Characterization of MT3-MMP Production by Embryonic Tendon Progenitor Cells during Development and the Influence of Substrate Elastic Modulus on MT3-MMP Production

A thesis

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Abstract

Tendons are frequently injured, but current treatment strategies aimed at repairing or regenerating tendon have remain insufficient due to a limited understanding of which factors regulate cell behavior, matrix assembly, and mechanical properties. By elucidating these factors, we will gain valuable insights into how tendons heal and develop, which may allow for improved tendon tissue engineering strategies. Tendon healing and development are characterized by substantial extracellular matrix turnover, which is thought to be driven by matrix metalloproteinases, including MT3-MMP. MT3-MMP has been shown to be present in embryonic tendon, but only at late stages of development by Western blot. MT3-MMP has been implicated as a possible regulator of tissue development, but its role in tendon development has not yet been revealed. Here, a detailed description of MT3-MMP production during embryonic tendon development is presented, and substrate elastic modulus is explored as a potential regulator of MT3-MMP production. Immunofluorescent staining and image analysis were utilized to measure MT3-MMP protein distribution during tendon development. Embryonic tendon progenitor cells were cultured on polyacrylamide gels of different elastic moduli to determine the effect of substrate elastic modulus of MT3-MMP production. Our results showed that MT3-MMP protein is present throughout tendon development, and protein production decreases in response to increased substrate elastic modulus, possibly through the actin cytoskeleton.

Acknowledgments

I would like to thank my advisor, Dr. Catherine Kuo, for her guidance and support over the past two years. Her unwavering determination to produce quality work and advance the field of tendon tissue engineering was contagious and significantly advanced my level of scientific thinking. I would also like to thank Dr. Qiaobing Xu and Dr. Mitch McVey for serving on my thesis committee and providing me with additional support and encouragement. The product of this thesis was truly a team effort and I would not be graduating without the enormous amount of help from my colleagues in the Kuo lab; Dr. Nathan Schiele, Dr. Matteo Stoppato, Zach Glass, Kaori Graybeal, Nicole Danek, Fritz von Flotow, Zach Tochka, and Faith Karanja. I am so grateful to have shared the lab with these individuals, and have learned something from each and every one of them. Lastly, I would like to thank my friends and family for their unconditional love and support throughout all my years of schooling.

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Chapter 1: Introduction

Tendons are frequently injured, but our poor understanding of how tendon cells mediate natural tendon development has prevented effective therapies from being developed. In place of surgical intervention, a novel therapeutic approach is to completely remove the damaged tendon and replace it with a suitable replacement grown in a laboratory. In order to proceed with this approach, we must first gain a better understanding of which factors regulate cell behavior, matrix assembly, and mechanical properties during normal tendon development and maturation. One potentially important contributor to tendon development is MT3-MMP but this protein has, so far, been only briefly studied. This thesis describes a more thorough characterization of MT3-MMP behavior during tendon development and explores elastic modulus as a potential regulator of expression. The following is a brief outline of the chapters contained herein:

Chapter 2: This introductory chapter provides background information on tendon development, tendon tissue engineering, and MMPs, including MT3-MMP. Important pieces of literature are reviewed and rationale for the forthcoming experiments is provided.

Chapter 3: This chapter describes the characterization of normal MT3-MMP protein spatiotemporal distribution during tendon development. Immunofluorescent staining results are presented to show MT3-MMP protein

production in embryonic tendon and by isolated tendon progenitor cells of several developmental stages. These experiments provide foundation data necessary for later studies in the behavior of MT3-MMP.

Chapter 4: This chapter begins with a detailed description of the two-dimensional polyacrylamide gel system and how the experimental elastic modulus values were achieved. Next, data is presented describing the effect of substrate elastic modulus on MT3-MMP gene expression and protein production. Additionally, actin fibers are imaged and a correlation between actin fiber alignment and MT3-MMP protein production is explored.

Chapter 5: This chapter describes the effect of disrupting the actin cytoskeleton on MT3-MMP protein production. Blebbistatin is used to disrupt the actin cytoskeleton and changes in MT3-MMP protein production are measured by immunofluorescent staining. These data further support our hypothesis that MT3-MMP is regulated by substrate elastic modulus through a mechanism involving the actin cytoskeleton.

Chapter 6: This final chapter serves as a summary of all described experiments and draws conclusions based on the presented data. Small and large scale implications are discussed and future directions are suggested.

Chapter 2: Background

2.1 Tendon background

Tendons are connective tissues which transduce muscular forces to skeletal motion. Composed primarily of highly aligned collagen type I fibers, tendons routinely experience high loads resulting from normal motion. Tendons are characterized by high uniaxial tensile strength but relatively weak lateral strength, which frequently leads to chronic and acute injuries. In many cases, it is the existing chronic tendon injury, such as tendonitis, that precedes and foreshadows an acute injury, like an abrupt tendon tear. Each year in the United States alone, there are 16.4 million tendon and ligament injuries and incidence is expected to rise due to an aging population [1]. Accordingly, there is a great need for tendon injury treatment strategies, but very few effective methods currently exist due to poor understanding of normal tendon development and healthy tendon structure at the cellular level. Specifically, we have not yet elucidated which factors regulate cell behavior, matrix assembly, and mechanical properties during normal tendon development and maturation. Minor tendon injuries can be treated with rest, ice, and anti-inflammatories, but more serious injuries may require invasive surgery. Surgical methods vary with the type and severity of the tendon injury, but common techniques include re-attachment and grafting surgery [2]. Today's gold standard of treatment for severe tendon injuries is autograft surgery, which still comes with a host of serious side effects [2]. These treatment methods include complications such as low donor availability (allograft), donor

site morbidity (autograft), and infection. While embryonic tendons heal perfectly, adult tendons heal in a scarred manner, resulting in a permanently weaker tissue and an increased risk of re-injuring of the tendon (Figure 2-1) [3]. Consequently, there exists a substantial need to advance the field of tendon tissue engineering to better treat these injuries.

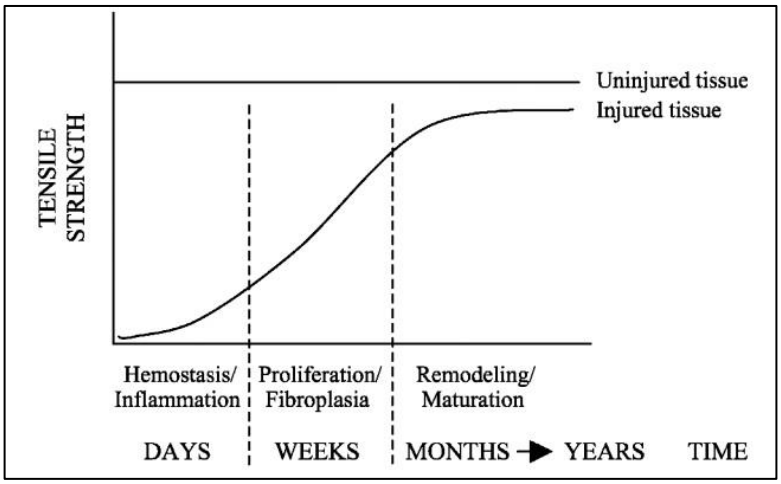


Figure 2-1: Post-injury, tendons undergo a series of healing steps which are marked by inflammation, proliferation, and remodeling. Even years after the injury, adult tendons never regain their original tensile strength [3].

2.2 Tendon tissue engineering

The field of tendon tissue engineering is still very much in its infancy. Tendons are complex tissues that have nonlinear mechanical properties and must be able to withstand extreme forces without rupturing. Biomaterials such as silk have been used in attempts to mimic the natural tendon, but so far, nothing has

proved successful as a clinically acceptable tendon replacement. A substantial roadblock in the progress of tendon tissue engineering is our lack of understanding of how tendons develop, and how injuries begin. Our only direct exposure to living human tendons occurs after the injury has already caused significant pain, and since it is unethical to invasively study healthy human tendons, we are quite limited in our ability to mimic tendon healing. By employing relevant animal models, researchers have made great strides in understanding tendon development and healing, but there is still much to be learned. Further study of tendon development at the cellular and molecular level is imperative to better inform tendon tissue engineering strategies.

2.3 Embryonic tendon development

Tendons are formed through a hierarchical structure in which collagen fibers are grouped with tendon fibroblasts to form fascicles, and fascicles are bound together by the connective endotenon to form the complete tendon (Figure 2-2) [4]. Studies have shown that there are three distinct steps involved in collagen fibrillogenesis. 1) Collagen molecules assemble extracellularly to form fibril intermediates. 2) Fibril intermediates link end-to-end to form longer, more mature fibrils. 3) Collagen fibrils undergo lateral growth to generate large-diameter fibrils [5]. Throughout these stages, tendons undergo substantial extracellular matrix turnover, characterized by a decrease in cell density, an increase in collagen type I content, and an increase in elastic modulus.

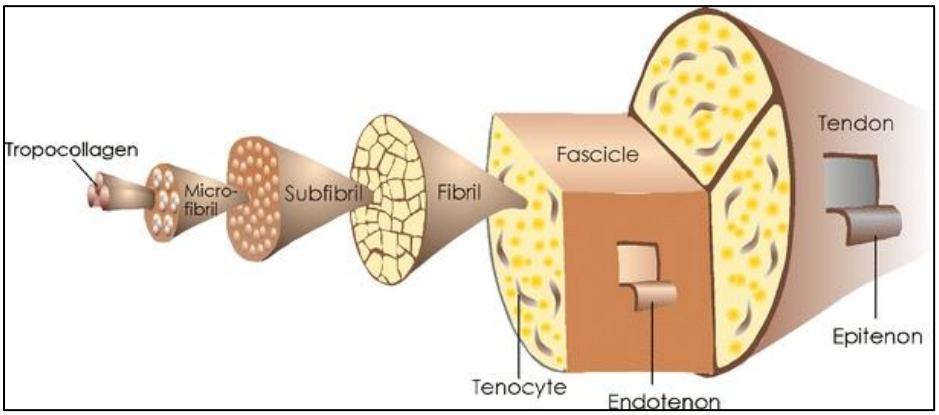


Figure 2-2: A. Hierarchical structure of mature tendon. Individual collagen fibers are bundled together to form fascicles, which are again bundled to form the full tendon [4].

By understanding the molecular structure of tendon, we can appreciate why tendon is so strong along its long axis, but relatively weak along its short axis. The collagen fibers of mature tendon are long, heavily cross-linked, and uniaxially *crimped* in a way that allows considerable stretching of the tendon before rupturing (Figure 2-3). Collagen fibers, instead of being taut at basal levels of tension or strain, exist in a wave-like pattern, which permeates the length of the tendon. The toe-in region of the stress/strain curve for tendon is thought to be a result of uncramping of the collagen fibers. In the toe-in region, the tendon can absorb the greatest amount of strain before sustaining any significantly increased stress [6]. Upon lengthening, collagen crimp can absorb about the first 2% of the strain, but with increased stretch beyond 2%, collagen fibers straighten and will eventually rupture with high strain.

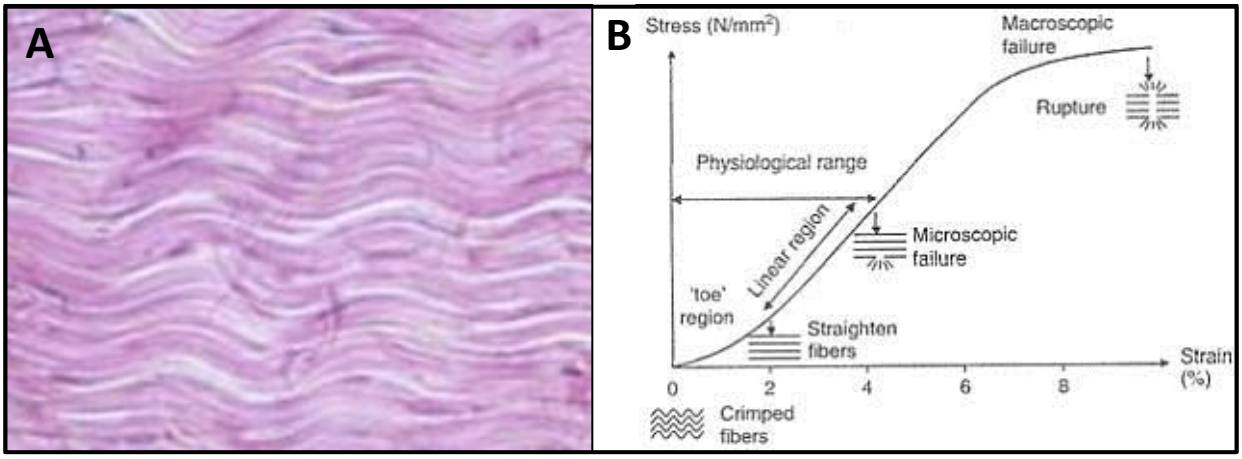


Figure 2-3: A. Tendon features characteristic collagen crimp, which allows the tissue to withstand substantial uniaxial loads (Nelson, 2012). **B.** Stress-strain curve for tendon shows how the force of the initial tendon extension (0-2% strain) may be absorbed by the straightening of the collagen crimp, beyond which point the collagen fibers are linearly stressed and eventually rupture with high strain [6].

During tendon development, collagen fibers undergo covalent intra- and intermolecular cross-linking mediated by lysyl oxidase, a protein which increases in content throughout development [7-8]. This lysyl oxidase-mediated collagen crossing contributes significantly to embryonic tendon elastic modulus at late stages of development [8]. Collagen fibers also branch in a way that allows individual fibers to separate from one fascicle, travel through the endotenon, and join another fascicle [5]. Despite these two mechanisms of stabilization, tendons are still relatively weak along their short axis. Indeed, tendons are commonly injured from lateral forces, such as a torn quadriceps tendon resulting from a blow to the side of the knee.

2.4 Matrix metalloproteinases (MMPs)

During development, tendons undergo significant changes in extracellular matrix (ECM) composition. Early embryonic tendon is composed of a wide variety of ECM molecules such as collagen type III, fibronectin, and decorin, all of which decrease in content throughout development [10]. Through matrix turnover, collagen type I content increases from undetectable levels to the point of composing 85% of the adult tendon's dry weight. While there are certainly many factors that contribute to matrix turnover, important contributors are the matrix metalloproteinase family. Matrix metalloproteinases (MMPs) are a group of 25 classified zinc-dependent endopeptidases capable of digesting a wide array of substrates. MMPs can break down nearly all structural ECM proteins as well as proteinases, proteinase inhibitors, latent growth factors, growth factor-binding receptors, chemokines, and cell adhesion molecules [11]. These functions allow MMPs to be influential in regulating biological processes such as cell migration, proliferation, adhesion, and apoptosis, all of which may be crucial to tendon development [12]. MMPs are generally expressed at low levels, but protein levels are known to rise rapidly when tissues undergo remodeling during embryonic development, inflammation, and wound repair [11]. Higher MMP activity during these processes suggests an important role for MMPs during tendon development.

Often, in order for a population of cells to grow and proliferate, it must first create space in the surrounding tissue by breaking down and removing the impeding matrix molecules. It is in this situation, especially, that the cell's arsenal of MMPs becomes invaluable. Based on the composition of the surrounding

ECM, the cell may freely release secreted MMPs, which can digest ECM components. Matrix turnover is a major step in tendon development, and it is thought that MMPs are influential in these processes of removing certain matrix components.

2.5 Membrane-type matrix metalloproteinases (MT-MMPs)

Most proteins of the MMP family are secreted freely outside the cell into the ECM, where they can actively degrade ECM molecules or interact with other proteins. A smaller group of six MMPs is known as the membrane-type matrix metalloproteinases (MT-MMPs), which are integrated into the cell membrane. Compared to MMPs, MT-MMPs are less well-characterized in terms of function and behavior. Like secreted MMPs, transmembrane MT-MMPs are synthesized as inactive zymogens and are later activated by furin-like enzymes in the *trans*-Golgi network or by plasmin at the cell surface [13]. While MMPs are released from the cell via exocytosis, MT-MMPs are inserted into the plasma membrane in an orientation that positions the large catalytic domain on the extracellular side of the cell and the smaller cytoplasmic domain just inside the cell membrane (Figure 2-4) [13-14]. This orientation allows MT-MMPs to interact with substrates outside of the cell membrane and subsequently pass this information to the cell nucleus by initiating a signaling cascade inside the cell.

