M. Li, C. Shen, A. Hu, R.C. Haydon, R. Kang, J. Mok, M.J. Lee, H.L. Luu, L.L. Shi, Bone Morphogenetic Protein (BMP) signaling in development and human diseases, Genes & diseases 1(1) (2014) 87-105.

[70] N.M. Wolfman, G. Hattersley, K. Cox, A.J. Celeste, R. Nelson, N. Yamaji, J.L. Dube, E. DiBlasio-Smith, J. Nove, J.J. Song, J.M. Wozney, V. Rosen, Ectopic Induction of Tendon and Ligament in Rats by Growth and Differentiation Factors 5, 6, and 7, Members of the TGF-b Gene Family, The Journal of clinical investigation 100(2) (1997) 321-330.

[71] H. Shen, R.H. Gelberman, M.J. Silva, S.E. Sakiyama-Elbert, S. Thomopoulos, BMP12 induces tenogenic differentiation of adipose-derived stromal cells, PloS one 8(10) (2013) e77613.

[72] J.Y. Lee, Z. Zhou, P.J. Taub, M. Ramcharan, Y. Li, T. Akinbiyi, E.R. Maharam, D.J. Leong, D.M. Laudier, T. Ruike, P.J. Torina, M. Zaidi, R.J. Majeska, M.B. Schaffler, E.L. Flatow, H.B. Sun, BMP-12 treatment of adult mesenchymal stem cells in vitro augments tendon-like tissue formation and defect repair in vivo, PloS one 6(3) (2011) e17531.

[73] C.I. Lorda-Diez, J.A. Montero, S. Choe, J.A. Garcia-Porrero, J.M. Hurle, Ligand- and Stage-Dependent Divergent Functions of BMP Signaling in the Differentiation of Embryonic Skeletogenic Progenitors In Vitro, 29(3) (2014) 735-748.

[74] G. Yang, B.B. Rothrauff, H. Lin, R. Gottardi, P.G. Alexander, R.S. Tuan, Enhancement of tenogenic differentiation of human adipose stem cells by tendon-derived extracellular matrix, Biomaterials 34 (2013) 9295-9306.

[75] C.-Y.C. Yeung, L.A.H. Zeef, C. Lallyett, Y. Lu, E.G. Canty-Laird, K.E. Kadler, Chick tendon fibroblast transcriptome and shape depend on whether the cell has made its own collagen matrix, Sci Rep 5(1) (2015) 13555.

[76] A.P. Breidenbach, N.A. Dyment, Y. Lu, M. Rao, J.T. Shearn, D.W. Rowe, K.E. Kadler, D.L. Butler, Fibrin gels exhibit improved biological, structural, and mechanical properties compared with collagen gels in cell-based tendon tissue-engineered constructs, Tissue Eng Part A 21(3-4) (2015) 438-50.

[77] J.C. Jung, P.X. Wang, G. Zhang, Y. Ezura, M.E. Fini, D.E. Birk, Collagen fibril growth during chicken tendon development: matrix metalloproteinase-2 and its activation, Cell Tissue Res 336(1) (2009) 79-89.

[78] S.S. Apte, N. Fukai, D.R. Beier, B.R. Olsen, The matrix metalloproteinase-14 (MMP-14) gene is structurally distinct from other MMP genes and is co-expressed with the TIMP-2 gene during mouse embryogenesis, J Biol Chem 272(41) (1997) 25511-7.

[79] H. Kinoh, H. Sato, Y. Tsunezuka, T. Takino, A. Kawashima, Y. Okada, M. Seiki, MT-MMP, the cell surface activator of proMMP-2 (pro-gelatinase A), is expressed with its substrate in mouse tissue during embryogenesis, J Cell Sci 109 (Pt 5) (1996) 953-9.

[80] L.V. Gulotta, D. Kovacevic, S. Montgomery, J.R. Ehteshami, J.D. Packer, S.A. Rodeo, Stem Cells Genetically Modified With the Developmental Gene MT1-MMP Improve Regeneration of the Supraspinatus Tendon-to-Bone Insertion Site, Am J Sport Med 38(7) (2010) 1429-1437.
[81] R.I. Sharma, J.G. Snedeker, Biochemical and biomechanical gradients for directed bone marrow stromal cell differentiation toward tendon and bone, Biomaterials 31(30) (2010) 7695-704.
[82] A.J. Engler, S. Sen, H.L. Sweeney, D.E. Discher, Matrix Elasticity Directs Stem Cell Lineage Specification, Cell 126(4) (2006) 677-689.
[83] D.J. McBride, R.L. Trelstad, F.H. Silver, Structural and Mechanical Assessment of Developing Chick Tendon, Int J Biol Macromol 10(4) (1988) 194-200.
[84] N.S. Kalson, D.F. Holmes, A. Herchenhan, Y. Lu, T. Starborg, K.E. Kadler, Slow stretching that mimics embryonic growth rate stimulates structural and mechanical development of tendon-like tissue in vitro, Dev Dyn 240(11) (2011) 2520-8.
[85] J.E. Marturano, N.R. Schiele, Z.A. Schiller, T.V. Galassi, M. Stoppato, C.K. Kuo, Embryonically inspired scaffolds regulate tenogenically differentiating cells, J Biomech 49(14) (2016) 3281-3288.
[86] Z. Yin, J.J. Hu, L. Yang, Z.F. Zheng, C.R. An, B.B. Wu, C. Zhang, W.L. Shen, H.H. Liu, J.L. Chen, B.C. Heng, G.J. Guo, X. Chen, H.W. Ouyang, Single-cell analysis reveals a nestin+ tendon stem/progenitor cell population with strong tenogenic potentiality, Science advances 2(11) (2016) e1600874.
[87] J.H. Wang, Mechanobiology of tendon, J Biomech 39(9) (2006) 1563-82.
[88] J.A. Kluge, G.G. Leisk, R.D. Cardwell, A.P. Fernandes, M. House, A. Ward, A.L. Dorfmann, D.L. Kaplan, Bioreactor System Using Noninvasive Imaging and Mechanical Stretch for Biomaterial Screening, Ann Biomed Eng 39(5) (2011) 1390-1402.
[89] A.R. Raveling, S.K. Theodossiou, N.R. Schiele, A 3D printed mechanical bioreactor for investigating mechanobiology and soft tissue mechanics, MethodsX (2018).
[90] D.W. Youngstrom, I. Rajpar, D.L. Kaplan, J.G. Barrett, A Bioreactor System for In Vitro Tendon Differentiation and Tendon Tissue Engineering, Journal of Orthopaedic Research 33(6) (2015) 911-918.
[91] A. Scott, P. Danielson, T. Abraham, G. Fong, A.V. Sampaio, T.M. Underhill, Mechanical force modulates scleraxis expression in bioartificial tendons, J Musculoskelet Neuronal Interact 11(2) (2011) 124-32.

[92] M.L. Bayer, C.Y.C. Yeung, K.E. Kadler, K. Qvortrup, K. Baar, R.B. Svensson, S.P. Magnusson, M. Krogsgaard, M. Koch, M. Kjaer, The initiation of embryonic-like collagen fibrillogenesis by adult human tendon fibroblasts when cultured under tension, Biomaterials 31(18) (2010) 4889-4897.
[93] K. Chokalingam, N. Juncosa-Melvin, S.A. Hunter, C. Gooch, C. Frede, J. Florert, G. Bradica, R. Wenstrup, D.L. Butler, Tensile stimulation of murine stem cell-collagen sponge constructs increases collagen type I gene expression and linear stiffness, Tissue Eng Part A 15(9) (2009) 2561-70.
[94] N. Juncosa-Melvin, K.S. Matlin, R.W. Holdcraft, V.S. Nirmalanandhan, D.L. Butler, Mechanical stimulation increases collagen type I and collagen type III gene expression of stem cell-collagen sponge constructs for patellar tendon repair, Tissue Eng 13(6) (2007) 1219-26.
[95] J.T. Shearn, N. Juncosa-Melvin, G.P. Boivin, M.T. Galloway, W. Goodwin, C. Gooch, M.G. Dunn, D.L. Butler, Mechanical stimulation of tendon tissue engineered constructs: Effects on construct stiffness, repair biomechanics, and their correlation, J Biomech Eng-T Asme 129(6) (2007) 848-854.
[96] V. Hamburger, H.L. Hamilton, A Series of Normal Stages in the Development of the Chick Embryo, Journal of Morphology 88(1) (1951) 49-92.
[97] G. Kardon, Muscle and tendon morphogenesis in the avian hind limb, Development 125(20) (1998) 4019-32.
[98] M. Kieny, A. Chevallier, Autonomy of tendon development in the embryonic chick wing, J Embryol Exp Morphol 49 (1979) 153-65.
[99] J.E. Marturano, J.F. Xylas, G.V. Sridharan, I. Georgakoudi, C.K. Kuo, Lysyl oxidase-mediated collagen crosslinks may be assessed as markers of functional properties of tendon tissue formation, Acta Biomater 10(3) (2014) 1370-9.
[100] E.A. Makris, D.J. Responte, N.K. Paschos, J.C. Hu, K.A. Athanasiou, Developing functional musculoskeletal tissues through hypoxia and lysyl oxidase-induced collagen cross-linking, PNAS (2014) E4832-E4841.
[101] A.G. Schwartz, J.H. Lipner, J.D. Pasteris, G.M. Genin, S. Thomopoulos, Muscle loading is necessary for the formation of a functional tendon enthesis, Bone 55(1) (2013) 44-51.
[102] S. Thomopoulos, H.M. Kim, S.Y. Rothermich, C. Biederstadt, R. Das, L.M. Galatz, Decreased muscle loading delays maturation of the tendon enthesis during postnatal development, J Orthop Res 25(9) (2007) 1154-63.
[103] Y. Liu, A.G. Schwartz, V. Birman, S. Thomopoulos, G.M. Genin, Stress amplification during development of the tendon-to-bone attachment, Biomech Model Mechanobiol 13 (2014) 973-983.