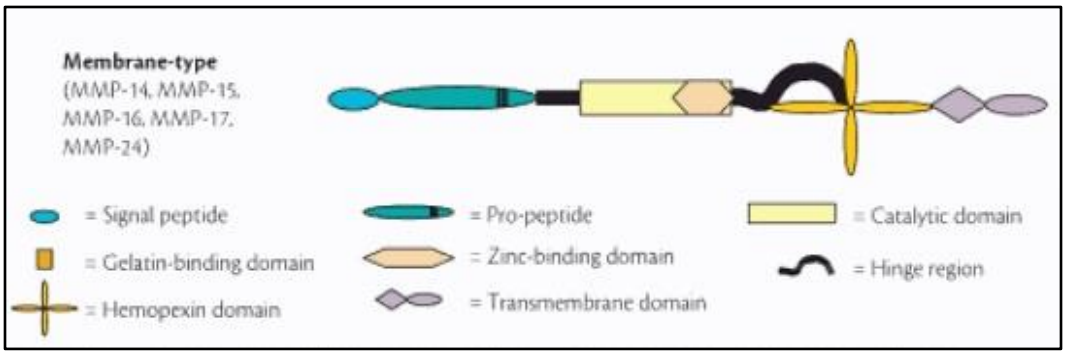


Figure 2-4: Conserved structure of MT-MMPs. After translation of the inactive pro-MT-MMP (pictured), the protein is directed to the Golgi via the signal peptide, and is then activated through cleavage at the furin-like motif by a furin-like enzyme [15].

2.6 MMPs and TIMPs

MMPs and MT-MMPs exist in a state of equilibrium with tissue inhibitors of metalloproteinases (TIMPs), which bind and block the catalytic domain of MMPs and alter the interactions between MMPs and other factors [11]. Four TIMPs have been identified and have been shown to maintain some level of specificity. For example, TIMP-1 effectively binds soluble MMPs, but shows no activity against MT-MMPs, including MT3-MMP. Furthermore, TIMP-2 and TIMP-3 inhibit soluble MMPs *and* MT-MMPs in a 1:1 molar ratio [18]. TIMPs prevent MMPs from binding and degrading ECM components, but are also thought to be involved in the process of MMP activation by MT-MMPs. A model has been proposed in which an MT-MMP first forms a complex with TIMP-2, then this complex binds MMP-2. Next, another free TIMP-2 molecule cleaves the pro-domain of MMP-2, thus activating it [11]. Although not widely studied, the complex interactions between MMPs and TIMPs may have significant

implications in tendon development, and may be an interesting topic for future studies.

2.7 MT3-MMP in tendon development

MT3-MMP (also referred to as MMP-16) is an MT-MMP that may play an important role in matrix turnover. MT3-MMP is known to be expressed in the heart, brain, lung, muscle, and skin [16], and was more recently found in tendon, when it was identified as an activator of MMP-2 [17] The ability of MT3-MMP to activate MMP-2 gives further weight to MT3-MMP's potential role in tendon development, since MMP-2 is known to regulate tendon fibril growth and degrade a wide array of tendon ECM molecules [17]. Substrates of MT3-MMP include collagen type I (partial degradation), collagen type III, cartilage proteoglycan, gelatin, fibronectin, vitronectin, and laminin-1 [18], all of which are ECM molecules present in tendon. Despite evidence that MT3-MMP may play an important role in tendon development, the behavior of the protein in tendon has only been briefly studied. It has been shown that MT3-MMP is expressed by embryonic chick limbs throughout development, but expression in tendon was not studied specifically (Figure 2-5) [16]. Another study used Western blot to show that the MT3-MMP protein is produced by embryonic chick tendon [17]. Additionally, it has been shown that the MT3-MMP gene is expressed in adult mouse tendon cells [18], but still, a thorough characterization in embryonic tendon has yet to be presented. The first objective of this thesis was to characterize MT3-MMP gene expression and protein spatiotemporal distribution throughout embryonic tendon development (Chapter 3).

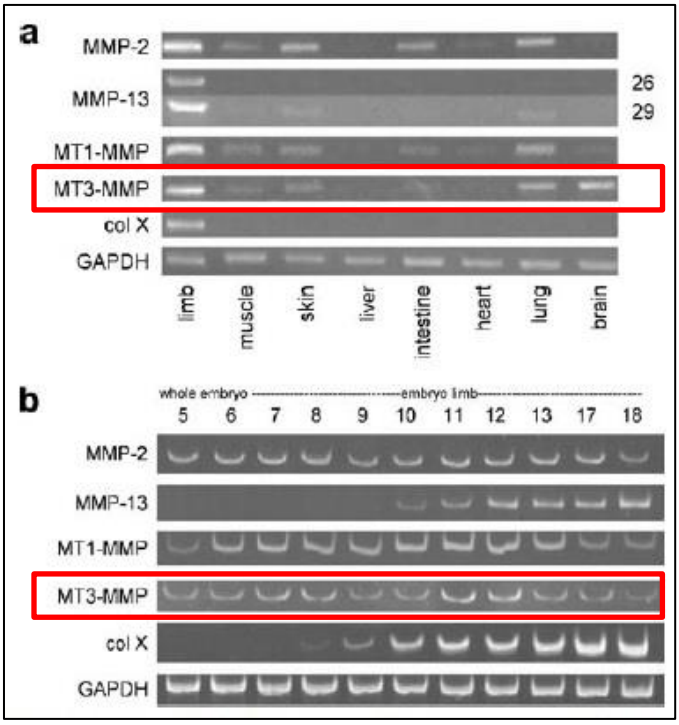


Figure 2-5: A. MT3-MMP is expressed by several tissues in chicken including limb, muscle, skin, intestine, lung, and brain. B. MT3-MMP is expressed in the limb throughout embryonic chick development, with expression peaking at days 11 and 12 (HH 37 and 38, respectively) [16].

One of the most effective ways to identify the function of a particular protein is to knock out its production and observe the effects compared to control samples. In 2008, a study was published describing the effects of knocking out MT1-MMP and MT3-MMP in mouse [20]. Mutant mouse strains were produced that were deficient in the MT1-MMP gene, the MT3-MMP gene, or a combination of the two. These mice were produced by selective breeding, meaning the mice were lacking the activity of MT1-MMP and/or MT3-MMP from conception. Data showed that the lack of MT3-MMP resulted in truncated and malformed bones, increased osteoblast apoptosis, and decreased chondrocyte proliferation, suggesting that MT3-MMP is required for unimpeded remodeling of

the extracellular matrix (Figure 2-6) [20]. Mice deficient in MT1-MMP and heterozygous for the MT3-MMP gene showed significantly reduced life span (16 days) compared to MT1-MMP-deficient/MT3-MMP-wild type mice (60 days), illustrating the importance of even a single functional MT3-MMP allele.

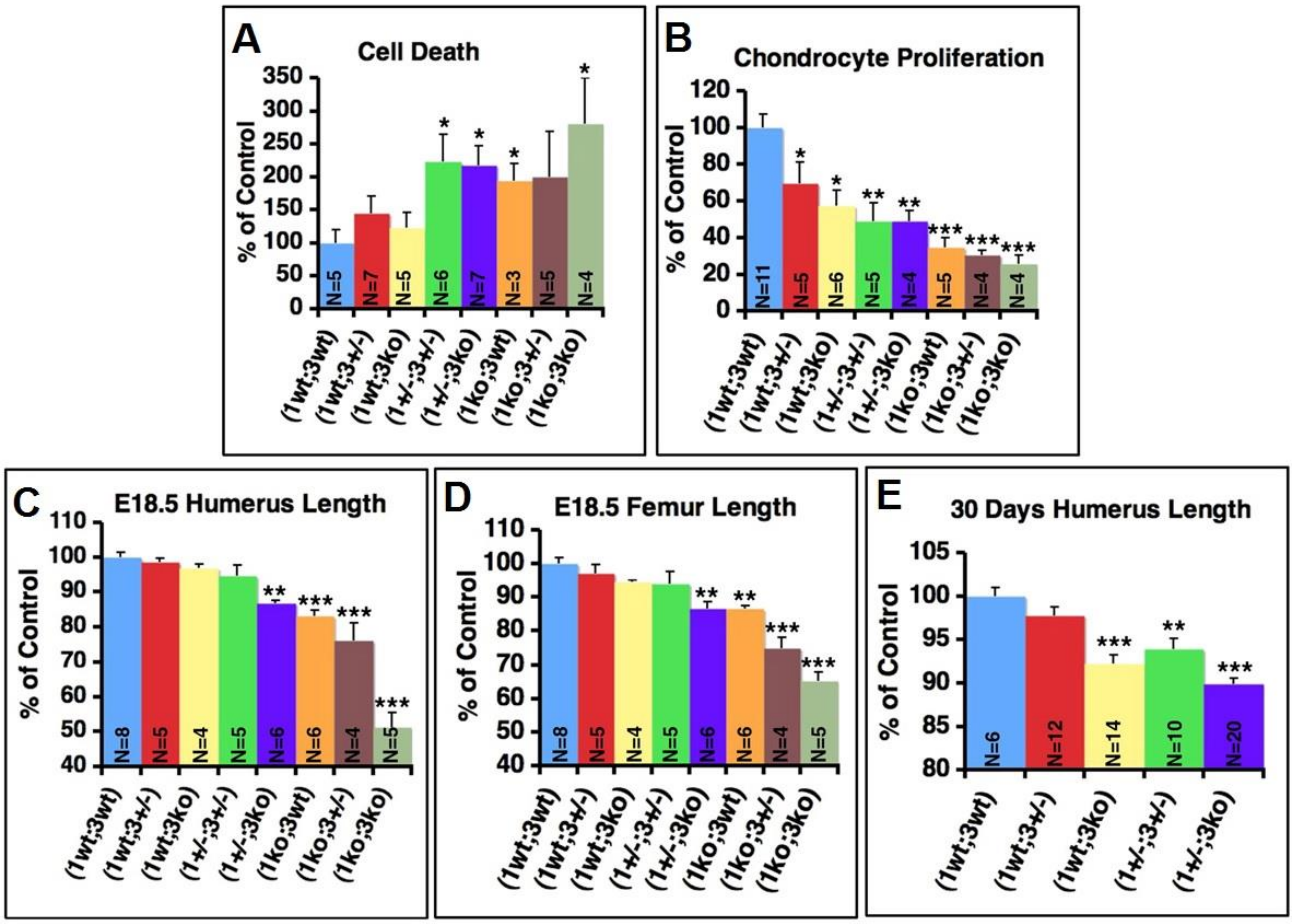


Figure 2-6: MT1-MMP and/or MT3-MMP deficiency leads to severe developmental deformities in mice that impact **A.** cell death, **B.** chondrocyte proliferation, **C.** embryonic humerus length, **D.** embryonic femur length, and **E.** postnatal humerus length. (Abbreviations: 1; MT1-MMP, 3; MT3-MMP, wt; wildtype, ko; knockout, +/-; heterozygous) [20].

Mice deficient in both MT1-MMP and MT3-MMP showed the most substantial developmental deformities. Double knockout mice showed severe craniofacial dysmorphism, clefting of the palate, stunted extremities, and failed to survive past the first day after birth [20]. Additionally, osteoblast apoptosis was higher and chondrocyte proliferation was lower than that of the single MT3-MMP knockout mice. These results clearly show the influence of MT1-MMP and MT3-MMP in development. Serious deformities were shown in bone, cartilage, and connective tissues, both at the tissue and cellular level. Unfortunately, this study did not examine the effects on tendon, although given the wide scope of tissue deformities, it would be quite surprising if tendons were not affected by the loss of MT3-MMP.

This study went on to investigate the ability of MT3-MMP to degrade collagen type I. Previous studies showed that while MT3-MMP is very effective at breaking down collagen type III (5-fold more efficient than MT1-MMP), it can only partially break down collagen type I into characteristic ¾ and ¼ fragments – [18]. In the Shi study, MT1-MMP-deficient (collagenase insufficient) mammary epithelial cells were infected with an *mt3-mmp* lentiviral expression vector or an empty vector as a negative control. Cells were plated on a high-density fibrillar collagen type I substrate and allowed to degrade the collagen over time. Cells were removed and the collagenolytic activity was visualized by staining the matrix with Coomassie blue. Results showed that while MT1-MMP-deficient cells expectedly did not degrade the substrate, cells infected with the MT3-MMP-expressing virus showed robust collagenolytic activity (Figure 2-7) [20]. This

study was repeated using a collagen type II substrate and again, MT3-MMP was shown to be effective in breaking down this form of collagen. Taken together, these results show that MT3-MMP is an effective collagenolytic enzyme capable of digesting collagen type I and collagen type II.

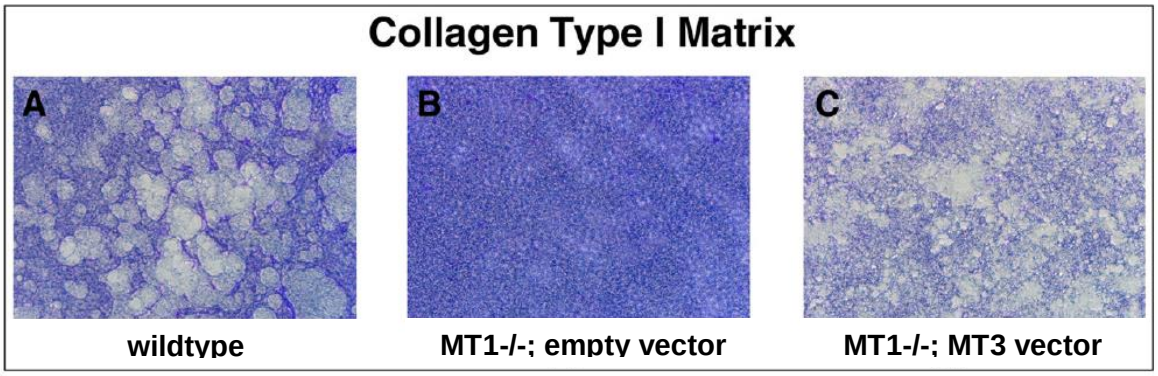


Figure 2-7: MT3-MMP can degrade collagen type I. **A.** Wildtype (MT1-MMP sufficient) mammary epithelial cells digest a high-density fibrillar collagen type I matrix. Following removal of the cells, the matrix was stained with Coomassie blue, which shows areas of degraded matrix as clear lytic zones. **B.** Cells deficient in MT1-MMP and infected with an empty lentiviral vector show no ability to degrade collagen type I. **C.** When MT1-MMP deficient cells are infected with the MT3-MMP-expressing lentivirus, they regain the ability to degrade fibrillar collagen type I, demonstrating that MT3-MMP is a collagenolytic enzyme [20].

In summary, it has been shown that MT3-MMP is expressed in tendon, digests a number of tendon ECM molecules, and may play an important role in embryonic development. However, a detailed characterization of MT3-MMP

expression during tendon development has not been addressed. In this thesis, MT3-MMP gene expression and protein spatiotemporal distribution were studied in order to investigate the role of MT3-MMP in tendon development. Successful outcomes may improve our understanding of MT3-MMP as well as better inform tendon tissue engineering strategies.

Chapter 3: MT3-MMP Gene Expression and Protein Spatiotemporal Distribution at Different Stages of Embryonic Tendon Development

3.1 Background:

3.1.1 MT3-MMP in tendon literature

MT3-MMP has been previously identified in tissues such as heart, brain, and lung, but was more recently discovered in tendon. In 2009, a study focused on the activity of MMP-2 during embryonic chick tendon development identified MT3-MMP as an activator of MMP-2. Although these data were sufficient to establish this relationship, results only showed MT3-MMP protein production at HH40 and 45 (Figure 3-1, A) [17]. Furthermore, the study did not examine tissue distribution or substrate specificity, and did not investigate other potential functions of MT3-MMP. A later study showed MT3-MMP gene expression in isolated adult mouse tendon cells within the context of examining targets of TGF-β1 in tendon healing (Figure 3-1, B) [19]. These studies left much to be desired in terms of a thorough characterization of MT3-MMP expression. Accordingly, we aimed to study MT3-MMP gene and protein production throughout multiple stages of tendon development by immunofluorescent (IF) staining and quantitative polymerase chain reaction (qPCR).

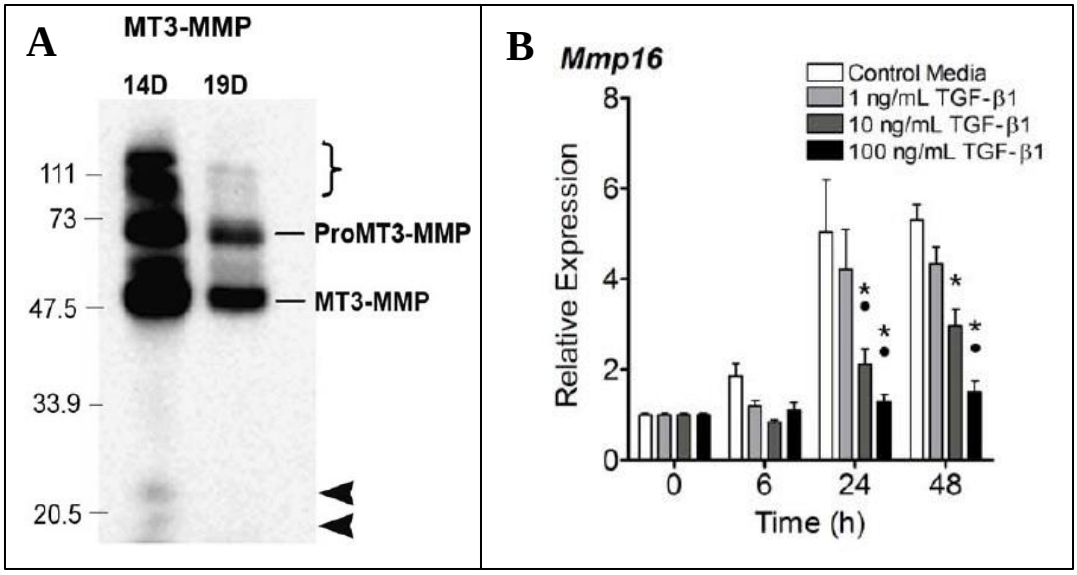


Figure 3-1: A. MT3-MMP protein production in chick tendon is greater at embryonic day 14 (HH 40) than at embryonic day 19 (HH 45) [17]. **B.** MT3-MMP gene expression in 7-month old mouse tendon is decreased in a dose-dependent response to TGF-β1 [19].

3.1.2 MT3-MMP is an activator of MMP-2

MT3-MMP has been shown to activate MMP-2, a protein known to promote tendon fibril growth [17]. Gelatin zymography revealed similar ratios of full-length proMT3-MMP/ truncated (active) MT3-MMP and full length proMMP-2/ truncated (active) MMP-2 throughout the later stages of chick tendon development. Higher activity of both MMP-2 and MT3-MMP were observed prior to and during fibril growth than at later stages, suggesting the MMP-2/MT3-MMP complex is active in the initiation and progression of normal fibril growth, matrix assembly, and tissue development [17].

In the same study, HH 40 chick tendons were isolated in MMP-2-containing medium or control medium (minimal essential medium). Tendon fibrils were analyzed by transmission electron microscopy and it was determined that MMP-2-treated tendons had significantly larger fibril diameters than tendons isolated in control medium. Furthermore, tendons isolated in the presence of MMP-2 were observed to be in the advanced lateral growth stage of tendon development, whereas the control tendons had not yet begun fibril growth [17]. In addition to its activity in promoting fibril growth, MMP-2 has been shown to break down a number of important ECM molecules such as gelatin, collagen types I, III, IV, V, VII, X, laminin, aggrecan, vitronectin, and decorin [17, 20-21] In effect, these ECM molecules are indirect substrates of MT3-MMP, since MT3-MMP activates MMP-2. In summary, MT3-MMP activates MMP-2, and MMP-2 has been shown to be a driving factor in tendon development and maturation.

3.1.3 Substrates of MT3-MMP

One of the primary functions of MT3-MMP is to degrade extracellular matrix molecules. Studies have shown that MT3-MMP is capable of breaking down many ECM molecules and non-ECM molecules, many of which are present in tendon (Table 3-1) [11, 18]. Early embryonic tendon is composed of an assorted set of ECM molecules such as fibronectin, collagen type III, and decorin, all of which decrease in content throughout tendon development [10]. In fact, by the time the tendon is fully matured it is composed of 85% collagen type I. It has been shown that MT3-MMP partially cleaves collagen type I and is five times more effective at cleaving collagen type III than a closely related protein, MT1-

MMP. The substrates for MT3-MMP activity suggest it may play an important role in tendon ECM turnover [18].

ECM molecules	Non-ECM molecules
Collagen type I	MMP-2
Collagen type II	α2-macroglobulin
Collagen type III	CD44
Fibronectin	Syndecan
Vitronectin	α1-antiproteinase
Decorin	Tissue transglutaminase
Laminin	β glycan
Aggrecan	Low density lipoprotein receptor related protein (LRP)
Cartilage proteoglycan	

Table 3-1: Substrates of MT3-MMP [11, 18]

3.1.4 MT3-MMP expression mechanism

In order to produce a functional protein, the gene DNA is first transcribed to create an mRNA copy. The two strands of DNA are temporarily separated and can each serve as the template for thousands of mRNA molecules. Next, the mRNA is translated by ribosomes to a sequence of amino acids, which is then folded into the protein form. These processes are tightly controlled by the cell, and can affected by a number of factors, such as substrate elastic modulus Transcription levels can be enhanced by activators or reduced by repressors, and translation can be changed through protein interactions. Furthermore, once the protein is properly folded, its activity can change again through post-translational modifications such as glycosylation and methylation. MT3-MMP is first

translated in the inactive pro-form, in which a signal peptide and pro-peptide are attached to the catalytic domain, blocking its activity (Figure 3-2). The signal peptide, which contains the conserved Tyr^{42}-Gly^{43}-Tyr^{44}-Leu^{45} motif, directs MT3-MMP to the Golgi apparatus. In the *trans* Golgi network, pro-protein convertases recognize the furin-like motif, Arg^{108}-Arg^{109}-Lys^{110}-Arg^{111}, and remove the signal peptide and the pro-peptide, thus activating the MT3-MMP protein [10].

3.1.5 Experimental Overview

One of the primary focuses of the Kuo lab is the study of embryonic tendon development. Before we, as a scientific community, can more effectively treat tendon injuries, we need to first understand in greater capacity how tendons develop. We use embryonic mice and chickens as model tendon systems, which were carefully selected to provide a logical link to human tendons. The embryonic chick model is well-established in tendon literature, and chickens have large legs,

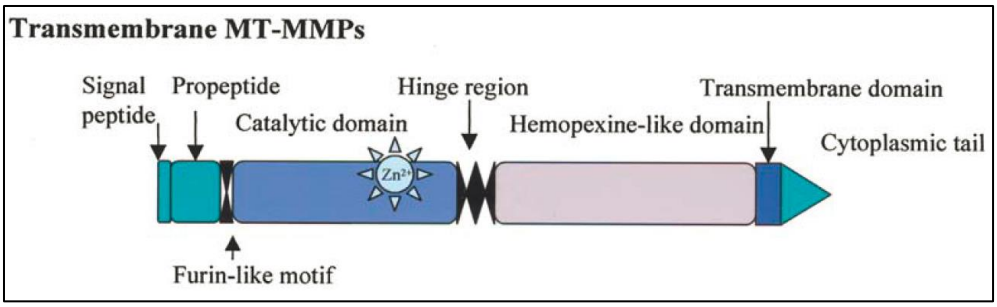


Figure 3-2: Conserved structure of inactive MT-MMP proteins. After translation of the pro-MT-MMP (pictured), the protein is directed towards the Golgi via the signal peptide, and is then activated through cleavage at the furin-like motif by a furin-like enzyme [10].

allowing for easy dissection and the study their whole tendons. The embryonic chick model is useful in proof-of-concept studies and for comparing our results to other pieces of literature, but does not provide a functional link to human tendon studies. The mammalian embryonic mouse model is also used, which is closer in lineage to humans and therefore more appropriate in applying our results to human tendon tissue engineering strategies. In the following studies, chick tendons were sectioned and tendon progenitor cells (TPC) were isolated for immunofluorescent (IF) staining. Mouse TPCs were isolated and cultured for IF staining, qPCR, and elastic modulus studies. Significant changes in gene or protein levels as a function of developmental stage may suggest an important role for MT3-MMP in tendon development.

3.2 Methods:

3.2.1 Embryonic mouse tendon progenitor cell (TPC) isolation

Scleraxis-GFP-positive mice were paired using a timed mating system that allowed us to accurately determine the developmental stage of the embryos within a 12-hour window. The pregnant mother mouse was sacrificed and the embryos were removed from the womb, washed with warm PBS, and checked for developmental stage using published guidelines and images. Limbs from all mice in the litter were detached, pooled, and minced in a 1mg/ml solution of type II collagenase in PBS (Gibco). Tendon/collagenase solution was placed in a 50-ml conical tube with 6ml additional collagenase solution and was shaken at 200 rpm

at 37˚C for one hour or more. Tendons were broken up further by pipetting the solution up and down several times. The solution was passed through a 40μm cell strainer, quenched with PBS, and centrifuged. The supernatant was removed, the cell pellet was resuspended in PBS, and centrifuged again. Cells were counted and seeded in a TCP culture flask at 20,000-30,000 cells/cm^2. Upon reaching 90% confluency, cells were detached using 0.05% trypsin-EDTA, and resuspended in a glass vial. Cells were sorted by FACS, which separated GFP-positive tendon cells from peripheral muscle and connective tissue cells. The end product of this isolation was a pool of mouse embryonic tendon progenitor cells, which were then seeded on an appropriate culture substrate and grown for 48 hours.

3.2.2 Embryonic chick tendon progenitor cell isolation

Fertilized chicken eggs were purchased and incubated in a humid 37˚C incubator until the appropriate time. Embryonic chicks were removed from their eggs, washed with warm PBS, and checked for developmental stage using published guidelines and images. Tendons were pooled and minced in a 1mg/ml solution of type II collagenase in PBS (Gibco). Tendon/collagenase solution was placed in a 50-ml conical tube with 6ml additional collagenase solution and was shaken at 200 rpm at 37˚C for one hour or more. Tendons were broken up further by pipetting the solution up and down several times. The solution was passed through a 40μm cell strainer, quenched with PBS, and centrifuged. The supernatant was removed, the cell pellet was resuspended in PBS, and centrifuged again. Cells were counted and seeded in a TCP culture flask at 20,000-30,000 cells/cm^2.

3.2.3 Embryonic chick tendon isolation

Embryonic chickens were sacrificed using the appropriate staging system. Embryonic chick calcaneus tendons were isolated and immediately placed in 4% paraformaldehyde (PFA), and incubated overnight at 4˚C. After three washes with PBS to remove the PFA, the tissue was transferred to a 25% sucrose-PBS solution, and incubated at room temperature for two hours. Next, the tendon was transferred to a 50% sucrose-PBS solution, and incubated at room temperature for two hours. Next, the tendon was transferred to a 1:1 (50% sucrose: 50% OCT in PBS) solution for two hours. Half of this solution was removed and replaced with an equal volume of 100% OCT, and incubated at room temperature for two hours. The tendon was removed and transferred to a 100% OCT solution and incubated at room temperature for two hours. Finally, the tendon was placed in an embedding block filled with 100% OCT and was frozen on dry ice. Tendon samples were sectioned longitudinally using a cryostat (Leica CM 1950 Cryostat) at a thickness of 10μm and stored at -80˚C.

3.2.4 Immunofluorescent Staining

Embryonic tendon progenitor cells (E14.5, 16.5, 18) were seeded on sterile glass coverslips at a density of 20,000 cells/cm^2. Cells were cultured for 48 hours in normal growth media (DMEM, 10% fetal bovine serum, 1% penicillin/streptomycin), which was changed after 24 hours. Cells were washed with warm PBS, fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature then washed 3x (5 minutes each) with HBSS. Chick tendon sections

have been previously fixed. Prior to staining, sections were thawed to room temperature and washed with PBS to remove the OCT solution. 60 µL blocking solution (10% goat serum in PBST (0.1% Triton X-100)) was applied for 1 hour at room temperature. Blocking solution was removed and replaced with 60 µL primary antibody solution (Abcam rabbit anti-MMP16; product code ab73877 lot# GR99235-2), used at 1:1000 and incubated overnight at 4˚C. The following day, the cells were washed 3 times (5 minutes each) with PBST (0.1% Triton X-100), then the secondary antibody (Invitrogen Alexa 488 goat anti-rabbit (FITC); catalog number A-11034, lot#1212189) was applied at 1:1000 for 1 hour at room temperature (DAPI was included in this step at 1:2000). Cells were washed 3 times (5 minutes each) with PBST (0.1% Triton X-100) then inverted on a glass coverslide with 5µl mounting media (ProLong Gold antifade reagent) and sealed with clear nail polish. Samples were visualized with a Leica TCS SP2 AOBS scanning confocal microscope. Negative control samples used no primary antibody, and showed no signs of background fluorescence.

3.2.5 Image Analysis

ImageJ software was used to measure the integrated density of IF images, which is defined as the product of selected area and mean gray value. The area was selected to best encompass the area of a single cell. Images in which only one cell was in the frame were not cropped, and the entire 50x50µm image was analyzed. Mean gray value is calculated by converting all red, green, and blue pixels to gray and averaging their intensity. Only green pixels were present in the MT3-MMP fluorescence images, so red and blue pixels were naturally

disregarded. Statistical differences were evaluated for significance with a one-way ANOVA and t-test, using $p < 0.05$.

3.3 Results:

3.3.1 MT3-MMP protein is produced by embryonic mouse and chick TPCs

To examine baseline levels of MT3-MMP protein production by mouse and chick tendon progenitor cells, TPCs of various stages were cultured on glass coverslips for 48 hours. E14.5, 16.5, and 18 mouse TPCs (n= 2 biological replicates; independent cell pools were used with each pool containing TPCs from one whole litter and 2 experimental replicates; cells from each pool were cultured on two coverslips) and HH35 and 43 chick TPCs (n= 1 biological replicate; cell pool was used with each pool containing TPCs from 3-6 embryos and 2 experimental replicates; cells were cultured on two coverslips) were fixed and stained for MT3-MMP protein production, then visualized by confocal microscopy. At least 6 images per condition were selected and averaged for pixel intensity with ImageJ software. Results showed no statistically significant differences in MT3-MMP protein production between developmental stages of mouse (p= 0.494) or chick (p=0.08) TPCs (Figures 3-4 and 3-5).

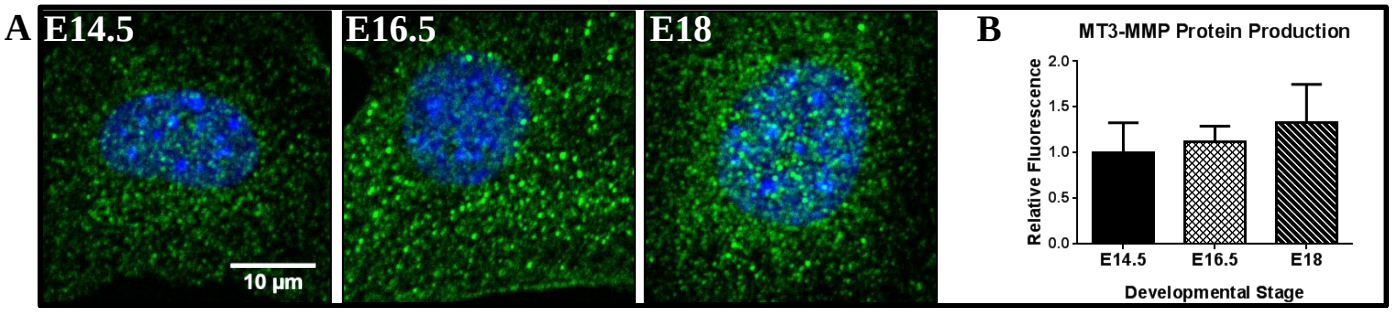


Figure 3-4: **A.** MT3-MMP protein production (green) by embryonic mouse TPCs. Cell nuclei were co-stained with DAPI (blue). **B.** Image analysis revealed no significant differences in MT3-MMP protein production levels across these stages of development. Data is normalized to E14.5 protein production.