[104] N.A. Dyment, A.P. Breidenbach, A.G. Schwartz, R.P. Russell, L. Aschbacher-Smith, H. Liu, Y. Hagiwara, R. Jiang, S. Thomopoulos, D.L. Butler, D.W. Rowe, Gdf5 progenitors give rise to fibrocartilage cells that mineralize via hedgehog signaling to form the zonal enthesis, 405(1) (2015) 96-107.
[105] L.J. Ng, S. Wheatley, G.E. Muscat, J. Conway-Campbell, J. Bowles, E. Wright, D.M. Bell, P.P. Tam, K.S. Cheah, P. Koopman, SOX9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse, Dev Biol 183(1) (1997) 108-21.
[106] A.G. Schwartz, F. Long, S. Thomopoulos, Enthesis fibrocartilage cells originate from a population of Hedgehog-responsive cells modulated by the loading environment, Development 142(1) (2015) 196-206.
[107] P.P. Provenzano, R. Vanderby, Jr., Collagen fibril morphology and organization: implications for force transmission in ligament and tendon, Matrix Biol 25(2) (2006) 71-84.
[108] R.B. Svensson, A. Herchenhan, T. Starborg, M. Larsen, K.E. Kadler, K. Qvortrup, S.P. Magnusson, Evidence of structurally continuous collagen fibrils in tendons, Acta Biomater 50 (2017) 293-301.
[109] N.J. Lemme, N.Y. Li, S.F. DeFroda, J. Kleiner, B.D. Owens, Epidemiology of Achilles Tendon Ruptures in the United States: Athletic and Nonathletic Injuries From 2012 to 2016, Orthopaedic journal of sports medicine 6(11) (2018) 2325967118808238.
[110] L.S. Oh, B.R. Wolf, M.P. Hall, B.A. Levy, R.G. Marx, Indications for rotator cuff repair: a systematic review, Clin Orthop Relat Res 455 (2007) 52-63.
[111] P.B. Voleti, M.R. Buckley, L.J. Soslowsky, Tendon healing: repair and regeneration, Annual review of biomedical engineering 14 (2012) 47-71.
[112] S.P. Arnoczky, M. Lavagnino, M. Egerbacher, The mechanobiological aetiopathogenesis of tendinopathy: is it the over-stimulation or the under-stimulation of tendon cells?, International Journal of Experimental Pathology 88(4) (2007) 217-226.
[113] M. Kjaer, Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading, Physiol Rev 84(2) (2004) 649-98.
[114] S.P. Magnusson, M. Kjaer, The impact of loading, unloading, ageing and injury on the human tendon, J Physiol 597(5) (2019) 1283-1298.
[115] M.L. Killian, L. Cavinatto, L.M. Galatz, S. Thomopoulos, The role of mechanobiology in tendon healing, J Shoulder Elbow Surg 21(2) (2012) 228-37.

[116] K.M. Heinemeier, D. Skovgaard, M.L. Bayer, K. Qvortrup, A. Kjaer, M. Kjaer, S.P. Magnusson, M. Kongsgaard, Uphill running improves rat Achilles tendon tissue mechanical properties and alters gene expression without inducing pathological changes, J Appl Physiol 113 (2012) 827-836.
[117] S.A. Jelinsky, S.A. Rodeo, J. Li, L.V. Gulotta, J.M. Archambault, H.J. Seeherman, Regulation of gene expression in human tendinopathy, BMC Muskuloskeletal Disorders 12(86) (2011) 1-12.

[118] R.C. Dirks, J.S. Richard, A.M. Fearon, A. Scott, L.G. Koch, S.L. Britton, S.J. Warden, Uphill treadmill running does not induce histopathological changes in the rat Achilles tendon, BMC musculoskeletal disorders 14 (2013) 90.
[119] L.J. Soslowsky, S. Thomopoulos, S. Tun, C.L. Flanagan, C.C. Keefer, J. Mastaw, J.E. Carpenter, Neer Award 1999 - Overuse activity injures the supraspinatus tendon in an animal model: A histologic and biomechanical study, J Shoulder Elb Surg 9(2) (2000) 79-84.
[120] J.M. Archambault, S.A. Jelinsky, S.P. Lake, A.A. Hill, D.L. Glaser, L.J. Soslowsky, Rat Supraspinatus Tendon Expresses Cartilage Markers with Overuse, J Orthop Res 25(5) (2006) 617-624.
[121] G.Y. Ng, P.Y. Chung, J.S. Wang, R.T. Cheung, Enforced Bipedal Downhill Running Induces Achilles Tendinosis in Rats, Connect Tissue Res 52(6) (2011) 466-471.
[122] A. Arampatzis, K. Karamanidis, K. Albracht, Adaptational responses of the human Achilles tendon by modulation of the applied cyclic strain magnitude, Journal of Experimental Biology 210 (2007) 2743-2753.
[123] A. Arampatzis, A. Peper, S. Bierbaum, K. Albracht, Plasticity of human Achilles tendon mechanical and morphological properties in response to cyclic strain, J Biomech 43 (2010) 3073-3079.
[124] D.T. Fung, V.M. Wang, D.M. Laudier, J.H. Shine, J. Basta-Pljakic, K.J. Jepsen, M.B. Schaffler, E.L. Flatow, Subrupture Tendon Fatigue Damage, J Orthop Res 27 (2009) 264-273.
[125] H.B. Sun, Y. Li, D.T. Fung, R.J. Majeska, M.B. Schaffler, E.L. Flatow, Coordinate regulation of IL-1beta and MMP-13 in rat tendons following subrupture fatigue damage, Clin Orthop Relat Res 466(7) (2008) 1555-61.
[126] C.T. Thorpe, S. Chaudhry, I.I. Lei, A. Varone, G.P. Riley, H.L. Birch, P.D. Clegg, H.R. Screen, Tendon overload results in alterations in cell shape and increased markers of inflammation and matrix degradation, Scand J Med Sci Sports 25 (2015) e381-e391.

[127] E.M. Spiesz, C.T. Thorpe, S. Chaudhry, G.P. Riley, H.L. Birch, P.D. Clegg, H.R. Screen, Tendon extracellular matrix damage, degradation and inflammation in response to in vitro overload exercise, J Orthop Res 33 (2015) 889-897.
[128] S.A. Goodman, S.A. May, D. Heinegard, R.K. Smith, Tenocyte response to cyclical strain and transforming growth factor beta is dependent upon age and site of origin., Biorheology 41(5) (2004) 613-628.
[129] I.K.Y. Lo, L.L. Marchuk, R. Hollinshead, D.A. Hart, C.B. Frank, Matrix metalloproteinase and tissue inhibitor of matrix metalloproteinase mRNA levels are specifically altered in torn rotator cuff tendons., Am J Sports Med 32(5) (2004) 1223-1229.
[130] M. Lavagnino, S.P. Arnoczky, In vitro alterations in cytoskeletal tensional homeostasis control gene expression in tendon cells, J Orthop Res 23 (2005) 1211-1218.
[131] S.L. Woo, M.A. Gomez, T.J. Sites, P.T. Newton, C.A. Orlando, W.H. Akeson, The Biomechanical and Morphological Changes in the MCL of the Rabbit After Immobilization and Remobilization., J Bone Joint Surg 69A (1987) 1200-1211.
[132] M. Lavagnino, S.P. Arnoczky, T. Tian, Z. Vaupel, Effect of amplitude and frequency of cyclic tensile strain on the inhibition of MMP-1 mRNA expression in tendon cells: an in vitro study, Connect Tissue Res 44(3-4) (2003) 181-7.
[133] S.P. Arnoczky, T. Tian, M. Lavagnino, K. Gardner, Ex vivo static tensile loading inhibits MMP-1 expression in rat tail tendon cells through a cytoskeletally based mechanotransduction mechanism, J Orthop Res 22(2) (2004) 328-33.
[134] S.P. Arnoczky, M. Lavagnino, M. Egerbacher, O. Caballero, K. Gardner, Matrix metalloproteinase inhibitors prevent a decrease in the mechanical properties of stress-deprived tendons - An in vitro experimental study, Am J Sport Med 35(5) (2007) 763-769.
[135] K. Gardner, S.P. Arnoczky, O. Caballero, M. Lavagnino, The effect of stress-deprivation and cyclic loading on the TIMP/MMP ratio in tendon cells: an in vitro experimental study, Disability and rehabilitation 30(20-22) (2008) 1523-9.
[136] A.P. Bhole, B.P. Flynn, M. Liles, N. Saeidi, C.A. Dimarzio, J.W. Ruberti, Mechanical strain enhances survivability of collagen micronetworks in the presence of collagenase: implications for load-bearing matrix growth and stability, Philosophical transactions. Series A, Mathematical, physical, and engineering sciences 367(1902) (2009) 3339-62.
[137] R.J. Camp, M. Liles, J. Beale, N. Saeidi, B.P. Flynn, E. Moore, S.K. Murthy, J.W. Ruberti, Molecular mechanochemistry: low force switch slows enzymatic cleavage of human type I collagen monomer, J Am Chem Soc 133(11) (2011) 4073-8.

[138] S.W. Chang, B.P. Flynn, J.W. Ruberti, M.J. Buehler, Molecular mechanism of force induced stabilization of collagen against enzymatic breakdown, Biomaterials 33(15) (2012) 3852-9.
[139] B.P. Flynn, A.P. Bhole, N. Saeidi, M. Liles, C.A. Dimarzio, J.W. Ruberti, Mechanical strain stabilizes reconstituted collagen fibrils against enzymatic degradation by mammalian collagenase matrix metalloproteinase 8 (MMP-8), PloS one 5(8) (2010) e12337.
[140] B.P. Flynn, G.E. Tilburey, J.W. Ruberti, Highly sensitive single-fibril erosion assay demonstrates mechanochemical switch in native collagen fibrils, Biomech Model Mechanobiol 12(2) (2013) 291-300.
[141] K.E. Wyatt, J.W. Bourne, P.A. Torzilli, Deformation-dependent enzyme mechanokinetic cleavage of type I collagen, J Biomech Eng 131(5) (2009) 051004.
[142] S. Thomopoulos, G.R. Williams, L.J. Soslowsky, Tendon to bone healing: differences in biomechanical, structural, and compositional properties due to a range of activity levels, J Biomech Eng 125 (2003) 106-113.
[143] B.R. Freedman, J.J. Sarver, M.R. Buckley, P.B. Voleti, L.J. Soslowsky, Biomechanical and structural response of healing Achilles tendon to fatigue loading following acute injury, J Biomech 47 (2014) 2028-2034.
[144] B.R. Freedman, G.W. Fryhofer, N.S. Salka, H.A. Raja, C.D. Hillin, C.A. Nuss, D.C. Farber, L.J. Soslowsky, Mechanical, histological, and functional properties remain inferior in conservatively treated Achilles tendons in rodents: Long term evaluation, J Biomech 56 (2017) 55-60.
[145] K. Gardner, M. Lavagnino, M. Egerbacher, S.P. Arnoczky, Re-establishment of cytoskeletal tensional homeostasis in lax tendons occurs through an actin-mediated cellular contraction of the extracellular matrix, J Orthop Res 30(11) (2012) 1695-701.
[146] M. Lavagnino, A. Bedi, C.P. Walsh, E.R.S. Enselman, S. Sheibani-Rad, S.P. Arnoczky, Tendon Contraction After Cyclic Elongation Is an Age-Dependent Phenomenon In Vitro and In Vivo Comparisons, Am J Sport Med 42(6) (2014) 1471-1477.
[147] H.L. Moser, A.P. Doe, K. Meier, S. Garnier, D. Laudier, H. Akiyama, M.A. Zumstein, L.M. Galatz, A.H. Huang, Genetic lineage tracing of targeted cell populations during enthesis healing, J Orthop Res 36 (2018) 3275-3284.
[148] R. Yoshida, F. Alaee, F. Dyrna, M.S. Kronenberg, P. Maye, I. Kalajzic, D.W. Rowe, A.D. Mazzocca, N.A. Dyment, Murine supraspinatus tendon injury model to identify the cellular origins of rotator cuff healing, Connect Tissue Res 57(6) (2016) 507-515.

[149] K. Legerlotz, E.R. Jones, H.R. Screen, G.P. Riley, Increased expression of IL-6 family members in tendon pathology, Rheumatology (Oxford) 51(7) (2012) 1161-5.

[150] G. Riley, Tendinopathy—from basic science to treatment, Nat Clin Pract Rheumatol 4(2) (2008) 82-89.
[151] Y. Ueda, A. Inui, Y. Mifune, F. Takase, T. Kataoka, T. Kurosawa, K. Yamaura, T. Kokubu, R. Kuroda, Molecular changes to tendons after collagenase-induced acute tendon injury in a senescence-accelerated mouse model, BMC Muskuloskeletal Disorders 20(120) (2019) 1-7.
[152] K. Legerlotz, G.C. Jones, H.R. Screen, G.P. Riley, Cyclic loading of tendon fascicles using a novel fatigue loading system increases interleukin-6 expression by tenocytes, Scand J Med Sci Sports 23(1) (2013) 31-7.
[153] C.N. Manning, N. Havlioglu, E. Knutsen, S.E. Sakiyama-Elbert, M.J. Silva, S. Thomopoulos, R.H. Gelberman, The early inflammatory response after flexor tendon healing: a gene expression and histological analysis, J Orthop Res 32(5) (2014) 645-52.
[154] G. Yang, H.J. Im, J.H. Wang, Repetitive mechanical stretching modulates IL-1beta induced COX-2, MMP-1 expression, and PGE2 production in human patellar tendon fibroblasts, Gene 363 (2005) 166-72.
[155] P.K. Beredjiklian, M. Favata, J.S. Cartmell, C.L. Flanagan, T.M. Crombleholme, L.J. Soslowsky, Regenerative versus reparative healing in tendon: a study of biomechanical and histological properties in fetal sheep, Ann Biomed Eng 31(10) (2003) 1143-52.
[156] M. Favata, P.K. Beredjiklian, M.H. Zgonis, D.P. Beason, T.M. Crombleholme, A.F. Jawad, L.J. Soslowsky, Regenerative properties of fetal sheep tendon are not adversely affected by transplantation into an adult environment, J Orthop Res 24(11) (2006) 2124-32.
[157] K. Howell, C. Chien, R. Bell, D. Laudier, S.F. Tufa, D.R. Keene, N. Andarawis-Puri, A.H. Huang, Novel Model of Tendon Regeneration Reveals Distinct Cell Mechanisms Underlying Regenerative and Fibrotic Tendon Healing, Sci Rep 7 (2017) srep45238.
[158] A. McClellan, R. Evans, C. Sze, S. Kan, Y. Paterson, D. Guest, A novel mechanism for the protection of embryonic stem cell derived tenocytes from inflammatory cytokine interleukin 1 beta, Sci Rep 9(2755) (2019).
[159] D.J. Berkoff, S.A. Kallianos, S.M. Eskildsen, P.S. Weinhold, Use of an IL1-receptor antagonist to prevent the progression of tendinopathy in a rat model, J Orthop Res 34(4) (2016) 616-622.
[160] J. Li, M. Stoppato, N.R. Schiele, K.L. Graybeal, P.K. Nguyen, C.K. Kuo, Embryonic and postnatal tendon cells respond differently to interleukin-1β, Annals of the New York Academy of Sciences (2019) 1-10.

[161] D. Lin, P. Alberton, M.D. Caceres, E. Volkmer, M. Schieker, D. Docheva, Tenomodulin is essential for prevention of adipocyte accumulation and fibrovascular scar formation during early tendon healing, Cell death & disease 8 (2017) e3116.
[162] A. Chhabra, D. Tsou, R.T. Clark, V. Gaschen, E.B. Hunziker, B. Mikic, GDF-5 deficiency in mice delays Achilles tendon healing, J Orthop Res 21(5) (2003) 826-35.
[163] A.A. Dunkman, M.R. Buckley, M.J. Mienaltowski, S.M. Adams, S.J. Thomas, L. Satchell, A. Kumar, L. Pathmanathan, D.P. Beason, R.V. Iozzo, D.E. Birk, L.J. Soslowsky, Decorin expression is important for age-related changes in tendon structure and mechanical properties, Matrix Biol 32 (2013) 3-13.
[164] P.S. Robinson, T.F. Huang, E. Kazam, R.V. Iozzo, D.E. Birk, L.J. Soslowsky, Influence of decorin and biglycan on mechanical properties of multiple tendons in knockout mice, J Biomech Eng 127(1) (2005) 181-5.
[165] A.A. Dunkman, M.R. Buckley, M.J. Mienaltowski, S.M. Adams, S.J. Thomas, L. Satchell, A. Kumar, L. Pathmanathan, D.P. Beason, R.V. Iozzo, D.E. Birk, L.J. Soslowsky, The Tendon Injury Response Is Influenced by Decorin and Biglycan, Ann Biomed Eng 42(3) (2014) 619-630.
[166] A.A. Dunkman, M.R. Buckley, M.J. Mienaltowski, S.M. Adams, S.J. Thomas, A. Kumar, D.P. Beason, R.V. Iozzo, D.E. Birk, L.J. Soslowsky, The Injury Response of Aged Tendons in the Absence of Biglycan and Decorin, Matrix Biol 35 (2014) 232-238.
[167] T. Maeda, T. Sakabe, A. Sunaga, K. Sakai, A.L. Rivera, D.R. Keene, T. Sasaki, E. Stavnezer, J. Iannotti, R. Schweitzer, D. Ilic, H. Baskaran, T. Sakai, Conversion of mechanical force into TGF-beta-mediated biochemical signals, Curr Biol 21(11) (2011) 933-41.
[168] M.L. Killian, L. Cavinatto, S.A. Shah, E.J. Sato, S.R. Ward, N. Havlioglu, L.M. Galatz, S. Thomopoulos, The effects of chronic unloading and gap formation on tendon-to-bone healing in a rat model of massive rotator cuff tears, J Orthop Res 32(3) (2014) 439-47.
[169] R.T. Benson, S.M. McDonnell, H.J. Knowles, J.L. Rees, A.J. Carr, P.A. Hulley, Tendinopathy and tears of the rotator cuff are associated with hypoxia and apoptosis, J Bone Joint Surg Br 92(3) (2010) 448-453.
[170] H. Tempfer, A. Traweger, Tendon Vasculature in Health and Disease, Front Physiol 6 (2015) 1-7.
[171] K.J. Sikes, J. Li, S. Gao, Q. Shen, J.D. Sandy, A. Plaas, V.M. Wang, TGF-b1 or hypoxia enhance glucose metabolism and lactate production via HIF1A signaling in tendon cells, Connect Tissue Res 59(5) (2018) 458-471.

[172] H. Alfredson, D. Bjur, K. Thorsen, R. Lorentzon, P. Sandstrom, High intratendinous lactate levels in painful chronic Achilles tendinosis. An investigation using microdialysis technique, Journal of Orthopaedic Research 20(5) (2002) 934-938.
[173] H. Sano, I. Wakabayashi, E. Itoi, Stress distribution in the supraspinatus tendon with partial-thickness tears: An analysis using two-dimensional finite element model, J Shoulder Elbow Surg 15(1) (2006) 100-105.

[174] M. Lavagnino, S.P. Arnoczky, N. Elvin, J. Dodds, Patellar Tendon Strain Is Increased at the Site of the Jumper's Knee Lesion During Knee Flexion and Tendon Loading, Am J Sports Med 36(11) (2008) 2110-2118.
[175] M. Lavagnino, S.P. Arnoczky, J. Dodds, N. Elvin, Infrapatellar Straps Decrease Patellar Tendon Strain at the Site of the Jumper's Knee Lesion: A Computational Analysis Based on Radiographic Measurements, Sports Health 3(3) (2011) 296-302.

[176] B.P. Ingalls, Mathematical modeling in systems biology: an introduction, The MIT Press, Cambridge, MA, 2013.
[177] S. Goutelle, M. Maurin, F. Rougier, X. Barbaut, L. Bourguignon, M. Ducher, P. Maire, The Hill equation: a review of its capabilities in pharmacological modelling, Fundam Clin Pharmacol 22 (2008) 633-648.
[178] S.R. Young, B. Gardiner, A. Mehdizadeh, J. Rubenson, B. Umberger, D.W. Smith, Adaptive Remodeling of Achilles Tendon: A Multi-scale Computational Model, PLoS computational biology 12(9) (2016) e1005106.
[179] A. Mehdizadeh, B.S. Gardiner, M. Lavagnino, D.W. Smith, Predicting tenocyte expression profiles and average molecular concentrations in Achilles tendon ECM from tissue strain and fiber damage, Biomech Model Mechanobiol 16 (2017) 1329-1348.
[180] H.L. Ansorge, S. Adams, A.F. Jawad, D.E. Birk, L.J. Soslowsky, Mechanical Property Changes During Neonatal Development and Healing Using a Multiple Regression Model, J Biomech 45(7) (2012) 1288-1292.
[181] W.J. Richardson, B. Kegerreis, S. Thomopoulos, J.W. Holmes, Potential strain-dependent mechanisms defining matrix alignment in healing tendons, Biomech Model Mechanobiol 17 (2018) 1569-1580.
[182] K. Chen, X. Hu, S.S. Blemker, J.W. Holmes, Multiscale computational model of Achilles tendon wound healing: Untangling the effects of repair and loading, PLoS computational biology 14(12) (2018) e1006652.

[183] S. Duenwald-Kuehl, J. Kondratko, R.S. Lakes, R. Vanderby, Jr., Damage mechanics of porcine flexor tendon: mechanical evaluation and modeling, Ann Biomed Eng 40(8) (2012) 1692-707.
[184] M.R. Buckley, A.A. Dunkman, K.E. Reuther, A. Kumar, L. Pathmanathan, D.P. Beason, Validation of an Empirical Damage Model for Aging and In Vivo Injury of the Murine Patellar Tendon, J Biomech Eng 135 (2013) 41005-1-7.
[185] J.A. Gordon, B.R. Freedman, A. Zuskov, R.V. Iozzo, D.E. Birk, L.J. Soslowsky, Achilles tendons from decorin- and biglycan-null mouse models have inferior mechanical and structural properties predicted by an image-based empirical damage model, J Biomech 48(10) (2015) 2110-2115.

[186] A.S. LaCroix, S.E. Duenwald-Kuehl, R.S. Lakes, R. Vanderby, Jr., Relationship between tendon stiffness and failure: a metaanalysis, J Appl Physiol (1985) 115(1) (2013) 43-51.
[187] T.Y. Kostrominova, S. Calve, E.M. Arruda, L.M. Larkin, Ultrastructure of myotendinous junctions in tendon-skeletal muscle constructs engineered in vitro, Histol Histopathol 24(5) (2009) 541-550.
[188] X.H. Deng, A. Lebaschi, C.L. Camp, C.B. Carballo, N.W. Coleman, J. Zong, B.M. Grawe, S.A. Rodeo, Expression of Signaling Molecules Involved in Embryonic Development of the Insertion Site Is Inadequate for Reformation of the Native Enthesis: Evaluation in a Novel Murine ACL Reconstruction Model, J Bone Joint Surg 100(3102) (2018) 1-11.
[189] R. LaRanger, J.R. Peters-Hall, M. Coquelin, B.R. Alabi, C.T. Chen, W.E. Wright, J.W. Shay, Reconstituting Mouse Lungs with Conditionally Reprogrammed Human Bronchial Epithelial Cells, Tissue Eng Part A 24(7-8) (2018) 559-568.
[190] K. Schinnerling, C. Rosas, L. Soto, R. Thomas, J.C. Aguillon, Humanized Mouse Models of Rheumatoid Arthritis for Studies on Immunopathogenesis and Preclinical Testing of Cell-Based Therapies, Frontiers in immunology 10 (2019) 203.
[191] L.M. Galatz, L. Gerstenfeld, E. Heber-Katz, S.A. Rodeo, Tendon regeneration and scar formation: The concept of scarless healing, J Orthop Res 33(6) (2015) 823-31.
[192] R. Wilkins, L.J. Bisson, Operative versus nonoperative management of acute Achilles tendon ruptures: a quantitative systematic review of randomized controlled trials, Am J Sports Med 40(9) (2012) 2154-60.
[193] L.M. Galatz, C.M. Ball, S.A. Teefey, W.D. Middleton, K. Yamaguchi, The outcome and repair integrity of completely arthroscopically repaired large and massive rotator cuff tears, J Bone Joint Surg Am 86-A(2) (2004) 219-24.