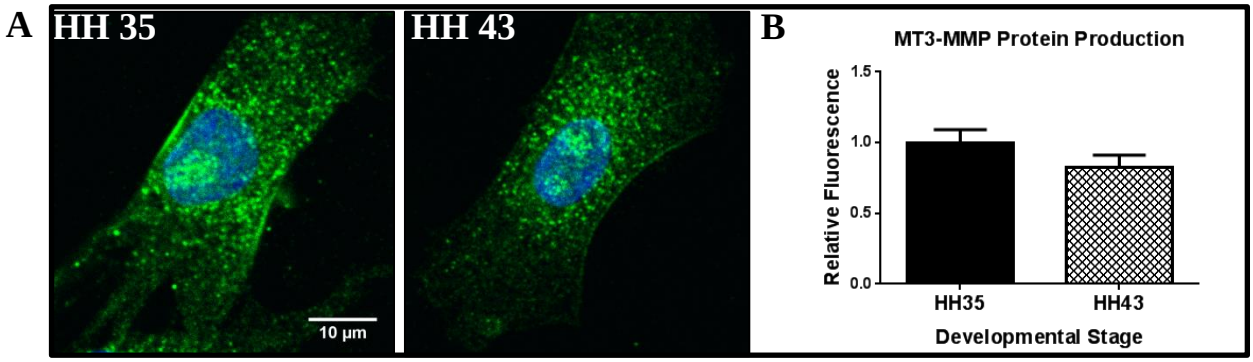


Figure 3-5: **A.** MT3-MMP protein production (green) by embryonic chick TPCs. Cell nuclei were co-stained with DAPI (blue). **B.** Image analysis revealed no significant differences in MT3-MMP protein production between these stages of development. Data is normalized to HH 35 protein production.

3.3.2 MT3-MMP is produced in embryonic chick tendon

To investigate TPC immunofluorescent staining *in vivo*, embryonic chick tendons and mouse limbs were sectioned (thickness= 10 µm) and stained for

MT3-MMP protein production, to better visualize *in vivo* spatiotemporal distribution and approximate levels of MT3-MMP. Protein production was visualized using the same IF staining protocol as in the previous experiment using embryonic TPCs. Here, four stages of chick tendon (HH 35, 37, 40, 43, n=3 tendons per stage) were stained for MT3-MMP protein production and visualized by confocal microscopy. At least 6 images of each condition were analyzed using ImageJ software, and measured for pixel intensity.

Image analysis revealed statistically significant differences in MT3-MMP protein production by HH 43 chick TPCs and each other stage tested (HH 35, 37, and 40, n= 2 biological replicates) (Figure 3-6, A). MT3-MMP protein production remained at a relatively high level during HH 35 through HH 40, but then decreased by approximately 50% during HH 43.

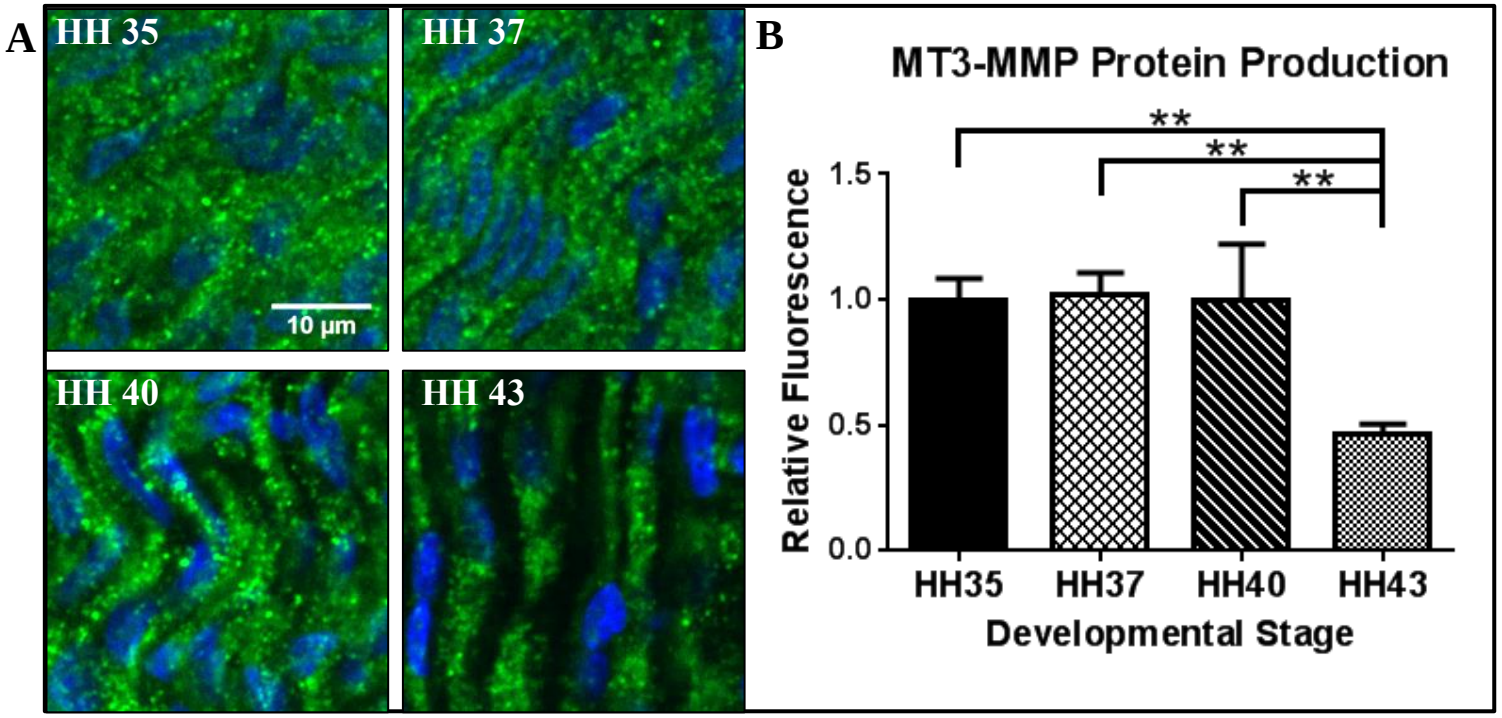


Figure 3-6: **A.** MT3-MMP protein production (green) in chick tendon. Cell nuclei were co-stained with DAPI (blue). **B.** Image analysis revealed significant differences in protein production during HH 43 and each other stage (HH35, 37, 40). Data is normalized to HH 35 protein production. (** = $p < 0.01$)

3.4 Discussion:

3.4.1 MT3-MMP primary antibody testing

Two positive controls were used to test the MT3-MMP primary antibody. We stained HeLa cells for MT3-MMP protein distribution and compared our results to an image supplied by Abcam (Figure 3-3). These images were very similar in terms of spatiotemporal patterns of MT3-MMP protein. Additionally, we stained E16.5 mouse TPCs and compared our results to a published image image of fibroblasts stained for MT1-MMP, which is known to be a very similar protein to MT3-MMP in terms of expression patterns [16]. Our results were very similar and showed comparable punctate staining patterns (Figure 3-3).

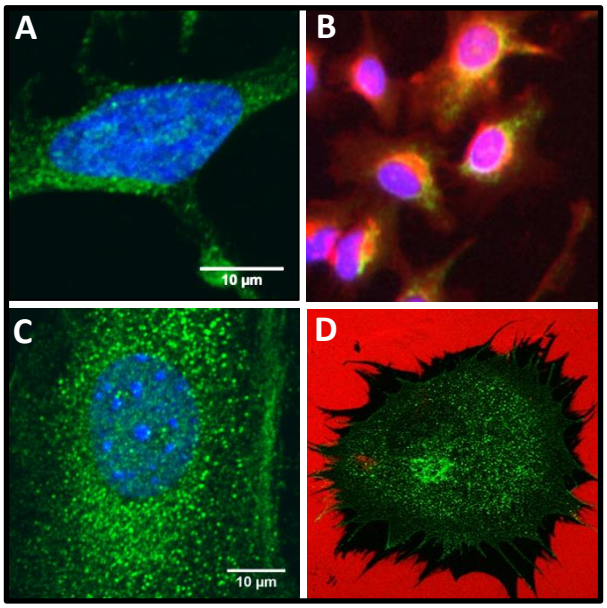


Figure 3-3: Testing of the MT3-MMP primary antibody. Our IF stain for MT3-MMP in HeLa cells (**A**) matched the equivalent image provided by the antibody manufacturer (**B**). Additionally, our IF stain for MT3-MMP in E16.5 mouse TPCs (**C**) matched a published image of the staining pattern of MT1-MMP (**D**), which is known to have a similar expression pattern to MT3-MMP [16].

Immunofluorescent staining was used to visualize MT3-MMP protein distribution patterns in embryonic mouse TPCs isolated at different stages of embryonic development. Using a timed mating system, mouse TPCs were isolated at various stages of embryonic development. Mouse cells staged at embryonic days E14.5, 16.5, and 18 were chosen because they span the early, intermediate and late stages of tendon development, the period during which the tendon micro- and macro-architecture are being established [26].

3.4.2 MT3-MMP protein production by embryonic TPCs

In the first characterization experiment, results showed no significant differences in MT3-MMP protein production by cultured mouse or chick TPCs of several developmental stages. Here, mouse and chick TPCs were cultured on glass coverslips, fixed, and stained for MT3-MMP protein production. After averaging the results from several replicates, image analysis revealed no statistically significant trend in MT3-MMP protein production as a function of developmental stage by mouse or chick TPCs. Although glass may not by an ideal substrate to culture mouse TPCs, this method was necessary in order to perform confocal imaging on the samples. This experiment showed that MT3-MMP protein production is conserved throughout multiple culture passages, and can therefore be used in forthcoming experiments. More importantly, it was discovered that MT3-MMP protein production by TPCs isolated at different developmental stages did not change.

3.4.3 MT3-MMP protein production by embryonic tendon

To better understand the expression patterns of MT3-MMP, we examined protein levels and distribution in tendon by dissecting and sectioning the tissue. Compared to isolating and culturing embryonic TPCs, this method gives a much more accurate of MT3-MMP protein production *in vivo*. Accordingly, we extracted embryonic chick tendon and mouse limb, fixed, and sectioned them. Sections were then stained for MT3-MMP protein distribution and visualized using a confocal microscope.

Results from embryonic chick tendon section staining showed an interesting trend in terms of MT3-MMP protein production. Averages from three biological replicates showed high levels of MT3-MMP protein production during HH 35, 37 and 40, but significantly lower levels during HH 43. This trend is consistent with a previous study, which showed by Western blot that MT3-MMP protein production is greater at HH 40 than HH 45 in embryonic chick tendon [17]. While analyzing these results, we sought to find an event during tendon developmental that coincided with the sudden drop in MT3-MMP protein production, and we saw two plausible explanations. This change may be due to a decrease in cell density associated with advanced tendon development [9]. With fewer cells at later stages, total MT3-MMP protein production in the tendon may be diminished. An alternative and more interesting explanation relates MT3-MMP production to changes in tendon elastic modulus. During chick tendon development, HH 40 marks the beginning of tendon fibril growth, which proceeds until HH 44 [17]. It is during this phase of embryonic development that the

intermediate tendon fibrils grow to form long fibrils more characteristic of mature tendon [17]. The process of fibril maturation results in increased elastic modulus, which was confirmed by a previous Kuo lab publication [9]. This study showed by force-volume atomic force microscopy that embryonic chick tendon elastic modulus remains around 20 kPa during HH 30 through HH 40, at which point the modulus increases to 33 kPa during HH 43 (Figure 3-7, A). Here, an inverse correlation between tendon elastic modulus and MT3-MMP protein production was shown with three biological replicates (Figure 3-7, B). Although interesting, these results only showed a correlation, not causation, between elastic modulus and MT3-MMP protein production, so further experimentation was required to better understand this relationship.

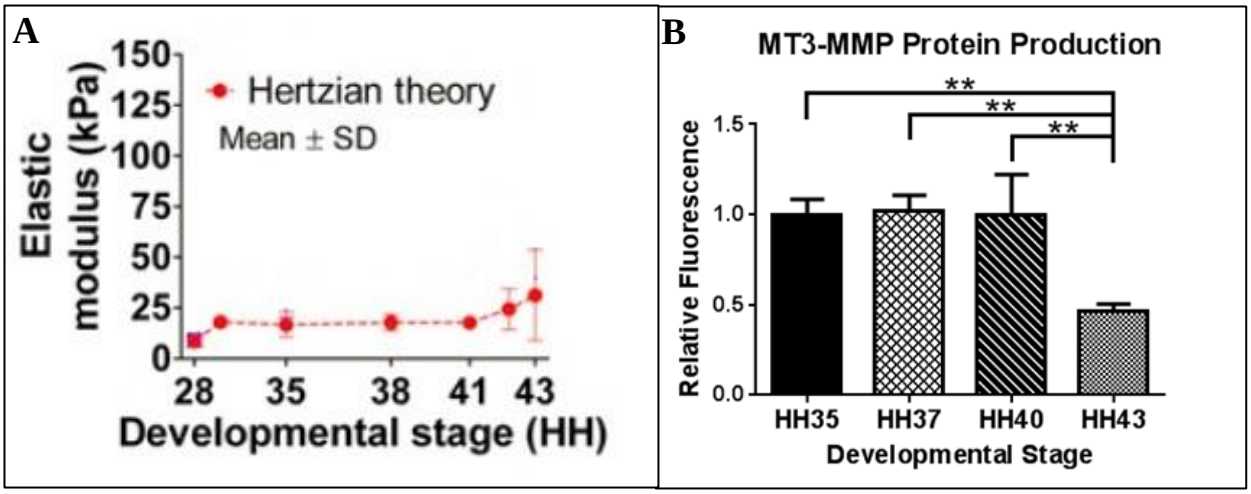


Figure 3-7: A. Elastic modulus of embryonic chick tendon changes as a function of developmental stage. As measured by force-volume AFM, tendon elastic modulus remains steady at 20 kPa during HH 28 through HH 41, at which point it increases to 30 kPa during HH 43 [9]. This trend is inversely correlated to **B,** MT3-MMP protein production levels in embryonic chick tendon.

Chapter 4: Effect of Substrate Elastic Modulus on MT3-MMP Expression

4.1 Background:

4.1.1 Effect of substrate elastic modulus on cell behavior

One important factor in regulating cell behavior is substrate elastic modulus. Tissues encompass a wide range of elastic moduli, ranging from as soft as the brain (1 kPa) to as hard as bone (100 kPa). Cells that adhere to these tissues perceive the elastic modulus through mechanotransduction, which is a system of integrin-mediated signal transduction. Based on changes to extracellular matrix (ECM) elastic modulus, cells can respond by altering their morphology, mobility, and protein production [5, 23, 27]. Changes in substrate elastic modulus have also been shown to direct stem cell differentiation [23]. In the absence of exogenous growth factors, human mesenchymal stem cells (MSC) grown on soft polyacrylamide gels (0.1-1 kPa) took on a neurological morphology. MSCs grown on polyacrylamide gels of intermediate modulus (8-17 kPa) took on a myoblast phenotype and cells grown on high modulus substrates (25-40 kPa) took on a polygonal morphology similar to that of osteoblasts (Figure 4-1) [23]. These results show that substrate elastic modulus alone has the potential to significantly alter cell behavior and function.