[194] P.R. Heuberer, D. Smolen, L. Pauzenberger, F. Plachel, S. Salem, B. Laky, B. Kriegleder, W. Anderl, Longitudinal Long-term Magnetic Resonance Imaging and Clinical Follow-up After Single-Row Arthroscopic Rotator Cuff Repair: Clinical Superiority of Structural Tendon Integrity, Am J Sports Med 45(6) (2017) 1283-1288.
[195] A. Subramanian, T.F. Schilling, Tendon development and musculoskeletal assembly: emerging roles for the extracellular matrix, Development 142 (2015) 4191-4204.
[196] J. Kastelic, A. Galeski, E. Baer, The multicomposite structure of tendon, Connect Tissue Res 6(1) (1978) 11-23.
[197] C.M. McNeilly, A.J. Banes, M. Benjamin, J.R. Ralphs, Tendon cells in vivo form a three dimensional network of cell processes linked by gap junctions, J Anat 189 (Pt 3) (1996) 593-600.
[198] M. Grinstein, H.L. Dingwall, L.D. O'Connor, K. Zou, T.D. Capellini, J.L. Galloway, A distinct transition from cell growth to physiological homeostasis in the tendon, eLife 8 (2019) e48689.
[199] D.E. Leckband, J. de Rooij, Cadherin adhesion and mechanotransduction, Annu Rev Cell Dev Biol 30 (2014) 291-315.
[200] K. Mubyana, D.T. Corr, Cyclic Uniaxial Tensile Strain Enhances the Mechanical Properties of Engineered, Scaffold-Free Tendon Fibers, Tissue Eng Part A 24(23&24) (2018) 1807-1817.
[201] Y.H. Li, M. Ramcharan, Z.P. Zhou, D.J. Leong, T. Akinbiyi, R.J. Majeska, H.B. Sun, The Role of Scleraxis in Fate Determination of Mesenchymal Stem Cells for Tenocyte Differentiation, Sci Rep 5 (2015).
[202] T.-W. Qin, Y.-L. Sun, A.R. Thoreson, S.P. Steinmann, P.C. Amadio, K.-N. An, C. Zhao, Effect of mechanical stimulation on bone marrow stromal cell seeded tendon slice constructs: A potential engineered tendon patch for rotator cuff repair, Biomaterials 51 (2015) 43-50.
[203] V.S. Nirmalanandhan, M.R. Dressler, J.T. Shearn, N. Juncosa-Melvin, M. Rao, C. Gooch, G. Bradica, D.L. Butler, Mechanical stimulation of tissue engineered tendon constructs: effect of scaffold materials, J Biomech Eng 129(6) (2007) 919-23.
[204] R.L. Stanley, R.A. Fleck, D.L. Becker, A.E. Goodship, J.R. Ralphs, J.C. Patterson-Kane, Gap junction protein expression and cellularity: comparison of immature and adult equine digital tendons, J Anat 211 (2007) 325-334.
[205] Y. Kimura, H. Matsunami, T. Inoue, K. Shimamura, N. Uchida, T. Ueno, T. Miyazaki, M. Takeichi, Cadherin-11 Expressed in Association with Mesenchymal Morphogenesis in the Head, Somite, and Limb Bud of Early Mouse Embryos, Dev Biol 169(1) (1995) 347-358.

[206] Y. Luo, M. Ferreira-Cornwell, H. Baldwin, I. Kostetskii, J. Lenox, M. Lieberman, G. Radice, Rescuing the N-cadherin knockout by cardiac-specific expression of N- or E-cadherin., Development 128(4) (2001) 459-469.
[207] Y. Luo, I. Kostetskii, G.L. Radice, N‐cadherin is not essential for limb mesenchymal chondrogenesis, Dev Dynam 232(2) (2005) 336-344.
[208] P. Hasson, A. DeLaurier, M. Bennett, E. Grigorieva, L.A. Naiche, V.E. Papaioannou, T.J. Mohun, M.P. Logan, Tbx4 and tbx5 acting in connective tissue are required for limb muscle and tendon patterning, Dev Cell 18(1) (2010) 148-56.
[209] F.H. Brembeck, M. Rosario, W. Birchmeier, Balancing cell adhesion and Wnt signaling, the key role of beta-catenin, Curr Opin Genet Dev 16(1) (2006) 51-59.
[210] B. Hinz, P. Pittet, J. Smith-Clerc, C. Chaponnier, J.J. Meister, Myofibroblast development is characterized by specific cell-cell adherens junctions, Mol Biol Cell 15(9) (2004) 4310-20.
[211] P. Pittet, K.M. Lee, A.J. Kulik, J.J. Meister, B. Hinz, Fibrogenic fibroblasts increase intercellular adhesion strength by reinforcing individual OB-cadherin bonds, J Cell Sci 121(6) (2008) 877-886.
[212] F. Helmbacher, Tissue-specific activities of the Fat1 cadherin cooperate to control neuromuscular morphogenesis, PLoS Biol 16(5) (2018) e2004734.
[213] A. Ahmed, C. de Bock, L. Lincz, J. Pundavela, I. Zouikr, E. Sontag, H. Hondermarck, R. Thorne, FAT1 cadherin acts upstream of Hippo signalling through TAZ to regulate neuronal differentiation, Cell. Mol. Life Sci. 72 (2015) 4653–69.
[214] L.G. Morris, A.M. Kaufman, Y. Gong, D. Ramaswami, L.A. Walsh, S. Turcan, S. Eng, K. Kannan, Y. Zou, L. Peng, V.E. Banuchi, P. Paty, Z. Zeng, E. Vakiani, D. Solit, B. Singh, I. Ganly, L. Liau, T.C. Cloughesy, P.S. Mischel, I.K. Mellinghoff, T.A. Chan, Recurrent somatic mutation of FAT1 in multiple human cancers leads to aberrant Wnt activation, Nat. Genet. 45(3) (2013) 253-261.
[215] E.D. Hay, The mesenchymal cell, its role in the embryo, and the remarkable signaling mechanisms that create it, Dev Dyn 233(3) (2005) 706-720.
[216] T.G. Smith, N. Van Hateren, C. Tickle, S.A. Wilson, The expression of Fat-1 cadherin during chick limb development, Int J Dev Biol 51(2) (2007) 173-6.
[217] D.G. Seidler, R. Dreier, Decorin and its galactosaminoglycan chain: Extracellular regulator of cellular function?, IUBMB Life 60(11) (2008) 729-733.
[218] C. Ruhland, E. Schonherr, H. Robenek, U. Hansen, R.V. Iozzo, P. Bruckner, D.G. Seidler, The glycosaminoglycan chain of decorin plays an important role in collagen fibril formation at the early stages of fibrillogenesis, FEBS J 274(16) (2007) 4246-55.

[219] C.I. Lorda-Diez, J.A. Garcia-Porrero, J.M. Hurle, J.A. Montero, Decorin gene expression in the differentiation of the skeletal connective tissues of the developing limb, Gene Expr Patterns 15 (2014) 52-60.
[220] H. Akiyama, M. Chaboissier, J.F. Martin, A. Schedl, B. De Crombrugghe, The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6, Gene Dev 16 (2002) 2813-2828.
[221] S. Yamada, S. Pokutta, F. Drees, W.I. Weis, W.J. Nelson, Deconstructing the Cadherin-Catenin-Actin Complex, Cell 123(5) (2005) 889-901.
[222] C.J. Gottardi, B.M. Gumbiner, Distinct molecular forms of β-catenin are targeted to adhesive or transcriptional complexes, J Cell Biol 167(2) (2004) 339-349.
[223] T. Valenta, G. Hausmann, K. Basler, The many faces and functions of β-catenin, The EMBO Journal 31(12) (2012) 2714-2736.
[224] E.M. Verheyen, C.J. Gottardi, Regulation of Wnt/β-Catenin Signaling by Protein Kinases, Dev Dyn 239(1) (2010) 34-44.
[225] Y. Kishimoto, B. Ohkawara, T. Sakai, M. Ito, A. Masuda, N. Ishiguro, C. Shukunami, D. Docheva, K. Ohno, Wnt/β-catenin signaling suppresses expressions of Scx, Mkx, and Tnmd in tendonderived cells, PloS one 12(7) (2017) e0182051.
[226] S. Miyabara, Y. Yuda, Y. Kasashima, A. Kuwano, K. Arai, Regulation of Tenomodulin Expression Via Wnt/beta-catenin Signaling in Equine Bone Marrow-derived Mesenchymal Stem Cells, Journal of equine science / Japanese Society of Equine Science 25(1) (2014) 7-13.
[227] N. Genet, N. Bhatt, A. Bourdieu, K.K. Hirschi, Multifaceted Roles of Connexin 43 in Stem Cell Niches, Current Stem Cell Reports 4(1) (2018) 1-12.
[228] L.I. Plotkin, J.P. Stains, Connexins and pannexins in the skeleton: gap junctions, hemichannels and more, Cellular and molecular life sciences : CMLS 72(15) (2015) 2853-67.
[229] J.R. Ralphs, M. Benjamin, A.D. Waggett, D.C. Russell, K. Messner, J. Gao, Regional differences in cell shape and gap junction expression in rat Achilles tendon: relation to fibrocartilage differentiation, J Anat 193 (1998) 215-222.
[230] A.D. Waggett, M. Benjamin, J.R. Ralphs, Connexin 32 and 43 gap junctions differentially modulate tenocyte response to cyclic mechanical load, European journal of cell biology 85(11) (2006) 1145-1154.
[231] V. Russo, A. Mauro, A. Martelli, O. Di Giacinto, L. Di Marcantonio, D. Nardinocchi, P. Berardinelli, B. Barboni, Cellular and molecular maturation in fetal and adult ovine calcaneal tendons, J Anat 226 (2015) 126-142.