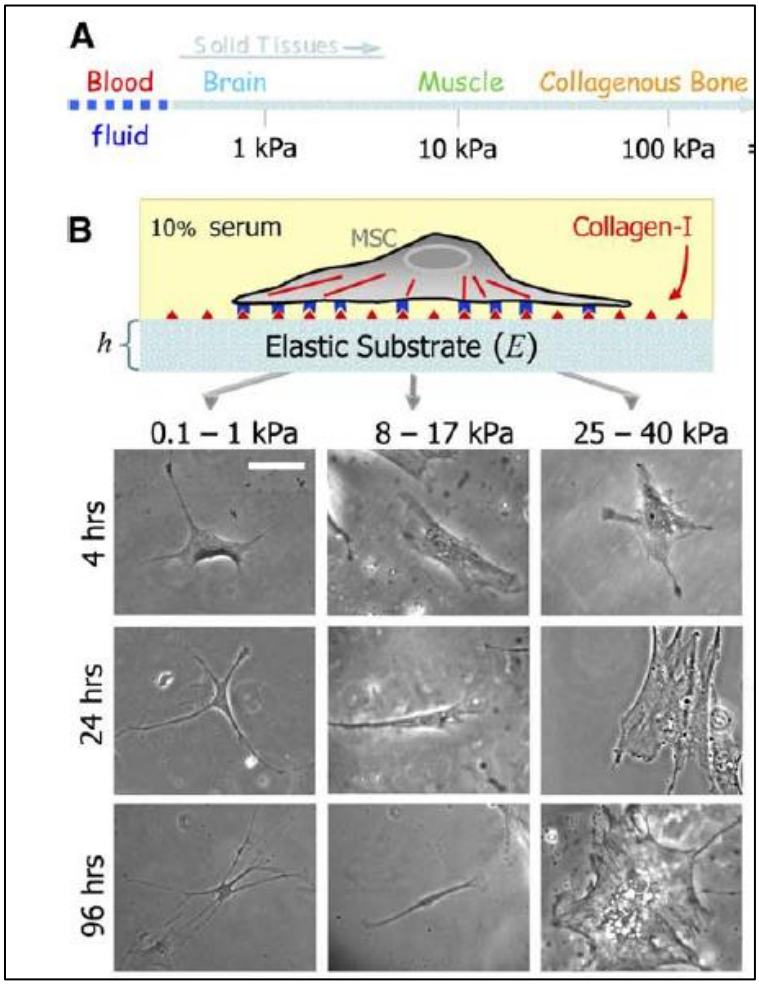


Figure 4-1: In the absence of exogenous growth factors, substrate elastic modulus directs MSC lineage towards three distinct morphologies. Human MSCs cultured on soft substrates display neuron morphology, cells cultured on intermediate gels take on myoblast morphology, and cells cultured on stiff substrates look like osteoblasts [23].

4.1.2 Two-dimensional cell substrates

The experiments described in this thesis involve the use of a two-dimensional (2D) polyacrylamide gel substrate for cell culture purposes. 2D substrates are commonly used in cell biology studies because they are well-

established in the literature and easy to control. 2D substrate systems allow for the fine-tuning of many parameters such as water content, protein composition, and elastic modulus as illustrated by the Engler paper [23]. The elastic modulus of polyacrylamide gels can range from 1 to over 120 kPa, which encompasses modulus values for many tissues. Many types of cells adhere and proliferate well on these substrates, making them valuable research tools with many applications.

4.1.3 MMPs are regulated by substrate elastic modulus

There is evidence to suggest that matrix metalloproteinases (MMPs) are regulated by substrate elastic modulus. In 2009, a study was published on the effects of substrate modulus on the regulation of ECM-degrading genes. Rat annulus fibrosus cells cultured on polyacrylamide gels (elastic moduli = 1, 32, and 63 kPa) show significantly increased MMP-3 and MMP-13 gene expression levels when grown on gels of greater elastic modulus (Figure 4-2) [5]. Although these proteins are secreted MMPs and degrade a different set of ECM molecules than MT3-MMP, there is much conservation between MMPs and MT-MMPs in terms of structure and behavior.

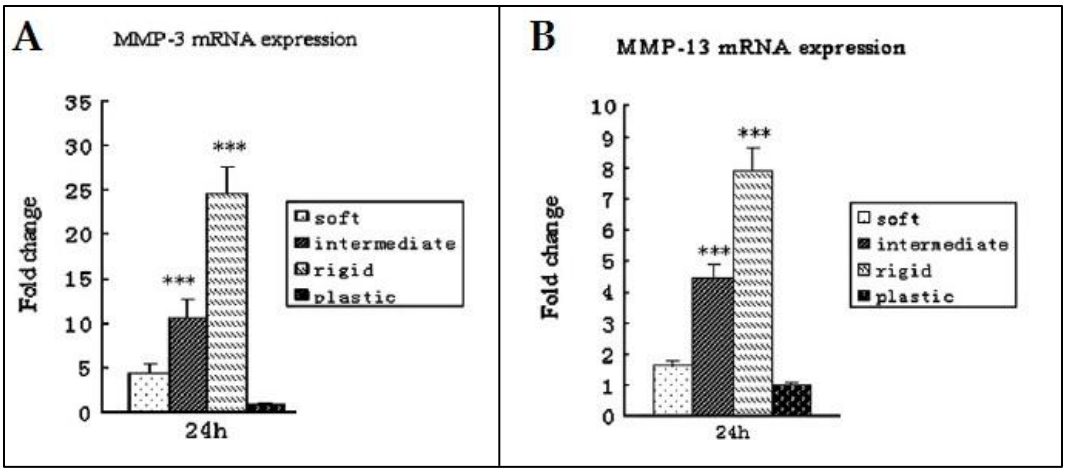


Figure 4-2: A, MMP-3 and **B,** MMP-13 gene expression is regulated by substrate elastic modulus in rat annulus cells cultured on polyacrylamide gels. PA gel elastic moduli = 1 kPa (soft), 32 kPa (intermediate), and 63 kPa (rigid) (*** = $p < 0.001$) [5].

4.1.4 Increased MT3-MMP protein production results in actin disorganization

Another study showed that MT3-MMP overexpression in baboon smooth muscle cells induces actin disorganization [24]. The actin cytoskeleton is a rigid protein network that allows cells to keep and change their shape under internal and external pressures. Actin filaments rapidly assemble and disassemble, which can result in changes in cell morphology or mobility. A relationship between MT3-MMP and the actin cytoskeleton may suggest that MT3-MMP is involved in mechanotransduction pathways during tendon development.

Smooth muscle cells subjected to adenoviral transfection result in the overproduction of MT3-MMP protein. Immunofluorescence staining results show poorly organized, clustered actin filaments (Figure 4-3, -BB94). When these cells

are treated with the broad-spectrum MMP-inhibitor BB94, actin organization is rescued to achieve a morphology similar to that of untreated cells which express MT3-MMP at a normal level (Figure 4-3, +BB94) [24]. This study suggests that there is a functional relationship between MT3-MMP and the actin cytoskeleton. Cytoskeletal actin filaments allow cells to balance forces applied by the ECM and keep their shape. In agreement with Newton's Third Law of Motion, any force exerted on the cell from must be balanced by an equivalent force, or else the cell would collapse. In this instance, the actin cytoskeleton provides a force that opposes the force applied by the ECM.

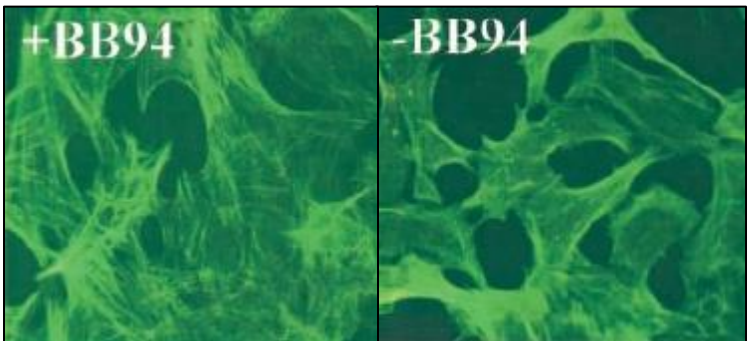


Figure 4-3: MT3-MMP overexpression results in disorganization of cytoskeletal actin in smooth muscle cells. Cells overexpressing MT3-MMP in the absence of MMP-inhibitor BB94 **(-BB94)** show clustered actin filaments. In the presence of BB94 **(+BB94)**, cells show a rescued actin morphology similar to that of untreated cells [24].

4.1.5 MT3-MMP overexpression results in increased focal adhesion kinase cleavage

The 2004 Shofuda study went on to more specifically show that MT3-MMP interacts with the focal adhesion complex (FAC), which is a protein group located immediately within the cell membrane. The FAC provides a physical link between transmembrane integrins and intracellular actin filaments, and in influential in allowing cells to sense ECM elastic modulus. One of the most important components of the FAC is the focal adhesion kinase (FAK) which, once activated, phosphorylates a tyrosine residue and transmits important signals downstream towards the cell nucleus [24]. By over-expressing MT3-MMP in smooth muscle cells, this study showed increased FAK cleavage, suggesting that MT3-MMP directly or indirectly cleaves FAK. These data provide evidence that MT3-MMP may be involved in mechanotransduction pathways and is regulated by the actin cytoskeleton.

4.1.6 Elastic modulus of tendon

The topic of mechanotransduction is especially important to the study of tendon development because the elastic modulus of embryonic tendon changes throughout development. During embryonic development, embryonic chick tendon microscale elastic modulus remains around 19 kPa during HH 30 through HH 40, at which point the modulus increases to 33 kPa during HH 43 [9]. This increase in elastic modulus coincides with the fibril growth stage of tendon development, when pre-formed fibril intermediates assemble end-to-end to form

longer fibrils consistent with mature, mechanically-functional fibrils [25]. Tendon elastic modulus continues to increase after birth as the collagen fibers increase in diameter and strength. If MT3-MMP is regulated by substrate elastic modulus, it may support the theory that the changing elastic modulus of embryonic tendon regulates MT3-MMP, suggesting that MT3-MMP expression has a role in matrix remodeling during tendon development.

In the forthcoming experiments, mouse embryonic tendon cells were used to investigate the effect of substrate elastic modulus on MT3-MMP expression. As a mammalian model system, mouse TPCs are more closely related to human tendon cells, as compared to chick TPCs. No previous studies have been published on the elastic modulus of embryonic mouse tendon throughout development, so the Marturano (2013) study on embryonic chick tendon characterization was referenced to select the moduli of the gels. Therefore, we used these elastic moduli to represent different stages of tendon throughout development. This is rationalized by the fact that both species have a 20-day gestation period and similar tendon development programs [26].

4.1.7 Experimental Overview

In the previous chapter, a possible correlation between tendon elastic modulus and MT3-MMP protein production was observed. Here, experiments were designed to investigate the validity of this relationship. We chose to utilize the well-characterized polyacrylamide gel system, which is a two-dimensional substrate that can be easily adjusted for elastic modulus by changing the

composition ratio of acrylamide to bis-acrylamide. Target polyacrylamide gel elastic modulus values were chosen based on the modulus of embryonic tendon, which spans from 19 kPa during intermediate tendon development to 33 kPa during late tendon development [9]. Additionally, a 120 kPa gel was fabricated, which may represent the elastic modulus of adult tendon. Mouse embryonic tendon progenitor cells were cultured on polyacrylamide gels of different elastic moduli and measured for changes in MT3-MMP gene expression and protein production. We hypothesize that MT3-MMP expression by mouse tendon progenitor cells decreases in response to increasing substrate elastic modulus.

4.2 Methods:

4.2.1 Polyacrylamide gel fabrication

Prior to fabricating the polyacrylamide gels, glass coverslips were chemically coated to promote gel attachment. Square glass coverslips (width = 22mm) were soaked in 0.5% 3-aminopropyltriethoxysilane (APTES) for 10 minutes, washed with distilled water, then heated at 80˚C for 45 minutes. Coverslips were cooled to room temperature, soaked in 0.5% glutaraldehyde for 30 minutes then washed with distilled water. Lastly, coverslips were soaked in 70% ethanol for 10 minutes and allowed to dry under UV light.

Polyacrylamide gels were composed of 4-15% acrylamide, 0.1-0.5% bis-acrylamide, 1x PBS, 400µg collagen type I (bovine), 0.2% tetramethylethylenediamine (TEMED), 1.4% 1N hydrochloric acid (HCl), 0.02%

acrylic acid N-hydroxysuccinimide ester (NHS-acrylate), and 0.05% ammonium persulfate. Gel suspensions were mixed thoroughly and pipetted in 60 μL aliquots on chemically-coated square coverslips. Sterile circular coverslips (diameter = 1.8cm) were carefully placed on top of gel suspensions, flattening them, and gels were allowed to solidify for 30 minutes at room temperature. Gels were washed with HBSS three times and incubated at 4˚C overnight prior to cell seeding.

4.2.2 Atomic force microscopy

Polyacrylamide gels were measured for elastic modulus using a Bruker Dimension 3100 Atomic Force Microscope with a low noise scanner. Gels were tested the day after fabrication, as this would be the day to seed the cells. A 5-μm radius SiO2 probe (Corpscular) was attached with epoxy to tipless probes with a 0.06-N/m spring constant (Bruker). Cantilever deflection was calibrated before each measurement using glass coverslides in HBSS. The force-volume mode was used to capture indentation force curves at nodes of a 2D array. Single indentation force curves were recorded over 10 x 10-μm gel areas in a 2 x 2 array, with 256 sampling points each. Indentation slope was converted to elastic modulus theoretically based on Hertzian indentation mechanics. In these measurements, a spherical tip indentation model was used.

$$E_{micro} = \frac{\sqrt{\pi}}{2}\left(1-\nu^2\right)\frac{S}{\sqrt{A}}$$

…where E_{micro} is elastic modulus calculated for microscale tips and A is the projected contact area of the spherical indenter at full indentation depth. The contact area A was estimated using a published method, where *r* is the radius of the indenter [9].

$$A = \pi \cdot (h(2r - h))$$

To track the stability of elastic modulus over time, three independent PA gel master mixes were made, and three gels from each mix were tested by AFM every other day for 8 days. Between measurements, gels were kept at 37˚C in normal growth media, just as they would if they were seeded with cells. Gels were placed in HBSS during AFM testing, and upon completion were placed back at in fresh growth media at 37˚C.

4.2.3 Embryonic mouse tendon progenitor cell (TPC) isolation

Scleraxis-GFP-positive mice were paired using a timed mating system that allowed us to accurately determine the developmental stage of the embryos within a 12-hour window. The pregnant mother mouse was sacrificed and the embryos were removed from the womb, washed with warm PBS, and checked for developmental stage using published guidelines and images. Limbs from all mice in the litter were detached, pooled, and minced in a 1mg/ml solution of type II collagenase in PBS (Gibco). Tendon/collagenase solution was placed in a 50 ml conical tube with 6 ml additional collagenase solution and was shaken at 200 rpm

at 37˚C for one hour or more. Tendons were broken up further by pipetting the solution up and down several times. The solution was passed through a 40 μm cell strainer, quenched with PBS, and centrifuged. The supernatant was removed, the cell pellet was resuspended in PBS, and centrifuged again. Cells were counted and seeded in a TCP culture flask at 20,000-30,000 cells/cm^2. Upon reaching 90% confluency, cells were detached using 0.05% trypsin-EDTA, and resuspended in a glass vial. Cells were sorted by FACS, which separated GFP-positive tendon cells from peripheral muscle and connective tissue cells. The end product of this isolation was a pool of mouse embryonic tendon progenitor cells, which were then seeded on an appropriate culture substrate and grown for 48 hours.

4.2.4 Polyacrylamide gel seeding

Polyacrylamide gels were seeded with mouse TPCs the day after gel fabrication. Cells were detached from TCP flasks using 0.05% trypsin-EDTA and centrifuged to pellet the cells. Cells were resuspended in warm growth media (DMEM, 10% fetal bovine serum, 1% penicillin/streptomycin), counted, and brought to a density of 333,000 cells/ml. HBSS was removed from the gels. 150μl of the cell suspension (20,000 cells/ cm^2) was pipetted on top of the gel and incubated at 37˚C for 2 hours, at which point 4ml of warm growth media was added to each gel.

4.2.5 Immunofluorescence Staining (MT3-MMP)

Cells were washed with warm PBS, fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature then washed 3x (5 minutes each) with

PBS. 60 µL blocking solution (10% goat serum in PBST (0.1% Triton X-100)) was applied for 1 hour at room temperature. Blocking solution was removed and replaced with 60 µL primary antibody solution (Abcam rabbit anti-MMP16; product code ab73877, lot#GR99235-2), used at 1:1000 and incubated overnight at 4˚C. The following day, the cells were washed 3 times (5 minutes each) with PBST (0.1% Triton X-100), then the secondary antibody (Invitrogen goat anti-rabbit (FITC); catalog number A-11034, lot#1212189) was applied at 1:1000 for 1 hour at room temperature (DAPI was included in this step at 1:2000). Cells were washed 3 times (5 minutes each) with PBST (0.1% Triton X-100) then inverted on a glass coverslide with 5µl mounting media (ProLong Gold antifade reagent) and sealed with clear nail polish. Samples were visualized with a Leica TCS SP2 AOBS scanning confocal microscope. Negative control samples used no primary antibody, and showed no signs of background fluorescence.

4.2.6 Immunofluorescence Staining (Actin)

Cells were washed with warm PBS, fixed with 4% PFA in PBS for 10 minutes at room temperature then washed 3x (5 minutes each) with PBS. 488 AlexaFluor Phalloidin (1:50) was applied for 1 hour at room temperature (DAPI was included in this step at 1:2000). Cells were washed 3 times (5 minutes each) with PBST (0.1% Triton X-100) then inverted on a glass coverslide with 5 µl mounting media (ProLong Gold antifade reagent) and sealed with clear nail polish. Samples were visualized with a Leica TCS SP2 AOBS scanning confocal microscope.