[232] C.M. Coleman, G.A. Loredo, C.W. Lo, R.S. Tuan, Correlation of GDF5 and Connexin 43 mRNA Expression During Embryonic Development, The Anatomical Record Part A 275A (2003) 1117-1121.
[233] S.K. Theodossiou, A.L. Bozeman, N. Burgett, M.R. Brumley, H.E. Swann, A.R. Raveling, J.J. Becker, N.R. Schiele, Onset of neonatal locomotor behavior and the mechanical development of Achilles and tail tendons, J Biomech 96 (2019) 109354.
[234] S.M. Smith, C.E. Thomas, D.E. Birk, Pericellular proteins of the developing mouse tendon: a proteomic analysis, Connect Tissue Res 53(1) (2012) 2-13.
[235] M.E. Wall, N.A. Dyment, J. Bodle, J. Volmer, E. Loboa, A. Cederlund, A.M. Fox, A.J. Banes, Cell Signaling in Tenocytes: Response to Load and Ligands in Health and Disease, in: P.W. Ackerman, D.A. Hart (Eds.), Metabolic Influences on Risk for Tendon Disorders, Springer International Publishing, Switzerland, 2016, pp. 79-95.
[236] J.R. Ralphs, A.D. Waggett, M. Benjamin, Actin stress fibres and cell-cell adhesion molecules in tendons: organisation in vivo and response to mechanical loading of tendon cells in vitro, Matrix Biol 21(1) (2002) 67-74.
[237] T.C. Keller, M.V. Hogan, G. Kesturu, R. James, G. Balian, A.B. Chhabra, Growth/differentiation factor-5 modulates the synthesis and expression of extracellular matrix and cell-adhesion-related molecules of rat Achilles tendon fibroblasts, Connect Tissue Res 52(4) (2011) 353-64.
[238] B. Mikic, B.J. Schalet, R.T. Clark, V. Gaschen, E.B. Hunziker, GDF-5 deficiency in mice alters the ultrastructure, mechanical properties and composition of the Achilles tendon, J Orthop Res 19(3) (2001) 365-71.
[239] M. Kuzma-Kuzniarska, C. Yapp, T.W. Pearson-Jones, A.K. Jones, P.A. Hulley, Functional assessment of gap junctions in monolayer and three-dimensional cultures of human tendon cells using fluorescence recovery after photobleaching, J Biomed Opt 19(1) (2014).
[240] E. Maeda, S. Kimura, M. Yamada, M. Tashiro, T. Ohashi, Enhanced gap junction intercellular communication inhibits catabolic and pro-inflammatory responses in tenocytes against heat stress, J Cell Commun Signal 11(4) (2017) 369-380.
[241] E. Maeda, S.J. Ye, W. Wang, D.L. Bader, M.M. Knight, D.A. Lee, Gap junction permeability between tenocytes within tendon fascicles is suppressed by tensile loading, Biomech Model Mechan 11(3-4) (2012) 439-447.
[242] E. Maeda, T. Ohashi, Mechano-regulation of gap junction communications between tendon cells is dependent on the magnitude of tensile strain, Biochem Bioph Res Co 465(2) (2015) 281-286.

[243] H.R.C. Screen, D.L. Bader, D.A. Lee, J.C. Shelton, Local Strain Measurement within Tendon, Strain 40 (2004) 157-163.
[244] E. Maeda, H. Pian, T. Ohashi, Temporal regulation of gap junctional communication between tenocytes subjected to static tensile strain with physiological and non-physiological amplitudes, Biochem Biophys Res Commun 482(4) (2017) 1170-1175.
[245] A.J. Banes, P. Weinhold, X. Yang, M. Tsuzaki, D. Bynum, M. Bottlang, T. Brown, Gap junctions regulate responses of tendon cells ex vivo to mechanical loading, Clin Orthop Relat R (367) (1999) S356-S370.
[246] M.E. Wall, C. Otey, J. Qi, A.J. Banes, Connexin 43 is localized with actin in tenocytes, Cell Motil Cytoskel 64(2) (2007) 121-130.
[247] J. Qi, A.M. Fox, L.G. Alexopoulos, L. Chi, D. Bynum, F. Guilak, A.J. Banes, IL-1beta decreases the elastic modulus of human tenocytes, J Appl Physiol 101(1) (2006) 189-95.
[248] M. Tsuzaki, G. Guyton, W. Garrett, J.M. Archambault, W. Herzog, L. Almekinders, D. Bynum, X. Yang, A.J. Banes, IL-1 beta induces COX2, MMP-1, -3 and -13, ADAMTS-4, IL-1 beta and IL-6 in human tendon cells, J Orthop Res 21(2) (2003) 256-64.
[249] J. Qi, L. Chi, D. Bynum, A.J. Banes, Gap junctions in IL-1β-mediated cell survival response to strain, J Appl Physiol (1985) 110(5) (2011) 1425-31.
[250] Y. Khaliq, G.G. Zhanel, Fluoroquinolone-Associated Tendinopathy: A Critical Review of the Literature, Clinical Infectious Diseases 36 (2003) 1404-1410.
[251] A. Menon, L. Pettinari, C. Martinelli, G. Colombo, N. Portinaro, I. Dalle-Donne, M.C. d'Agostino, N. Gagliano, New insights in extracellular matrix remodeling and collagen turnover related pathways in cultured human tenocytes after ciprofloxacin administration, Muscles, ligaments and tendons journal 3(3) (2013) 122-31.
[252] C. Zhang, Y. Zhang, B. Zhong, C.F. Luo, SMAD7 prevents heterotopic ossification in a rat Achilles tendon injury model via regulation of endothelial-mesenchymal transition, FEBS J 283(7) (2016) 1275-85.
[253] C.I. Lorda-Diez, J.A. Montero, M.J. Diaz-Mendoza, J.A. Garcia-Porrero, H.J. M., βig-h3 Potentiates the Profibrogenic Effect of TGFβ Signaling on Connective Tissue Progenitor Cells Through the Negative Regulation of Master Chondrogenic Genes, Tissue Eng Part A 19(3 and 4) (2013) 448-457.

[254] L.M. Sobral, P.F. Montan, K.G. Zecchin, H. Martelli-Junior, P.A. Vargas, E. Graner, R.D. Coletta, Smad7 Blocks Transforming Growth Factor‐β1‐Induced Gingival Fibroblast–Myofibroblast Transition via Inhibitory Regulation of Smad2 and Connective Tissue Growth Factor, Journal of Periodontology 82(4) (2011) 642-651.
[255] A. Khanna, M. Friel, N. Gougoulias, U.G. Longo, N. Maffulli, Prevention of adhesions in surgery of the flexor tendons of the hand: what is the evidence?, British medical bulletin 90 (2009) 85-109.
[256] S.H. Taylor, S. Al-Youha, T. Van Agtmael, Y. Lu, J. Wong, D.A. McGrouther, K.E. Kadler, Tendon is covered by a basement membrane epithelium that is required for cell retention and the prevention of adhesion formation, PloS one 6(1) (2011) e16337.
[257] A.I. Goncalves, M.T. Rodrigues, S.J. Lee, A. Atala, J.J. Yoo, R.L. Reis, M.E. Gomes, Understanding the role of growth factors in modulating stem cell tenogenesis, PloS one 8(12) (2013) e83734.
[258] C. Chien, B. Pryce, S.F. Tufa, D.R. Keene, A.H. Huang, Optimizing a 3D model system for molecular manipulation of tenogenesis, Connect Tissue Res 59(4) (2018) 295-308.
[259] A.M. Delise, R.S. Tuan, Analysis of N-cadherin function in limb mesenchymal chondrogenesis in vitro, Dev Dyn 225(2) (2002) 195-204.
[260] S.A. Oberlender, R.S. Tuan, Expression and functional involvement of N-cadherin in embryonic limb chondrogenesis, Development 120(1) (1994) 177-87.
[261] J. Kawaguchi, Y. Azuma, K. Hoshi, I. Kii, S. Takeshita, T. Ohta, H. Ozawa, M. Takeichi, O. Chisaka, A. Kudo, Targeted disruption of cadherin-11 leads to a reduction in bone density in calvaria and long bone metaphyses, Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 16(7) (2001) 1265-71.
[262] S. Alimperti, S.T. Andreadis, CDH2 and CDH11 act as regulators of stem cell fate decisions, Stem Cell Res 14(3) (2015) 270-82.
[263] A.R. Haas, R.S. Tuan, Chondrogenic differentiation of murine C3H10T1/2 multipotential mesenchymal cells: II. Stimulation by bone morphogenetic protein-2 requires modulation of N-cadherin expression and function, Differentiation 64(2) (1999) 77-89.
[264] A. Woods, G. Wang, H. Dupuis, Z. Shao, F. Beier, Rac1 Signaling Stimulates N-cadherin Expression, Mesenchymal Condensation, and Chondrogenesis, J Biol Chem 282(32) (2007) 23500-23508.

[265] R. Tuli, S. Tuli, S. Nandi, X. Huang, P.A. Manner, W.J. Hozack, K.G. Danielson, D.J. Hall, R.S. Tuan, Transforming growth factor-beta-mediated chondrogenesis of human mesenchymal progenitor cells involves N-cadherin and mitogen-activated protein kinase and Wnt signaling cross-talk, J Biol Chem 278(42) (2003) 41227-36.
[266] L. Gao, R. McBeath, C.S. Chen, Stem Cell Shape Regulates a Chondrogenic Versus Myogenic Fate Through Rac1 and N-Cadherin, Stem Cells 28(3) (2010) 564-572.
[267] D. Kim, S.S. Kang, E.J. Jin, Alterations in the temporal expression and function of cadherin-7 inhibit cell migration and condensation during chondrogenesis of chick limb mesenchymal cells in vitro, J Cell Physiol 221(1) (2009) 161-70.
[268] A. Di Benedetto, M. Watkins, S. Grimston, V. Salazar, C. Donsante, G. Mbalaviele, G.L. Radice, R. Civitelli, N-cadherin and cadherin 11 modulate postnatal bone growth and osteoblast differentiation by distinct mechanisms, J Cell Sci 123(Pt 15) (2010) 2640-8.
[269] M. Zhu, S. Lin, Y. Sun, Q. Feng, G. Li, L. Bian, Hydrogels functionalized with N-cadherin mimetic peptide enhance osteogenesis of hMSCs by emulating the osteogenic niche, Biomaterials 77 (2016) 44-52.
[270] L. Bian, M. Guvendiren, R.L. Mauck, J.A. Burdick, Hydrogels that mimic developmentally relevant matrix and N-cadherin interactions enhance MSC chondrogenesis, Proc Natl Acad Sci U S A 110(25) (2013) 10117-22.
[271] S.L. Cheng, F. Lecanda, M.K. Davidson, P.M. Warlow, S.F. Zhang, L. Zhang, S. Suzuki, T. St John, R. Civitelli, Human osteoblasts express a repertoire of cadherins, which are critical for BMP-2-induced osteogenic differentiation, Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 13(4) (1998) 633-44.
[272] L. Xu, F. Meng, M. Ni, Y. Lee, G. Li, N-cadherin regulates osteogenesis and migration of bone marrow-derived mesenchymal stem cells, Mol Biol Rep 40(3) (2013) 2533-9.
[273] H.P. Kiener, M.B. Brenner, Building the synovium: cadherin 11 mediates fibroblaset-like synoviocyte cell-cell adhesion, Arthritis Res Ther 7 (2005) 49-54.
[274] S.K. Agarwal, D.M. Lee, H.P. Kiener, M.B. Brenner, Coexpression of two mesenchymal cadherins, cadherin 11 and N-cadherin, on murine fibroblast-like synoviocytes, Arthritis Rheum-Us 58(4) (2008) 1044-1054.
[275] H.J. Donahue, R.W. Qu, D.C. Genetos, Joint diseases: from connexins to gap junctions, Nature Reviews 14 (2018) 42-51.

[276] M.D. Mayan, P. Carpintero-Fernandez, R. Gago-Fuentes, O. Martinez-de-Ilarduya, H. Wang, V. Valiunas, P. Brink, F.J. Blanco, Human Articular Chondrocytes Express Multiple Gap Junction Proteins, Am J Pathol 182(4) (2013) 1337-1346.
[277] C.M. Coleman, R.S. Tuan, Functional role of growth/differentiation factor 5 in chondrogenesis of limb mesenchymal cells, Mechanisms of development 120 (2003) 823-836.
[278] K. Schrobback, T. Klein, J. , T.B. Woodfield, The importance of connexin hemichannels during chondroprogenitor cell differentiation in hydrogel versus microtissue culture models., Tissue Eng Part A 21(11-12) (2015) 1785-94.
[279] Z. Li, Z. Zhou, M.M. Saunders, H.J. Donahue, Modulation of connexin 43 alters expression of osteoblastic differentiation markers., American journal of physiology. Cell physiology 290(4) (2006) 1248-1255.
[280] S.K. Grimston, M.P. Watkins, M.D. Brodt, M.J. Silva, R. Civitelli, Enhanced periosteal and endocortical responses to axial tibial compression loading in conditional connexin 43 deficient mice. , PloS one 7(9) (2012) e44222.
[281] M.C. Moorer, C. Hebert, R.E. Tomlinson, S.R. Iyer, M. Chason, J.P. Stains, Defective signaling, osteoblastogenesis and bone remodeling in a mouse model of connexin 43 C-terminal truncation, J Cell Sci 130(3) (2017) 531-540.
[282] S.K. Chang, Z.Z. Gu, M.B. Brenner, Fibroblast-like synoviocytes in inflammatory arthritis pathology: the emerging role of cadherin-11, Immunol Rev 233 (2010) 256-266.
[283] Y.E. Park, Y.J. Woo, S.H. Park, Y.M. Moon, H.J. Oh, J.I. Kim, H.S. Jin, S.H. Baek, G.T. Kim, J.H. Lee, M.L. Cho, S.I. Kim, IL-17 increases cadherin-11 expression in a model of autoimmune experimental arthritis and in rheumatoid arthritis, Immunol Lett 140(1-2) (2011) 97-103.
[284] L. Feng, Y. Zhang, X. Kou, R. Yang, D. Liu, X. Wang, Y. Song, H. Cao, D. He, Y. Gan, Y. Zhou, Cadherin-11 modulates cell morphology and collagen synthesis in periodontal ligament cells under mechanical stress, The Angle orthodontist 87(2) (2017) 193-199.
[285] P.F. Christopoulos, V.K. Bournia, S. Panopoulos, A. Vaiopoulos, M. Koutsilieris, P.P. Sfikakis, Increased messenger RNA levels of the mesenchymal cadherin-11 in the peripheral blood of systemic sclerosis patients correlate with diffuse skin involvement, Clin Exp Rheumatol 33(4) (2015) S36-S39.
[286] M.H. Wu, M. Pedroza, R. Lafyatis, A.T. George, M.D. Mayes, S. Assassi, F.K. Tan, M.B. Brenner, S.K. Agarwal, Identification of Cadherin 11 as a Mediator of Dermal Fibrosis and Possible Role in Systemic Sclerosis, Arthritis & Rheumatology 66(4) (2014) 1010-1021.
[287] M. Srinivas, V.K. Verselis, T.W. White, Human diseases associated with connexin mutations, Biochim Biophys Acta 1860(1) (2018) 192-201.

[288] A.A. Marino, D.D. Waddell, O.V. Kolomytkin, W.D. Meek, R. Wolf, K.K. Sadasivan, J.A. Albright, Increased intercellular communication through gap junctions may contribute to progression of osteoarthritis, Clin Orthop Relat Res 422 (2004) 224-232.
[289] C. Niger, F.D. Howell, J.P. Stains, Interleukin-1β Increases Gap Junctional Communication among Synovial Fibroblasts via the Extracellular Signal Regulated Kinase Pathway, Biol Cell 102(1) (2009) 37-49.
[290] Y. Wang, S. Zhang, D.S.W. Benoit, Degradable poly(ethylene glycol) (PEG)-based hydrogels for spatiotemporal control of siRNA/nanoparticle delivery, J Control Release 287 (2018) 58-66.
[291] A.K. Barry, H. Tabdili, I. Muhamed, J. Wu, N. Shashikanth, G.A. Gomez, A.S. Yap, C.J. Gottardi, J. de Rooij, N. Wang, D.E. Leckband, alpha-catenin cytomechanics--role in cadherin-dependent adhesion and mechanotransduction, J Cell Sci 127(Pt 8) (2014) 1779-91.
[292] R. Desai, R. Sarpal, N. Ishiyama, M. Pellikka, M. Ikura, U. Tepass, Monomeric alpha-catenin links cadherin to the actin cytoskeleton, Nat Cell Biol 15(3) (2013) 261-273.
[293] Y. Yu, R.C. Elble, Homeostatic Signaling by Cell–Cell Junctions and Its Dysregulation during Cancer Progression, J Clin Med 5(26) (2016) 5020026.
[294] K. Lundgreen, O. Lian, A. Scott, L. Engerbretsen, Increased levels of apoptosis and p53 in partial-thickness supraspinatus tendon tears, Knee Surg Sports Traumatol Arthrosc 21 (2013) 1636-1641.
[295] A.C. Abraham, S.A. Shah, M. Golman, L. Song, X. Li, I. Kurtaliaj, M. Akbar, N.L. Millar, Y. Abu-Amer, L.M. Galatz, S. Thomopoulos, Targeting the NF-kB signaling pathway in chronic tendon disease, Sci. Transl. Med. 11 (2019) eav4319.
[296] K.T. Best, F.K. Lee, E. Knapp, H.A. Awad, A.E. Loiselle, Deletion of NFKB1 enhances canonical NF-κB signaling and increases macrophage and myofibroblast content during tendon healing, Sci Rep 9 (2019) 10926.
[297] C.-K. Su, S.-C. Yen, T.-W. Li, Y.-C. Sun, Enzyme-Immobilized 3D-Printed Reactors for Online Monitoring of Rat Brain Extracellular Glucose and Lactate, Analytical Chemistry 88(12) (2016) 6265-6273.
[298] R.Y. Neches, K.J. Flynn, L. Zaman, E. Tung, N. Pudlo, On the Intrinsic Sterility of 3D Printing, Peer J 4(e2661) (2016).
[299] D.L. Butler, C. Gooch, K.R.C. Kinneberg, G.P. Boivin, M.T. Galloway, V.S. Nirmalanandhan, J.T. Shearn, N.A. Dyment, N. Juncosa-Melvin, The use of mesenchymal stem cells in collagen-based scaffolds for tissue-engineered repair of tendons, Nat Protoc 5(5) (2010) 849-863.

[300] R.A. Gould, K. Chin, T.P. Santisakultarm, A. Dropkin, J.M. Richards, C.B. Schaffer, J.T. Butcher, Cyclic strain anisotropy regulates valvular interstitial cell phenotype and tissue remodeling in three-dimensional culture., Acta Biomater 8(5) (2012) 1710-1719.
[301] D. Sakai, I. Kii, K. Nakagawa, H.N. Matsumoto, M. Takahashi, S. Yoshida, T. Hosoya, K. Takakuda, A. Kudo, Remodeling of Actin Cytoskeleton in Mouse Periosteal Cells under Mechanical Loading Induces Periosteal Cell Proliferation during Bone Formation, PloS one 6(9) (2011) e24847.
[302] J. Reuvers, A.R. Thoreson, C. Zhao, L. Zhang, G.D. Jay, K.N. An, M.L. Warman, P.C. Amadio, The mechanical properties of tail tendon fascicles from lubricin knockout, wild type and heterozygous mice, J Struct Biol 176(1) (2011) 41-5.
[303] K.A. Derwin, L.J. Soslowsky, A Quantitative Investigation of Structure-Function Relationships in a Tendon Fascicle Model, J Biomech Eng 121 (1999) 598.
[304] S.A. Ghodbane, M.G. Dunn, Physical and mechanical properties of linked type I collagen scaffolds derived from bovine, porcine, and ovine tendons, Journal of Biomedical Materials Research Part A 104A (2016) 2685-2692.
[305] A. Arivazhagan, S.H. Masood, Dynamic Mechanical Properties of ABS Material Processed by Fused Deposition Modelling, International Journal of Engineering Research and Applications (IJERA) 2(3) (2012) 2009-2014.
[306] T.A. Jarvinen, T.L. Jarvinen, P. Kannus, L. Jozsa, M. Jarvinen, Collagen fibres of the spontaneously ruptured human tendons display decreased thickness and crimp angle, J Orthop Res 22(6) (2004) 1303-9.
[307] J.K. de Jong, J.T. Nguyen, A.J. Sonnema, E.C. Nguyen, P.C. Amadio, S.L. Moran, The incidence of acute traumatic tendon injuries in the hand and wrist: a 10-year population-based study, Clinics in Orthopedic Surgery 6(2) (2014) 196-202.
[308] A.I. Caplan, Mesenchymal Stem Cells: Time to Change the Name!, Stem Cell Transl Med 6 (2017) 1445-1451.
[309] J.R. Ralphs, M. Benjamin, A.D. Waggett, D.W. Russell, K. Messner, J. Gao, Regional differences in cell shape and gap junction expression in rat Achilles tendon: relation to fibrocartilage differentiation, The Journal of Anatomy 193(2) (1998) 215-222.
[310] K.M. Chan, S.C. Fu, Y.P. Wong, W.C. Hui, Y.C. Cheuk, M.N.W. Wong, Expression of transforming growth factorbisoforms and theirroles in tendon healing, Wound Repair Regen 16 (2008) 399-407.