4.2.7 Image Analysis

ImageJ software was used to measure the integrated density of IF images, which is defined as the product of selected area and mean gray value. The area was selected to best encompass the area of a single cell. Images in which only one cell was in the frame were not cropped, and the entire 50x50 μm image was analyzed. Mean gray value is calculated by converting all red, green, and blue pixels to gray and averaging their intensity. Only green pixels were present in the MT3-MMP fluorescence images, so red and blue pixels were disregarded. Statistical differences were evaluated for significance with a one-way ANOVA and t-test, using $p < 0.05$.

4.2.8 Quantitative PCR

Samples were harvested 2 days after gel seeding in 1 mL of TRIzol reagent and total RNA was extracted according to chloroform extraction protocol. RNA concentration was assessed spectrophotometrically. Reverse transcription was performed using Superscript III First-Strand Synthesis System, with 2 μg total RNA reverse transcribed with oligo(dT) primers. qPCR was performed with Brilliant II SYBR Green qPCR Master Mix and the MX3000p qPCR System Primer pairs were designed and optimized for qPCR analysis of expression levels of mouse 18S rRNA (forward 5′-GACTCAACACGGGAAACCTCA-3′; reverse 5′-ACCAGACAAATCGCTCCACCAACT-3′) and MT3-MMP (forward 5′-TGATGGACCAACAGACCGAGATAAAGAAGG-3′; reverse 5′-

GGCCAAGATGCAGGGAATGACAATAGC-3′). Expression levels relative to 18S rRNA were calculated using the delta-delta cycle threshold method.

4.3 Results:

4.3.1 Polyacrylamide gel AFM testing

Force-volume atomic force microscopy was used to measure the elastic modulus of polyacrylamide gels. After testing several different polyacrylamide gel compositions, three gel compositions that matched in the intended elastic moduli values were achieved (Table 4-1). Nine gels of each composition were tested, and each gel was probed at two distinct points. Each of these points was the result of 256 measurements within a 10 x 10 μm grid.

Elastic Modulus (kPa)	Acrylamide	Bis-acrylamide
19	**4%**	**0.1%**
21	4.5%	0.1%
33	**5%**	**0.1%**
40	5.5%	0.1%
41	6%	0.1%
64	10%	0.1%
120	**15%**	**0.5%**

Table 4-1: Polyacrylamide gel elastic moduli and the corresponding acrylamide and bis-acrylamide percentages. PA gels used in experiments are bolded.

4.3.2 Polyacrylamide gel elastic modulus stability testing

Polyacrylamide gel elastic modulus stability was tested over 8 days (see methods). Results showed that the 4% and 5% acrylamide gels showed good elastic modulus stability, but the 15% acrylamide gel showed an increase in elastic modulus from day 0 to day 4, at which point it remained steady (Figure 4-4). Statistics showed no significant trends in the stability of the 4% and 5% acrylamide gels across 8 days ($p= 0.141$ and 0.223, respectively). The initial elastic modulus increase of the 120 kPa gel between days 0 and 4 resulted in a statistically significant increase ($p= 0.027$).

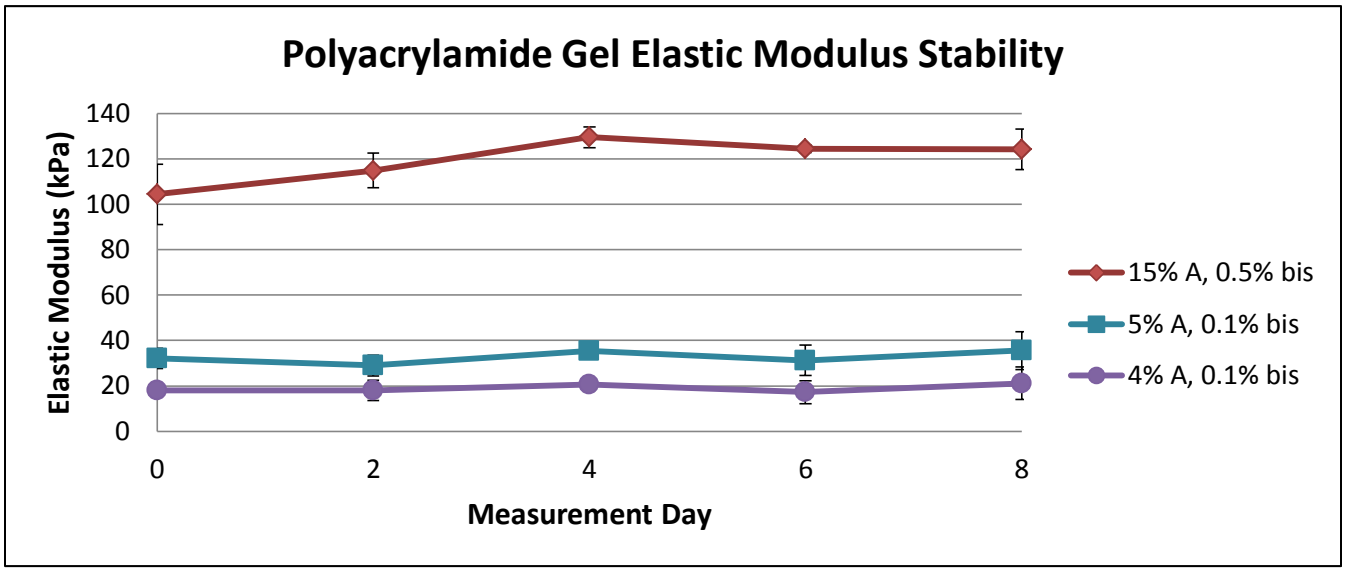


Figure 4-4: Elastic modulus of the 4 and 5% acrylamide gels remained steady through 8 days of testing, but the 15% acrylamide gel increased in elastic modulus through day 4, at which point it leveled out (A = acrylamide, bis = bis-acrylamide).

4.3.3 Substrate elastic modulus regulates TPC morphology

Prior to investigating the effect of substrate elastic modulus on the expression of MT3-MMP, elastic modulus effects on basic cell morphology were examined. Mouse TPCs of several developmental stages (E14.5, 16.5, and 18, n= 1 biological replicate, 3 experimental replicates) were seeded on polyacrylamide gels of various elastic moduli (19, 33, and 120 kPa) and cultured for 48 hours. Brightfield images were taken, which revealed apparent differences in the cells overall size and shape. TPCs grown on the 19 and 33 kPa gels appeared, by eye, to have a smaller surface area than those grown on the 120 kPa gel (Figure 4-5). These results showed that tendon progenitor cell morphology can be regulated by substrate elastic modulus.

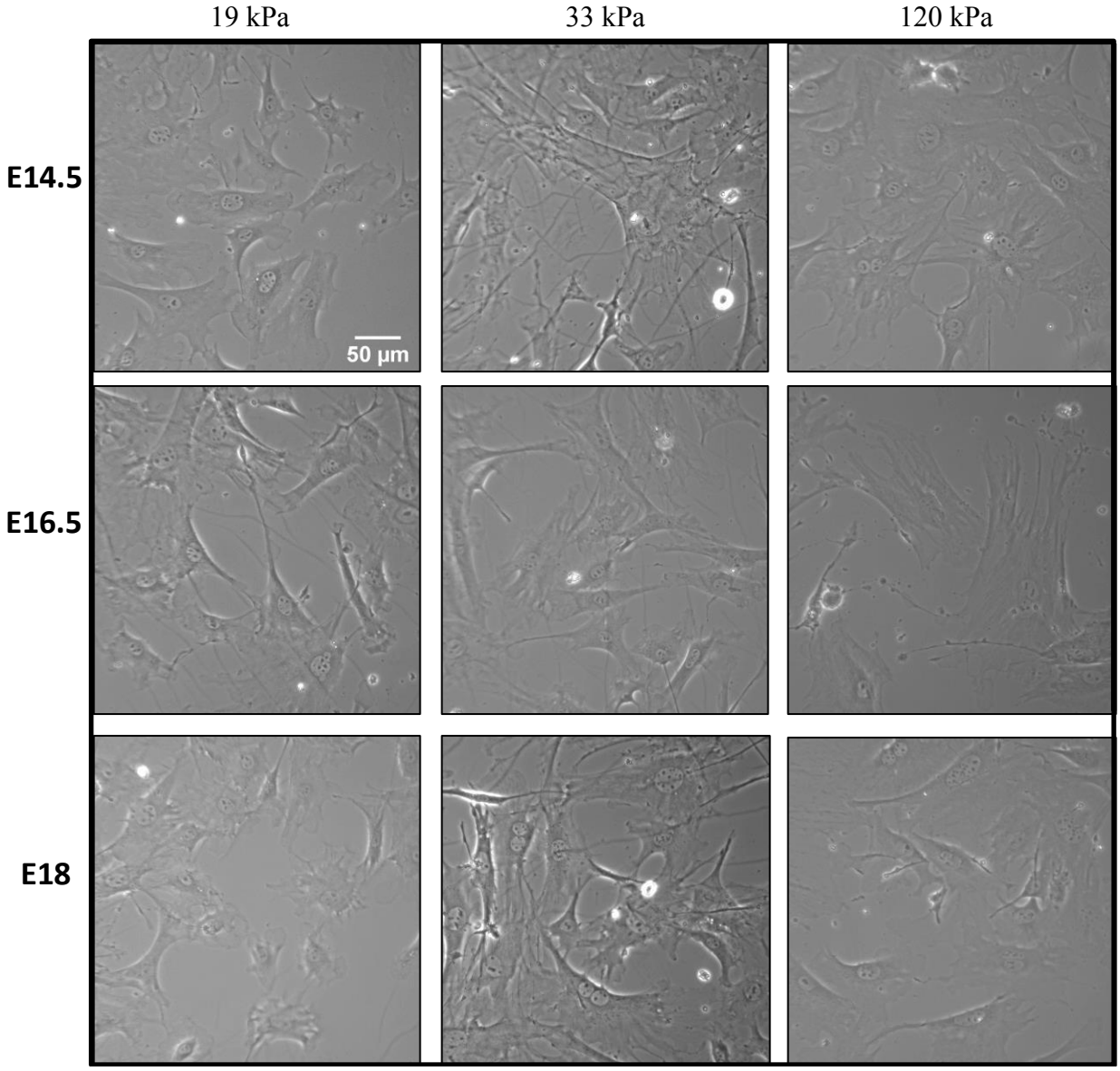
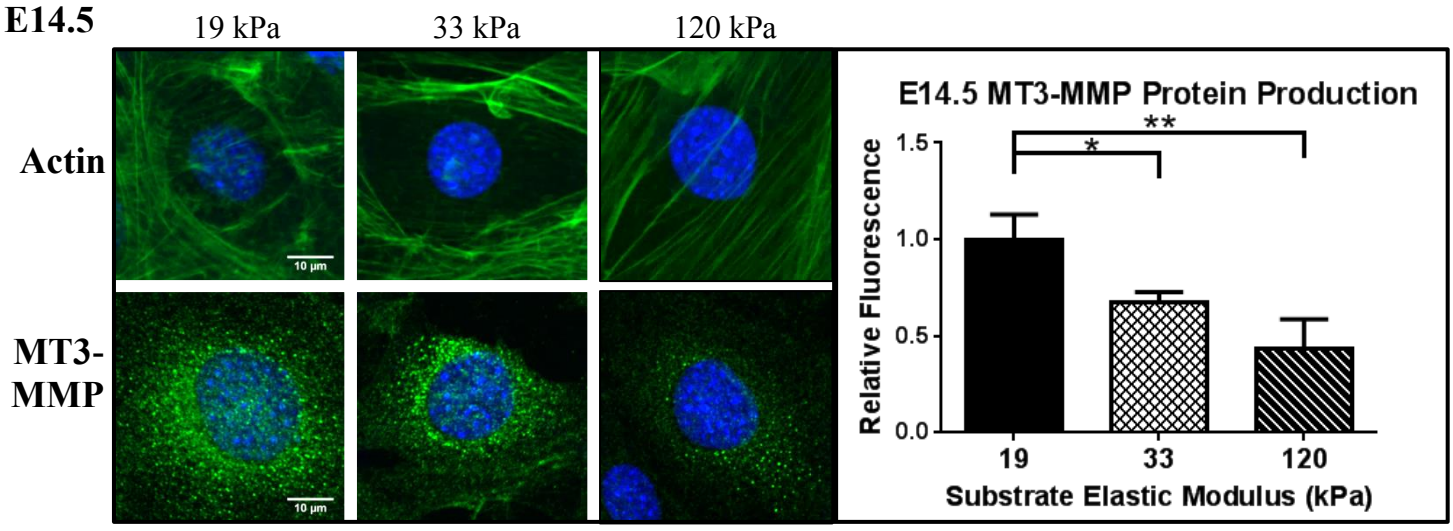


Figure 4-5: Changes in cell morphology were induced by substrate elastic modulus. Mouse TPCs cultured on polyacrylamide gels of greater elastic modulus appeared to have greater cell surface area. Images were taken 48 hours after cell seeding.

4.3.4 Substrate elastic modulus regulates MT3-MMP production

Mouse TPCs (E14.5, 16.5, and 18, n= 2 biological replicates, 2 experimental replicates) were grown on polyacrylamide gels of different elastic

moduli (19, 33, and 120 kPa) for 48 hours, and then actin morphology and MT3-MMP protein production were evaluated. For each developmental stage, two independent pools of embryonic TPCs were used. Cells of each biological sample were cultured on two gels per condition, and at least six confocal images were taken of cells from both gels. Actin filaments were visualized using phalloidin to evaluate whether the differences in polyacrylamide gel elastic modulus influenced actin morphology. As expected, representative images appeared to show an increase in uniaxial actin alignment with increased substrate stiffness (Figure 4-6,). Next, results showed MT3-MMP protein production by all developmental stages of mouse TPCs increased as a result of decreased substrate elastic modulus. Image analysis was used to measure protein production levels in a semi-quantitative manner, and changes in protein production were found to be statistically significant across many conditions. Statistically significant changes in levels of MT3-MMP protein production were achieved by TPCs grown on gels of low vs. moderate elastic modulus (E14.5, 16.5), on gels of low vs. high modulus (E14.5, 16.5, 18), and on gels of high vs. moderate modulus (E18).