[311] M.K. Murphy, D.J. Huey, J.C. Hu, K.A. Athanasiou, TGF-beta1, GDF-5, and BMP-2 stimulation induces chondrogenesis in expanded human articular chondrocytes and marrow-derived stromal cells, Stem Cells 33(3) (2015) 762-73.
[312] L. Bian, D.Y. Zhai, E. Tous, R. Rai, R.L. Mauck, J.A. Burdick, Enhanced MSC Chondrogenesis Following Delivery of TGF-β3 from Alginate Microspheres within Hyaluronic Acid Hydrogels In Vitro and In Vivo, Biomaterials 32(27) (2011) 6425-6434.
[313] R.L. Dahlin, M. Ni, V.V. Meretoja, F.K. Kasper, A.G. Mikos, TGF-beta3-induced chondrogenesis in co-cultures of chondrocytes and mesenchymal stem cells on biodegradable scaffolds, Biomaterials 35(1) (2014) 123-32.
[314] A.H. Huang, A. Stein, R.S. Tuan, R.L. Mauck, Transient exposure to transforming growth factor beta 3 improves the mechanical properties of mesenchymal stem cell-laden cartilage constructs in a density-dependent manner, Tissue Eng Part A 15(11) (2009) 3461-72.
[315] E. Havis, M.A. Bonnin, J. Esteves de Lima, B. Charvet, C. Milet, D. Duprez, TGFbeta and FGF promote tendon progenitor fate and act downstream of muscle contraction to regulate tendon differentiation during chick limb development, Development 143(20) (2016) 3839-3851.
[316] G.P. Riley, R.L. Harrall, T.E. Cawston, B.L. Hazleman, E.J. Mackie, Tenascin-C and human tendon degeneration, Am J Pathol 149(3) (1996) 933-943.
[317] F.S. Jones, P.L. Jones, The tenascin family of ECM glycoproteins: structure, function, and regulation during embryonic development and tissue remodeling, Dev Dyn 218(2) (2000) 235-59.
[318] B.K. Connizzo, S.M. Yannascoli, L.J. Soslowsky, Structure-function relationships of postnatal tendon development: A parallel to healing, Matrix Biol 32(2) (2013) 106-116.
[319] M. Benjamin, E. Kaiser, S. Milz, Structure-function relationships in tendons: a review, J Anat 212(3) (2008) 211-28.
[320] S. Torp, R.G.C. Arridge, C.D. Armeniades, E. Baer, Structure-property relationships in tendon as a function of age., Structure of Fibrous Biopolymers 26 (1975) 197-221.
[321] C.M. Waugh, A.J. Blazevich, F. Fath, T. Korff, Age-related changes in mechanical properties of the Achilles tendon, J Anat 220(2) (2012) 144-55.
[322] J. Kondratko-Mittnacht, S. Duenwald-Kuehl, R. Lakes, R. Vanderby, Jr., Shear load transfer in high and low stress tendons, J Mech Behav Biomed Mater 45 (2015) 109-120.
[323] H.R.C. Screen, S. Toorani, J.C. Shelton, Microstructural stress relaxation mechanics in functionally different tendons, Medical Engineering & Physics 35 (2013) 96-102.
[324] K.A. Derwin, L.J. Soslowsky, J.H. Kimura, A.H. Plaas, Proteoglycans and glycosaminoglycan fine structure in the mouse tail tendon fascicle, J Orthop Res 19(2) (2001) 269-77.

[325] A. Bruneau, N. Champagne, P. Cousineau-Pelletier, G. Parent, E. Langelier, Preparation of rat tail tendons for biomechanical and mechanobiological studies, Journal of visualized experiments : JoVE (41) (2010).
[326] M. Lavagnino, K. Malek, K.L. Gardner, S.P. Arnoczky, Thermal energy enhances cell-mediated contraction of lax rat tail tendon fascicles following exercise, Muscles, ligaments and tendons journal 5(1) (2015) 51-5.
[327] D.L. Butler, N. Juncosa-Melvin, G.P. Boivin, M.T. Galloway, J.T. Shearn, C. Gooch, H. Awad, Functional tissue engineering for tendon repair: A multidisciplinary strategy using mesenchymal stem cells, bioscaffolds, and mechanical stimulation, J Orthop Res 26(1) (2008) 1-9.
[328] B.K. Hall, S.W. Herring, Paralysis and growth of the musculoskeletal system in the embryonic chick, Journal of Morphology 206(1) (1990) 45-56.
[329] A. Hosseini, D.A. Hogg, The effects of paralysis on skeletal development in the chick embryo. I. General effects, J Anat 177 (1991) 159-68.
[330] K.J. Lamb, J.C. Lewthwaite, J.P. Lin, D. Simon, E. Kavanagh, C.P. Wheeler-Jones, A.A. Pitsillides, Diverse range of fixed positional deformities and bone growth restraint provoked by flaccid paralysis in embryonic chicks, Int J Exp Pathol 84(4) (2003) 191-9.
[331] J.E. Scott, M. Haigh, G.E. Neo, S. Gibson, The effect of muscle paralysis on the radial growth of collagen fibrils in developing tendon, Clin Sci (Lond) 72(3) (1987) 359-63.
[332] C. Beckham, R. Dimond, T.K. Greenlee, Jr., The role of movement in the development of a digital flexor tendon, Am J Anat 150(3) (1977) 443-59.
[333] B. Mikic, T.L. Johnson, A.B. Chhabra, B.J. Schalet, M. Wong, E.B. Hunziker, Differential effects of embryonic immobilization on the development of fibrocartilaginous skeletal elements, Journal of rehabilitation research and development 37(2) (2000) 127-33.
[334] S.F. Nemec, R. Hoftberger, U. Nemec, D. Bettelheim, P.C. Brugger, G. Kasprian, G. Amann, S. Rotmensch, J.M. Graham, Jr., D.L. Rimoin, D. Prayer, Fetal akinesia and associated abnormalities on prenatal MRI, Prenat Diagn 31(5) (2011) 484-90.
[335] F. Mersmann, S. Bohm, A. Schroll, H. Boeth, G.N. Duda, A. Arampatzis, Muscle and tendon adaptation in adolescent athletes: A longitudinal study, Scand J Med Sci Sports 27(1) (2017) 75-82.
[336] K. Kubo, H. Akima, M. Kouzaki, M. Ito, Y. Kawakami, H. Kanehisa, T. Fukunaga, Changes in the elastic properties of tendon structures following 20 days bed-rest in humans, European Journal of Applied Physiology 83(6) (2000) 463-468.

[337] A.A. De Aro, B. De Campos Vidal, A. Biancalana, F.T. Tolentino, L. Gomes, S.M. Mattiello, E.R. Pimentel, Analysis of the Deep Digital Flexor Tendon in Rats Submitted to Stretching after Immobilization, Connect Tissue Res 53(1) (2012) 29-38.
[338] T. Andersson, P. Eliasson, M. Hammerman, O. Sandberg, P. Aspenberg, Low-level mechanical stimulation is sufficient to improve tendon healing in rats, J Appl Physiol 113(9) (2012) 1398-1402.
[339] H.E. Swann, M.R. Brumley, Locomotion and posture development in immature male and female rats (Rattus norvegicus): Comparison of sensory-enriched versus sensory-deprived testing environments., J Comp Psychol Epub (2018).
[340] S. Lee, H. Chieh, C. Lin, I. Jou, Y. Sun, L. Kuo, P. Wu, F. Su, Characteristics of Sonography in a Rat Achilles Tendinopathy Model: Possible Non-invasive Predictors of Biomechanics, Sci Rep 7(5100) (2017).
[341] G. Parent, M. Cyr, F. Desbiens-Blais, E. Langelier, Bias and precision of algorithms in estimating the cross-sectional area of rat tail tendons, Meas Sci Technol 21 (2010) 125802.
[342] J.L. Chen, W. Zhang, Z.Y. Liu, T. Zhu, W.L. Shen, J.S. Ran, Q.M. Tang, X.N. Gong, L.J. Backman, X. Chen, X.W. Chen, F.Q. Wen, H.W. Ouyang, Characterization and comparison of post-natal rat Achilles tendon-derived stem cells at different development stages, Sci Rep 6 (2016).
[343] E.A. Makris, D.J. Responte, N.K. Paschos, J.C. Hu, K.A. Athanasiou, Developing functional musculoskeletal tissues through hypoxia and lysyl oxidase-induced collagen cross-linking, Proc Natl Acad Sci U S A 111(45) (2014) E4832-41.
[344] A.H. Lee, D.M. Elliott, Comparative multi-scale hierarchical structure of the tail, plantaris, and Achilles tendons in the rat, J Anat 234(2) (2019) 252-262.
[345] S. Calve, I.F. Lytle, K. Grosh, D.L. Brown, E.M. Arruda, Implantation increases tensile strength and collagen content of self-assembled tendon constructs, J Appl Physiol (1985) 108(4) (2010) 875-81.
[346] A.M. Pardes, B.R. Freedman, G.W. Fryhofer, N.S. Salka, P.R. Bhatt, L.J. Soslowsky, Males have Inferior Achilles Tendon Material Properties Compared to Females in a Rodent Model, Ann Biomed Eng 44(10) (2016) 2901-2910.
[347] B. Mikic, E. Amadei, K. Rossmeier, L. Bierwert, Sex matters in the establishment of murine tendon composition and material properties during growth, J Orthop Res 28(5) (2010) 631-8.
[348] M.J. Guerquin, B. Charvet, G. Nourissat, E. Havis, O. Ronsin, M.A. Bonnin, M. Ruggiu, I. Olivera-Martinez, N. Robert, Y.H. Lu, K.E. Kadler, T. Baumberger, L. Doursounian, F. Berenbaum, D. Duprez, Transcription factor EGR1 directs tendon differentiation and promotes tendon repair, Journal of Clinical Investigation 123(8) (2013) 3564-3576.

[349] V. Lejard, F. Blais, M.J. Guerquin, A. Bonnet, M.A. Bonnin, E. Havis, M. Malbouyres, C.B. Bidaud, G. Maro, P. Gilardi-Hebenstreit, J. Rossert, F. Ruggiero, D. Duprez, EGR1 and EGR2 involvement in vertebrate tendon differentiation, J Biol Chem 286(7) (2011) 5855-67.
[350] S.K. Theodossiou, J.B. Murray, N.R. Schiele, Cell-cell junctions in developing and adult tendons, Tissue Barriers 8(1) (2020).
[351] A.B. Berthet, C. Chen, K.B. Butcher, R.A. Schneider, T. Alliston, M. Amirtharajah, Smad3 Binds Scleraxis and Mohawk and Regulates Tendon Development, J Orthop Res 31(9) (2013) 1475-1483.
[352] G. Tan, B.A. Pryce, A. Stabio, J.V. Brigande, C. Wang, Z. Xia, S.F. Tufa, D.R. Keene, R. Schweitzer, TGFβ signaling is critical for maintenance of the tendon cell fate, eLife 9 (2020) e52695.
[353] A. Herchenhan, N.S. Kalson, D.F. Holmes, P. Hill, K.E. Kadler, L. Margetts, Tenocyte contraction induces crimp formation in tendon-like tissue, Biomech Model Mechanobiol 11(3-4) (2012) 449-59.
[354] R. Schweitzer, E. Zelzer, T. Volk, Connecting muscles to tendons: tendons and musculoskeletal development in flies and vertebrates, Development 137(17) (2010) 2807-17.
[355] H. Khayyeri, P. Blomgran, M. Hammerman, M.J. Turunen, A. Lowgren, M. Guizar-Sicairos, P. Aspenberg, H. Isaksson, Achilles tendon compositional and structural properties are altered after unloading by botox, Sci Rep 7 (2017) 13067.
[356] T.M. Doherty, A.L. Bozeman, T.L. Roth, M.R. Brumley, DNA methylation and behavioral changes induced by neonatal spinal transection, Infant Behav Dev 57 (2019) 101381.
[357] M. Javidi, C.P. McGowan, N.R. Schiele, D.C. Lin, Tendons from kangaroo rats are exceptionally strong and tough, Sci Rep 9(1) (2019) 8196.
[358] N.R. Schiele, J.E. Marturano, T. Thibideau, C.K. Kuo, The actin cytoskeleton may contribute to embryonic tendon mechanical properties, ORS Trans (2013).
[359] T. Shearer, C.T. Thorpe, H.R.C. Screen, The relative compliance of energy-storing tendons may be due to the helical fibril arrangement of their fascicles, J R Soc Interface 14(133) (2017).
[360] E.M. Spiesz, C.T. Thorpe, P.J. Thurner, H.R.C. Screen, Structure and collagen crimp patterns of functionally distinct equine tendons, revealed by quantitative polarised light microscopy (qPLM), Acta Biomater 70 (2018) 281-292.
[361] C.T. Thorpe, C. Klemt, G.P. Riley, H.L. Birch, P.D. Clegg, H.R.C. Screen, Helical sub-structures in energy-storing tendons provide a possible mechanism for efficient energy storage and return, Acta biomaterialia 9(8) (2013) 7948-7956.

[362] R.K. Choi, M.M. Smith, S. Smith, C.B. Little, E.C. Clarke, Functionally distinct tendons have different biomechanical, biochemical and histological responses to in vitro unloading, J Biomech 95 (2019) 109321.
[363] J.D. Eekhoff, F. Fang, L.G. Kahan, G. Espinosa, A.J. Cocciolone, J.E. Wagenseil, R.P. Mecham, S.P. Lake, Functionally Distinct Tendons From Elastin Haploinsufficient Mice Exhibit Mild Stiffening and Tendon-Specific Structural Alteration, J Biomech Eng 139(11) (2017).
[364] T.W. Herod, N.C. Chambers, S.P. Veres, Collagen fibrils in functionally distinct tendons have differing structural responses to tendon rupture and fatigue loading, Acta Biomater 42 (2016) 296-307.
[365] A.S. Quigley, S. Bancelin, D. Deska-Gauthier, F. Legare, L. Kreplak, S.P. Veres, In tendons, differing physiological requirements lead to functionally distinct nanostructures, Sci Rep 8(1) (2018) 4409.
[366] C. Popov, M. Burggraf, L. Kreja, A. Ignatius, M. Schieker, D. Docheva, Mechanical stimulation of human tendon stem/progenitor cells results in upregulation of matrix proteins, integrins and MMPs, and activation of p38 and ERK1/2 kinases, Bmc Mol Biol 16 (2015).
[367] M.S. Blumberg, D.E. Lucas, Dual mechanisms of twitching during sleep in neonatal rats, Behav Neuro 108(6) (1994) 1196-1202.
[368] E.D. Weber, D.J. Stelzner, Behavioral effects of spinal cord transection in the developing rat, Brain res 125(2) (1977) 241-255.
[369] Q. Yuan, H. Su, K. Chiu, W. Wu, Z. Lin, Contrasting neuropathology and functional recovery after spinal cord injury in developing and adult rats, Neurosci Bull 29(4) (2013) 509-516.
[370] M.M. Strain, S.D. Kauer, T. Kao, M.R. Brumley, Inter- and intralimb adaptations to a sensory perturbation during activation of the serotonin system after a low spinal cord transection in neonatal rats, Frontiers in neural circuits 8 (2014) 80.
[371] A.M. Pardes, Z.M. Beach, H. Raja, A.B. Rodriguez, B.R. Freedman, L.J. Soslowsky, Aging leads to inferior Achilles tendon mechanics and altered ankle function in rodents, J Biomech 60 (2017) 30-38.
[372] S. Deng, Z. Sun, C. Zhang, G. Chen, J. Li, Surgical Treatment Versus Conservative Management for Acute Achilles Tendon Rupture: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. Deng S1, Sun Z1, Zhang C1, Chen G1, Li J2., J Foot Ankle Surg 56(6) (2017).

[373] R.G. Young, D.L. Butler, W. Weber, A.I. Caplan, S.L. Gordon, D.J. Fink, Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair, Journal of Orthopaedic Research 16(4) (1998) 406-413.
[374] I. Grafe, S. Alexander, J.R. Peterson, T.N. Snider, B. Levi, B. Lee, Y. Mishina, TGF-β Family Signaling in Mesenchymal Differentiation, Cold Spring Harb Perspect Biol 10(5) (2018) a022202.
[375] Y.E. Zhang, Non-Smad Signaling Pathways of the TGF-β Family, Cold Spring Harb Perspect Biol 9 (2017) a022129.
[376] X.X. Cong, X.S. Rao, J.X. Lin, X.C. Liu, G.A. Zhang, X.K. Gao, M.Y. He, W.L. Shen, W. Fan, D. Pioletti, L.L. Zheng, H.H. Liu, Z. Yin, B.C. Low, R. Schweitzer, H. Ouyang, X. Chen, Y.T. Zhou, Activation of AKT-mTOR Signaling Directs Tenogenesis of Mesenchymal Stem Cells, Stem Cells (2018).
[377] X.X. Cong, X.S. Rao, J.X. Lin, X.C. Liu, G.A. Zhang, X.K. Gao, M.Y. He, W.L. Shen, W. Fan, D. Pioletti, L.L. Zheng, H.H. Liu, B.C. Low, R. Schweitzer, H. Ouyang, X. Chen, Y.T. Zhou, Activation of AKT-mTOR Signaling Directs Tenogenesis of Mesenchymal Stem Cells, Stem Cells 36 (2018) 527-539.
[378] I. Hers, E.E. Vincent, J.M. Tavare, Akt signalling in health and disease, Cell Signal. 23(10) (2011) 1515-1527.
[379] X. Guo, X.F. Wang, Signaling cross-talk between TGF-β/BMP and other pathways, Cell Res 19(1) (2009) 71-88.
[380] S.K. Madala, V. Sontake, R. Edukulla, C.R. Davidson, S. Schmidt, W.D. Hardie, Unique and Redundant Functions of p70 Ribosomal S6 Kinase Isoforms Regulate Mesenchymal Cell Proliferation and Migration in Pulmonary Fibrosis., Am J Respir Cell Mol Biol 55(6) (2016) 792-803.
[381] M.C. Wilkes, H. Mitchell, S.G. Penheiter, J.J. Doré, K. Suzuki, M. Edens, D.K. Sharma, R.E. Pagano, E.B. Leof, Transforming Growth Factor-β Activation of Phosphatidylinositol 3-Kinase Is Independent of Smad2 and Smad3 and Regulates Fibroblast Responses via p21-Activated Kinase-2, Cancer research 65(22) (2005) 10431-10440.
[382] G. Tan, B.A. Pryce, A. Stabio, J.V. Brigande, C. Wang, Z. Xia, S.F. Tufa, D.R. Keene, R. Schweitzer, TGF-β signaling is critical for maintenance of the tendon cell fate, eLife 9 (2020) e52695.
[383] Y. Asano, H. Ihn, K. Yamane, M. Jinnin, Y. Mimura, K. Tamaki, Phosphatidylinositol 3-kinase is involved in {alpha}2(I) collagen gene expression in normal and scleroderma fibroblasts., J Immunol 172 (2004) 7123-7135.

[384] K. Song, H. Wang, T.L. Krebs, D. Danielpour, Novel roles of Akt and mTOR in suppressing TGF- beta/ALK5-mediated Smad3 activation, EMBO J 25 (2006) 58-69.
[385] G.M. Nitulescu, M. Van De Venter, G. Nitulescu, A. Ungurianu, P. Juzenas, Q. Peng, O.T. Olaru, D. Gradinaru, A. Tsatsakis, D. Tsoukalas, D.A. Spandidos, D. Margina, The Akt pathway in oncology therapy and beyond, Int J Oncol. 53(6) (2018) 2319-2331.
[386] B.D. Hopkins, C. Pauli, X. Du, D.G. Wang, X. Li, D. Wu, S. Amadiume, M.D. Goncalves, C. Hodakoski, M.R. Lundquist, R. Bareja, Y. Ma, E.M. Harris, A. Sboner, H. Beltran, M.A. Rubin, S. Mukherjee, L.C. Cantley, Suppression of Insulin Feedback Enhances the Efficacy of PI3K Inhibitors, Nature 560(7719) (2018) 499-503.
[387] B.D. Manning, L.C. Cantley, AKT/PKB signaling: navigating downstream[Cell 129(7) (2007) 1261-1274.
[388] S. Lamouille, R. Derynck, Cell size and invasion in TGF-{beta} induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway, J Cell Biol 178 (2007) 437-451.
[389] E. Berthet, C. Chen, K. Butcher, R.A. Schneider, T. Alliston, M. Amirtharajah, Smad3 Binds Scleraxis and Mohawk and Regulates Tendon Matrix Organization, J Orthop Res 31 (2013) 1475-1483.
[390] R.A. Clark, G.A. McCoy, J.M. Folkvord, J.M. McPherson, TGF-beta 1 stimulates cultured human fibroblasts to proliferate and produce tissue-like fibroplasia: a fibronectin matrix-dependent event, J Cell Physiol 170(1) (1997) 69-80.
[391] Y.M. Farhat, A.A. Al-Maliki, T. Chen, S.C. Juneja, E.M. Schwarz, R.J. O'Keefe, H.A. Awad, Gene Expression Analysis of the Pleiotropic Effects of TGF-beta 1 in an In Vitro Model of Flexor Tendon Healing, PloS one 7(12) (2012).
[392] S.C. Juneja, E.M. Schwarz, R.J. O'Keefe, H.A. Awad, Cellular and Molecular Factors in Flexor Tendon Repair and Adhesions: A Histological and Gene Expression Analysis, Connect Tissue Res 54(3) (2013) 218-226.
[393] H.C.J. Goodier, A.J. Carr, S.J.B. Snelling, L. Roche, K. Wheway, B. Watkins, S.G. Dakin, Comparison of transforming growth factor beta expression in healthy and diseased human tendon, Arthritis Res Ther 18 (2016) 48.
[394] A.V. Bakin, A.K. Tomlinson, N.A. Bhowmick, H.L. Moses, C.L. Arteaga, Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration., J Biol Chem 275 (2000) 36803-36810.

[395] W. Wang, D. Rigueur, K.M. Lyons, TGFβ Signaling in Cartilage Development and Maintenance, Birth Defects Res C Embryo Today 102(1) (2014) 37-51.
[396] P. Xia, X. Wang, Y. Qu, Q. Lin, K. Cheng, M. Gao, S. Ren, T. Zhang, X. Li, TGF-β1-induced chondrogenesis of bone marrow mesenchymal stem cells is promoted by low-intensity pulsed ultrasound through the integrin-mTOR signaling pathway, Stem cell research & therapy 8 (2017) 281.
[397] B.A. Hocevar, T.L. Brown, P.H. Howe, TGF-β induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway, EMBO J 18(5) (1999) 1345-1356.
[398] D.A. Kaji, K.L. Howell, Z. Balic, D. Hubmacher, A.H. Huang, Tgfβ signaling is required for tenocyte recruitment and functional neonatal tendon regeneration, eLife 9 (2020) e51779.
[399] B. Dummler, O. Tschopp, D. Hynx, Z. Yang, S. Dirnhofer, B.A. Hemmings, Life with a Single Isoform of Akt: Mice Lacking Akt2 and Akt3 Are Viable but Display Impaired Glucose Homeostasis and Growth Deficiencies, Mol Cell Biol 26(21) (2006) 8042-8051.