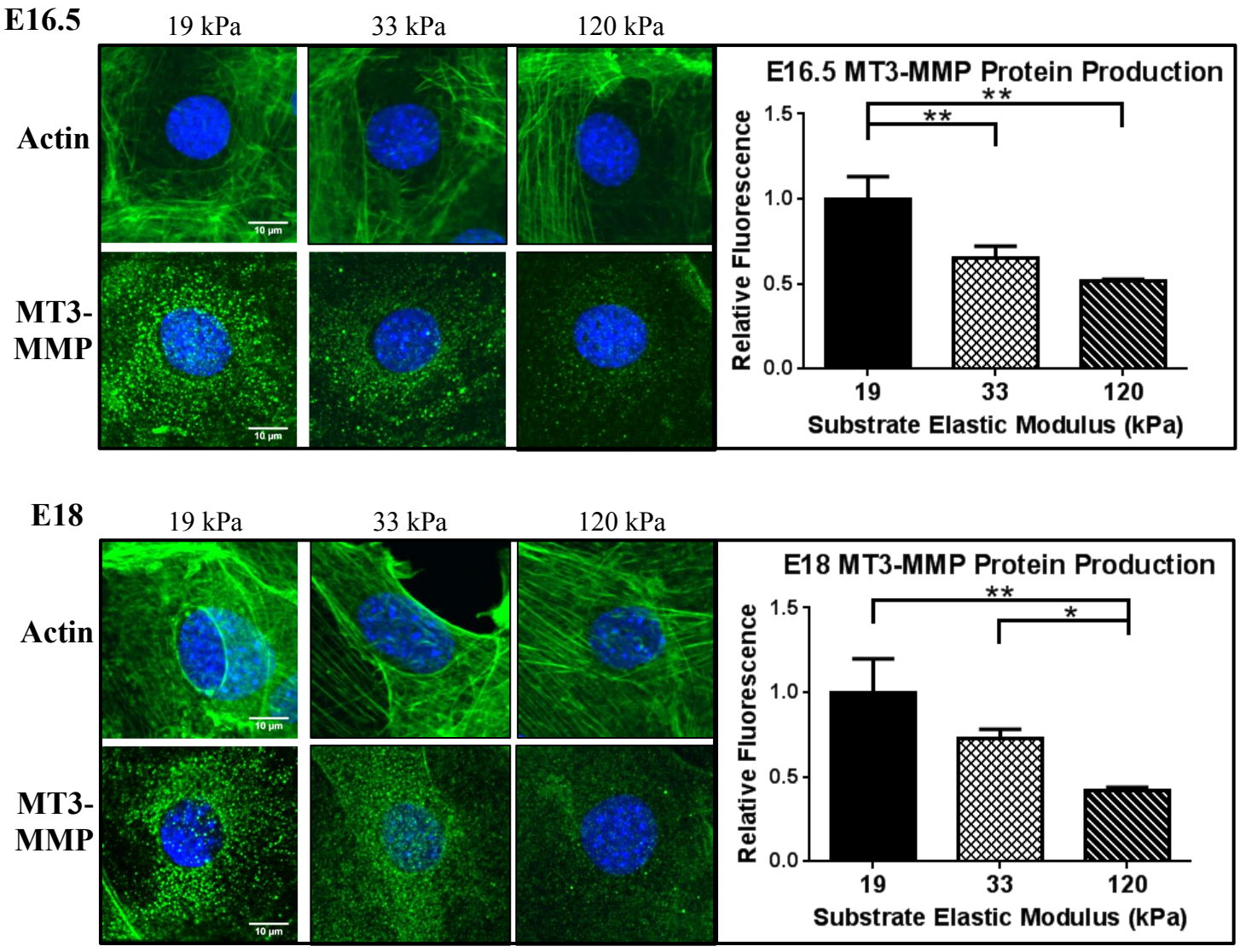


Figure 4-6: Cytoskeletal actin filaments (green) show increased organization with increased substrate elastic modulus in E14.5, 16.5, and 18 mouse TPCs. Actin organization appears to be inversely correlated with MT3-MMP protein production (green), which may be reduced due to increased substrate elastic modulus. Cell nuclei were co-stained with DAPI (blue). Image analysis reveals significant differences in MT3-MMP protein production due to increased substrate elastic modulus (* $p<0.05$, ** $p<0.01$).

4.3.5 Substrate elastic modulus does not regulate MT3-MMP expression

Results showed that MT3-MMP protein production was regulated by substrate elastic modulus. However, these results do not elucidate whether MT3-MMP is being regulated at the gene or protein level. To better understand how MT3-MMP is being regulated, quantitative PCR was performed to evaluate gene expression levels. Mouse TPCs (n=1 biological replicate, 3 experimental replicates) were cultured on polyacrylamide gels in an identical manner to the IF imaging experiments, and mRNA samples were collected for qPCR. Data revealed no significant changes in MT3-MMP gene expression in response to changes in substrate elastic modulus (Figure 4-7). Taken into consideration with the changes in protein levels, it appears that MT3-MMP is regulated by elastic modulus at the protein level only.

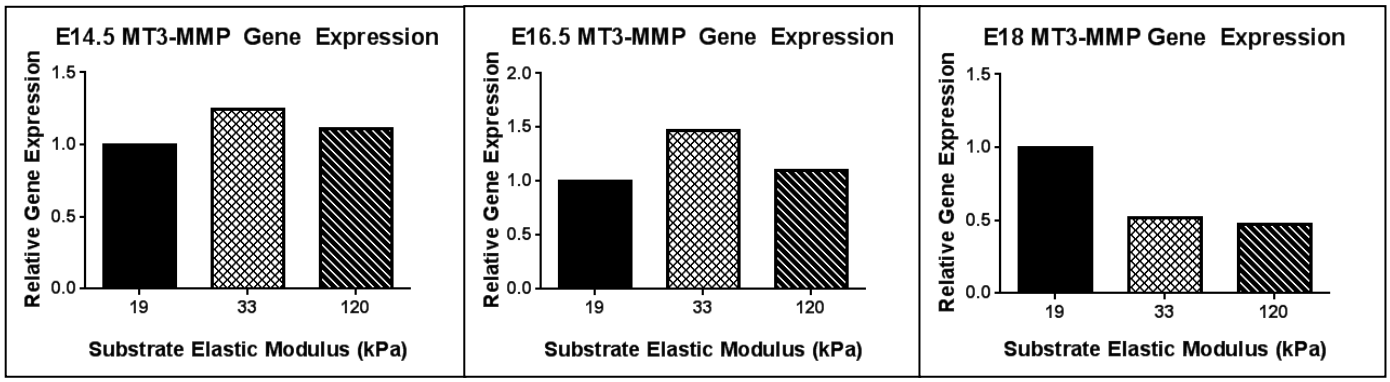


Figure 4-7: MT3-MMP gene expression by mouse TPCs cultured on PA gels appears to not be regulated by substrate elastic modulus.

4.4 Discussion:

The objective of these studies was to investigate the effect of substrate elastic modulus on the expression of MT3-MMP by mouse embryonic tendon progenitor cells. Polyacrylamide gels of different elastic moduli were used as a 2D cell culture substrate, on which cells were grown for 48 hours. Cells were fixed, and then stained for MT3-MMP protein and actin stress fibers. Results showed that in response to increased substrate elastic modulus, cytoskeletal actin organization increased, and MT3-MMP protein production decreased (Figure 4-6). Additionally, it was discovered that MT3-MMP gene expression may be not be regulated by substrate elastic modulus (Figure 4-7).

4.4.1 Mechanotransduction of substrate elastic modulus

Substrate elastic modulus is a regulator of many different cell processes such as morphology, differentiation, mobility, protein production, and apoptosis [27]. Cells sense the modulus of their surroundings through transmembrane integrin proteins, which perceive ECM characteristics and transfer that information to the cell nucleus via signal transduction cascades. The cell can then respond accordingly to better adapt to the environment. For example, fibroblastic cells may adopt a flat morphology and more aligned actin stress fibers when in contact with a stiff substrate [27].

4.4.2 MT3-MMP is regulated by substrate elastic modulus

Here, we showed that MT3-MMP is regulated by substrate elastic modulus at the protein level, and not at the gene level. By culturing embryonic

tendon cells on polyacrylamide gels of different moduli, MT3-MMP protein production decreased in response to increased substrate modulus (Figure 4-6). By repeating the same experiment and measuring the levels of MT3-MMP gene expression, results showed no significant trend in response to changes in substrate elastic modulus (Figure 4-7). Repeating these experiments with more biological replicates may provide more conclusive results regarding the effects of elastic modulus on MT3-MMP gene expression. Additionally, including more housekeeping genes, such as Ubiquitin C and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), may also reduce sources of error. Here, only the 18S ribosomal gene was used as a control, but we did not show that this gene is unaffected by substrate elastic modulus. Future studies should first investigate the effect of elastic modulus on housekeeping gene expression to ensure the reliability of the qPCR data.

Taken together, these data suggest that MT3-MMP gene is transcribed at the same rate, independent of substrate modulus, but a subsequent protein interaction decreases MT3-MMP protein production by TPCs.

4.4.3 Polyacrylamide gel system

The polyacrylamide gel system proved effective in the design of these experiments. Although a three-dimensional gel system would better mimic the *in vivo* tendon environment, this two-dimensional PA gel system was sufficient in these experiments to produce a positive result (the use of a 3D hydrogel system will be discussed as a future direction of this project). The three target elastic

moduli were carefully selected based on work done by a previous Kuo lab member [9]. In this work, embryonic chicks were carefully staged according to Hamburger-Hamilton guidelines and sacrificed at multiple stages of development. Calcaneus tendons were removed, fixed, and sectioned before being measured for elastic modulus by atomic force microscopy. Dr. Marturano found that the elastic modulus of embryonic chick tendon remains around 19 kPa during HH 30 through HH 41, at which point the modulus increases to above 30 during HH 43 (Figure 3-7) [9]. The two soft gels, 19 and 33 kPa, were selected to represent the elastic modulus of early and late-stage embryonic chick tendon. Even this relatively modest change in modulus proved to be enough to elicit statistically significant changes in MT3-MMP protein production by E14.5 ($*P < 0.05$) and 16.5 TPCs ($**P < 0.01$) (Figure 4-6).

4.4.4 AFM vs. tensile testing

The 120 kPa gel was used as an attempt to replicate the elastic modulus of adult tendon. The Marturano study did not include data on the elastic modulus of adult chicken tendon, but previous studies have reported on this. One report published the elastic modulus of adult (56 days old) chicken flexor tendons to be 335,000 kPa [28]. Instead of force-volume atomic force microscopy, this study measured elastic modulus by bulk property tissue tensile testing, which is known to result in much greater modulus readings, compared to AFM [29]. Although tensile testing is a common method to measure elastic modulus, AFM was chosen because it may be a more accurate measure of what individual cells sense, as opposed to bulk properties of the whole tissue. By AFM, elastic modulus is

measured by pressing a probe about half the diameter of the cell nucleus into the gel, which may be comparable to how cells perceive their surroundings through integrin-mediated mechanotransduction. Tensile testing stretches the entire tendon to the point of rupture, and elastic modulus is calculated form the resulting stress-strain curve. While useful in some cases, it was not deemed an appropriate measure of elastic modulus for these experiments.

Still, the elastic modulus of adult chicken tendon, as measured by AFM may be higher than 120 kPa, but the modulus capacity of the polyacrylamide gel system was limited by the reagent composition. Gels of greater modulus require a greater volume of acrylamide and bis-acrylamide, which results in a lower volume of water being added to reach an equivalent volume. The 120 kPa polyacrylamide gels used in these experiments were determined to approach to the maximum modulus because the high volume of acrylamide and bis-acrylamide displaced almost all of the water in the gel composition. Accordingly, results showed statistically significant changes in MT3-MMP protein production by cells cultured on the 120 kPa and 19 kPa gels at each developmental stage tested (Figure 4-6).

4.4.5 MT3-MMP gene expression is not regulated by elastic modulus

In addition to measuring MT3-MMP protein production, MT3-MMP gene expression was evaluated in response to changes in substrate elastic modulus. Mouse TPCs were cultured on polyacrylamide gels of different substrate moduli. RNA was extracted, reverse-transcribed to cDNA, and used as the template for qPCR. Each condition (3 developmental stages, 3 substrate elastic moduli) was

run on two gels, and matching samples were pooled. Interestingly, results showed no trend of MT3-MMP gene expression by cells of any developmental stage in response to changes in substrate elastic modulus (Figure 4-7). These data, taken together with the protein production results, suggest that the MT3-MMP gene is transcribed at the same rate independent of substrate modulus, and a later protein interaction causes changes in MT3-MMP protein levels. The specific pathway of regulation is not elucidated from these experiments, but is addressed in the following actin cytoskeleton disruption experiments.

Chapter 5: Effect of Actin Cytoskeletal Disruption on MT3-MMP Expression

5.1 Introduction:

5.1.1 MT3-MMP and the Actin Cytoskeleton

The actin cytoskeleton is a rigid protein network that allows cells to maintain their shape under extracellular or intracellular pressure. Actin monomers form long spiral chains that can quickly assemble and disassemble, allowing the cell to move or proliferate. In the previous chapter, both actin organization and MT3-MMP protein production were found to be regulated by substrate elastic modulus. Previous studies have suggested that there may be a functional relationship between MT3-MMP and actin fibers. A study published in 2004 showed that MT3-MMP overexpression in baboon smooth muscle cells induces actin disorganization [24]. Cells were subjected to adenoviral transfection which resulted in the overexpression of MT3-MMP protein, which resulted in poorly organized, clustered actin filaments, as visualized by immunofluorescent staining (Figure 5-1, -BB94). When these cells were treated with the broad-spectrum MMP-inhibitor BB94, MT3-MMP expression was reduced to a normal level, and actin morphology was observed to be similar in morphology to that of untreated cells (Figure 5-1, +BB94). This study suggests that there is a functional relationship between MT3-MMP and the actin cytoskeleton.

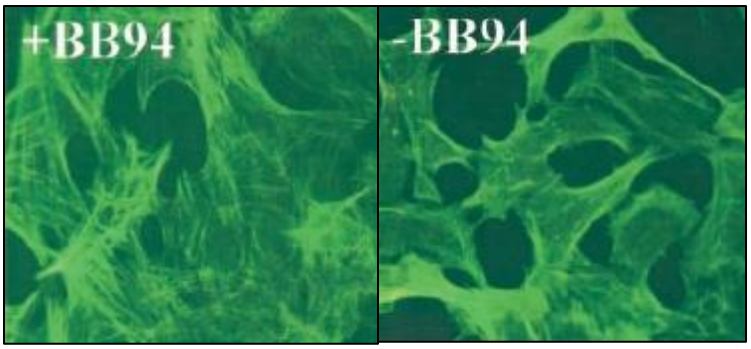


Figure 5-1: MT3-MMP overexpression results in disorganization of cytoskeletal actin in smooth muscle cells. Cells overexpressing MT3-MMP in the absence of MMP-inhibitor BB94 **(-BB94)** show clustered actin filaments. In the presence of BB94 **(+BB94)**, these same cells show a rescued actin morphology similar to that of untreated cells [24].

5.1.2 MT3-MMP and the Focal Adhesion Kinase

The 2004 Shofuda study went on to show a functional relationship between MT3-MMP and the focal adhesion complex (FAC), a protein group located immediately within the cell membrane. The FAC provides a physical link between transmembrane integrins and intracellular actin filaments, and is thus influential in mechanotransduction pathways, such as the perception of ECM elastic modulus. One of the most important components of the FAC is the focal adhesion kinase (FAK) which, once activated by ligation of integrin subunits to the ECM or by growth factors, phosphorylates a tyrosine residue and transmits a signal downstream to the cell nucleus [25]. By over-expressing MT3-MMP protein in smooth muscle cells, FAK cleavage was enhanced, suggesting that

MT3-MMP directly or indirectly cleaves FAK (Figure 5-2). FAK was immunoprecipitated from cell lysates with an anti-FAK polyclonal antibody and detected by immunoblotting with a monoclonal antibody [25]. Cells treated with BB94 (which returned MT3-MMP protein levels to normal) showed a strong band at 130 kDa, the correct weight of the full FAK protein. Interestingly, cells over-expressing MT3-MMP showed a decreased 130 kDa band and an increased band at 90 kDa, the correct weight of cleaved FAK [25]. This study provides evidence that MT3-MMP may be regulated by the actin cytoskeleton through an interaction with the focal adhesion kinase.

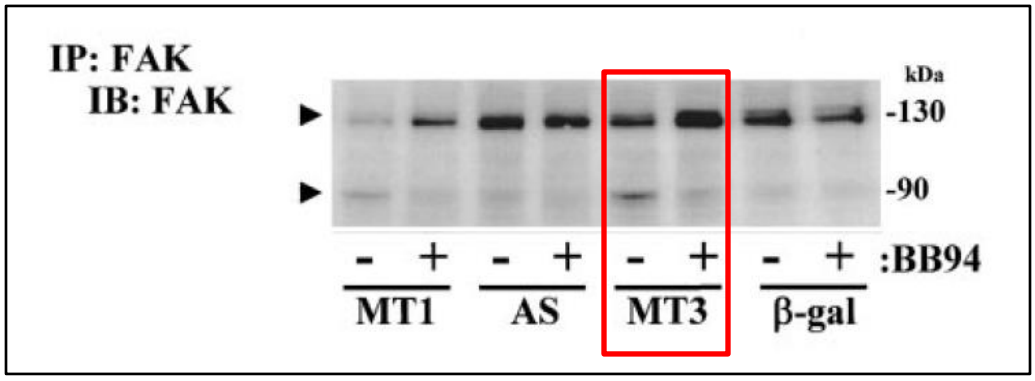


Figure 5-2: Focal adhesion kinase (FAK) cleavage is induced by MT3-MMP. Baboon smooth muscle cells overexpressing MT3-MMP (MT3 –BB94) shows a decreased 130 kDa band (full FAK protein) and increased 90 kDa band (cleaved FAK fragment) as compared to cells treated with BB94 (MT3 +BB94), an MMP-inhibitor, which returned MT3-MMP levels to normal [25].

A relationship between MT3-MMP and the focal adhesion complex further suggests that MT3-MMP is regulated by elastic modulus. This is relevant to the potential role of MT3-MMP in embryonic tendon development because tendon elastic modulus increases throughout development [9].

5.1.3 Blebbistatin Mechanism

To further investigate the potential interaction between the MT3-MMP protein and actin fibers, the actin cytoskeleton of TPCs were disrupted with blebbistatin (Figure 5-3), a small molecule drug that functions by blocking nonmuscle myosin II ATPase activity [30]. Blebbistatin binds to the myosin-ADP-P_i complex with high affinity and interferes with the phosphate release process. This interaction leaves myosin in the actin-detached state and prevents rigid actomyosin cross-linking (Figure 5-4) [31]. By disrupting the actin cytoskeleton and observing changes in MT3-MMP production, a better understanding of the relationship between the two proteins may be revealed.

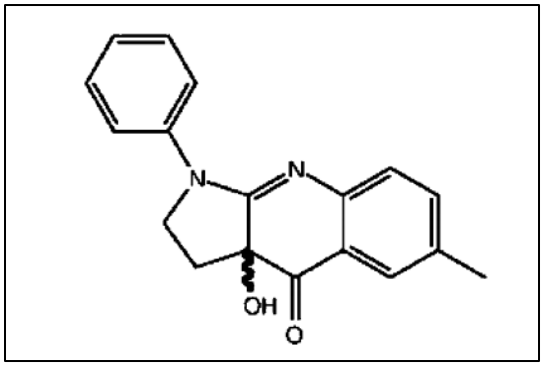


Figure 5-3: Chemical structure of blebbistatin [30].

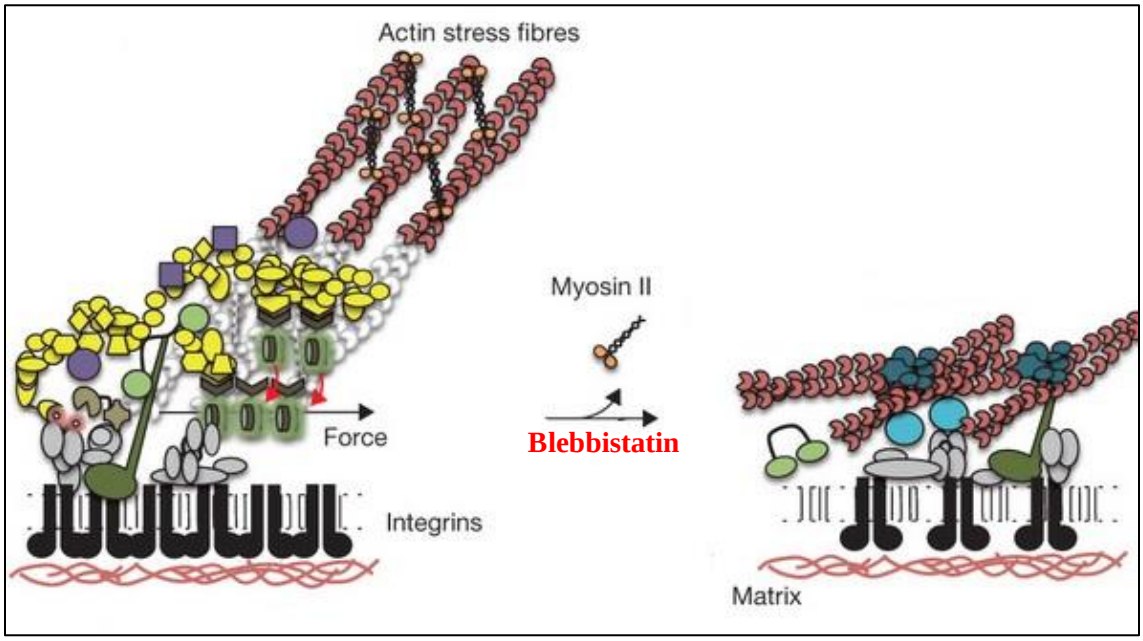


Figure 5-4: Mechanism of actin disruption by blebbistatin. Blebbistatin binds to the myosin-ADP-P_i complex with high affinity and interferes with the phosphate release process. This leaves myosin in an actin-detached state and prevents rigid actomyosin cross-linking [31-32].

5.1.4 Experimental Overview

In the previous chapter, data were presented to show that MT3-MMP protein production, and not gene expression, was regulated by substrate elastic modulus. These results suggested that the change in MT3-MMP production occurred at the protein level and may involve a post-translational modification or protein-protein interaction, but the specific mechanism of this interaction was not investigated. Here, the actin cytoskeleton was studied for a potential role in the regulation of MT3-MMP. Experiments were designed to disrupt the actin cytoskeleton using blebbistatin and changes in MT3-MMP protein production

were compared to vehicle control samples. Mouse TPCs were cultured on polyacrylamide gels of various elastic moduli for 24 hours in normal growth media, and then the media was changed to include either 25μM blebbistatin or 0.5% DMSO. MT3-MMP protein production was measured by immunofluorescent staining and image analysis was used to measure protein levels in a semi-quantitative manner. Samples were also stained with phalloidin to visualize the organization of the actin cytoskeleton with blebbistatin treatment. In the previous chapter, data showed that decreased substrate elastic modulus resulted in decreased actin organization and increased MT3-MMP protein production. Here, actin organization was disrupted by chemical treatment, so we hypothesize that MT3-MMP production is increased as a result of blebbistatin treatment.

5.2 Methods:

5.2.1 Polyacrylamide gel fabrication

Prior to fabricating the polyacrylamide gels, glass coverslips were chemically coated to promote gel attachment. Square glass coverslips (width = 22mm) were soaked in 0.5% 3-aminopropyltriethoxysilane (APTES) for 10 minutes, washed with distilled water, then heated at 80˚C for 45 minutes. Coverslips were cooled to room temperature, soaked in 0.5% glutaraldehyde for 30 minutes then washed with distilled water. Lastly, coverslips were soaked in 70% ethanol for 10 minutes and allowed to dry under UV light.

Polyacrylamide gels were composed of 4-15% acrylamide, 0.1-0.5% bis-acrylamide, 1x PBS, 400 μg collagen type I (bovine), 0.2% tetramethylethylenediamine (TEMED), 1.4% 1N hydrochloric acid (HCl), 0.02% acrylic acid N-hydroxysuccinimide ester (NHS-acrylate), and 0.05% ammonium persulfate. Gel suspensions were mixed thoroughly and pipetted in 60 μL aliquots on chemically-coated square coverslips. Sterile circular coverslips (diameter = 1.8 cm) were carefully placed on top of gel suspensions, flattening them, and gels were allowed to solidify for 30 minutes at room temperature. Gels were washed with HBSS three times and incubated at 4˚C overnight prior to cell seeding.

5.2.2 Atomic force microscopy

Polyacrylamide gels were measured for elastic modulus using a Bruker Dimension 3100 Atomic Force Microscope with a low noise scanner. Gels were tested the day after fabrication, as this would be the day to seed the cells. A 5 μm radius SiO2 probe (Corpscular) was attached with epoxy to tipless probes with a 0.06-N/m spring constant (Bruker). Cantilever deflection was calibrated before each measurement using glass coverslides in HBSS. The force-volume mode was used to capture indentation force curves at nodes of a 2D array. Single indentation force curves were recorded over 40 x 40-μm gel areas in a 2 x 2 array, with 128 sampling points each. Indentation slope was converted to elastic modulus theoretically based on Hertzian indentation mechanics. In these measurements, a spherical tip indentation model was used…

$$E_{micro} = \frac{\sqrt{\pi}}{2}\left(1-\nu^2\right)\frac{S}{\sqrt{A}}$$

…where E_{micro} is elastic modulus calculated for microscale tips and A is the projected contact area of the spherical indenter at full indentation depth. The contact area A was estimated using a published method, where *r* is the radius of the indenter.

$$A = \pi \cdot (h(2r-h))$$

5.2.3 Embryonic mouse tendon progenitor cell (TPC) isolation

Scleraxis-GFP-positive mice were paired using a timed mating system that allowed us to accurately determine the developmental stage of the embryos within a 12-hour window. The pregnant mother mouse was sacrificed and the embryos were removed from the womb, washed with warm PBS, and checked for developmental stage using published guidelines and images. Limbs from all mice in the litter were detached, pooled, and minced in a 1mg/ml solution of type II collagenase in PBS (Gibco). Tendon/collagenase solution was placed in a 50 ml conical tube with 6ml additional collagenase solution and was shaken at 200 rpm at 37˚C for one hour or more. Tendons were broken up further by pipetting the solution up and down several times. The solution was passed through a 40 μm cell strainer, quenched with PBS, and centrifuged. The supernatant was removed, the

cell pellet was resuspended in PBS, and centrifuged again. Cells were counted and seeded in a TCP culture flask at 20,000-30,000 cells/cm^2. Upon reaching 90% confluency, cells were detached using 0.05% trypsin-EDTA, and resuspended in a glass vial. Cells were sorted by FACS, which separated GFP-positive tendon cells from peripheral muscle and connective tissue cells. The end product of this isolation was a pool of mouse embryonic tendon progenitor cells, which were then seeded on an appropriate culture substrate and grown for 48 hours.

5.2.4 Polyacrylamide gel seeding

Polyacrylamide gels were seeded with mouse TPCs the day after gel fabrication. Cells were detached from TCP flasks using 0.05% trypsin-EDTA and centrifuged to pellet the cells. Cells were resuspended in warm growth media (DMEM, 10% fetal bovine serum, 1% penicillin/streptomycin), counted, and brought to a density of 333,000 cells/ml. HBSS was removed from the gels. 150 μl of the cell suspension (20,000 cells/ cm^2) was pipetted on top of the gel and incubated at 37˚C for 2 hours, at which point 4 ml of warm growth media was added to each gel.

5.2.5 Blebbistatin Treatment

Mouse TPCs were seeded on polyacrylamide gels and cultured for 24 hours, according to the methods described above. Normal growth media was removed and replaced with an equal volume of 25 μM blebbistatin (Sigma Aldrich, St. Louis, MO) in DMEM (10% FBS, 1% penicillin/streptomycin).

Vehicle control samples were treated with 0.5% DMSO. Cells were cultured for an additional 24 hours.

5.2.6 Immunofluorescence Staining (MT3-MMP)

Cells were washed with warm PBS, fixed with 4% paraformaldehyde (PFA) in PBS for 10 minutes at room temperature then washed 3x (5 minutes each) with PBS. 60 µL blocking solution (10% goat serum in PBST (0.1% Triton X-100)) was applied for 1 hour at room temperature. Blocking solution was removed and replaced with 60 µL primary antibody solution (Abcam rabbit anti-MMP16; ab73877 lot#GR99235-2), used at 1:1000 and incubated overnight at 4˚C. The following day, the cells were washed 3 times (5 minutes each) with PBST (0.1% Triton X-100), then the secondary antibody (Invitrogen goat anti-rabbit (FITC); catalog number A-11034, lot#1212189) was applied at 1:1000 for 1 hour at room temperature (DAPI was included in this step at 1:2000). Cells were washed 3 times (5 minutes each) with PBST then inverted on a glass coverslide with 5 µl mounting media (ProLong Gold antifade reagent) and sealed with clear nail polish. Samples were visualized with a Leica TCS SP2 AOBS scanning confocal microscope. Negative control samples used no primary antibody, and showed no signs of background fluorescence.

5.2.7 Immunofluorescence Staining (Actin)

Cells were washed with warm PBS, fixed with 4% PFA in PBS for 10 minutes at room temperature then washed 3x (5 minutes each) with PBS. 488 AlexaFluor Phalloidin (1:50) was applied at for 1 hour at room temperature

(DAPI was included in this step at 1:2000). Cells were washed 3 times (5 minutes each) with PBST (0.1% Triton X-100) then inverted on a glass coverslide with 5 µl mounting media (ProLong Gold antifade reagent) and sealed with clear nail polish. Samples were visualized with a Leica TCS SP2 AOBS scanning confocal microscope.

5.2.8 Image Analysis

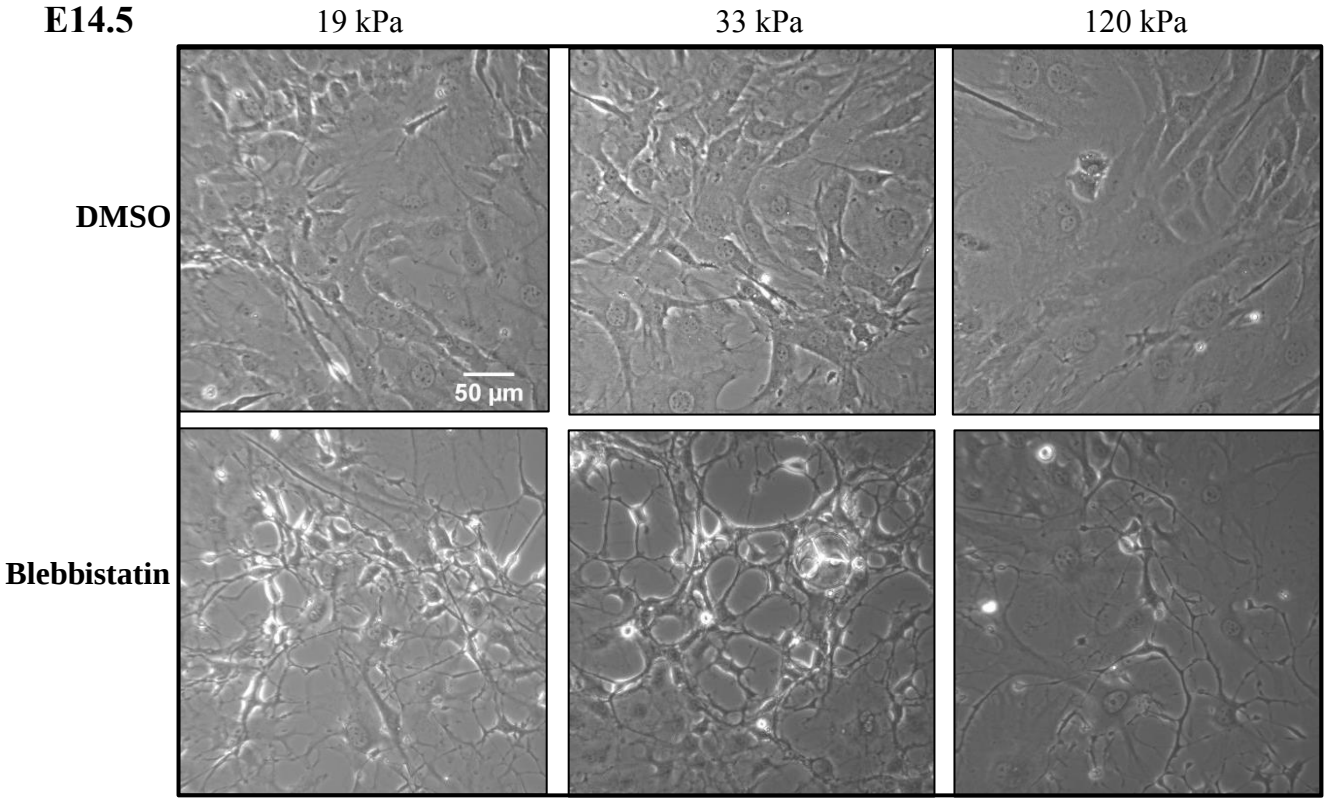
ImageJ software was used to measure the integrated density of IF images, which is defined as the product of selected area and mean gray value. The area was selected to best encompass the area of a single cell. Images in which only one cell was in the frame were not cropped, and the entire 50x50 µm image was analyzed. Mean gray value is calculated by converting all red, green, and blue pixels to gray and averaging their intensity. Only green pixels were present in the MT3-MMP fluorescence images, so red and blue pixels were disregarded. Statistical differences were evaluated for significance with a one-way ANOVA and t-test, using $p< 0.05$.

5.3 Results:

5.3.1 Blebbistatin alters TPC morphology

Mouse TPCs (E14.5, 16.5 and 18, n= 1 biological replicate, 3 experimental replicates) were seeded on polyacrylamide gels of various elastic moduli (19, 33, 120 kPa) and cultured for 24 hours in normal growth media

before being treated with 25μM blebbistatin or 0.5% DMSO as a vehicle control. Cells were cultured for an additional 24 hours. Results showed that cell morphology was changed dramatically in TPCs of all three developmental stages tested (Figure 5-5). Cells appeared to contract and become more spherical, which was evident from the increased light intensity around the edges of the cells.



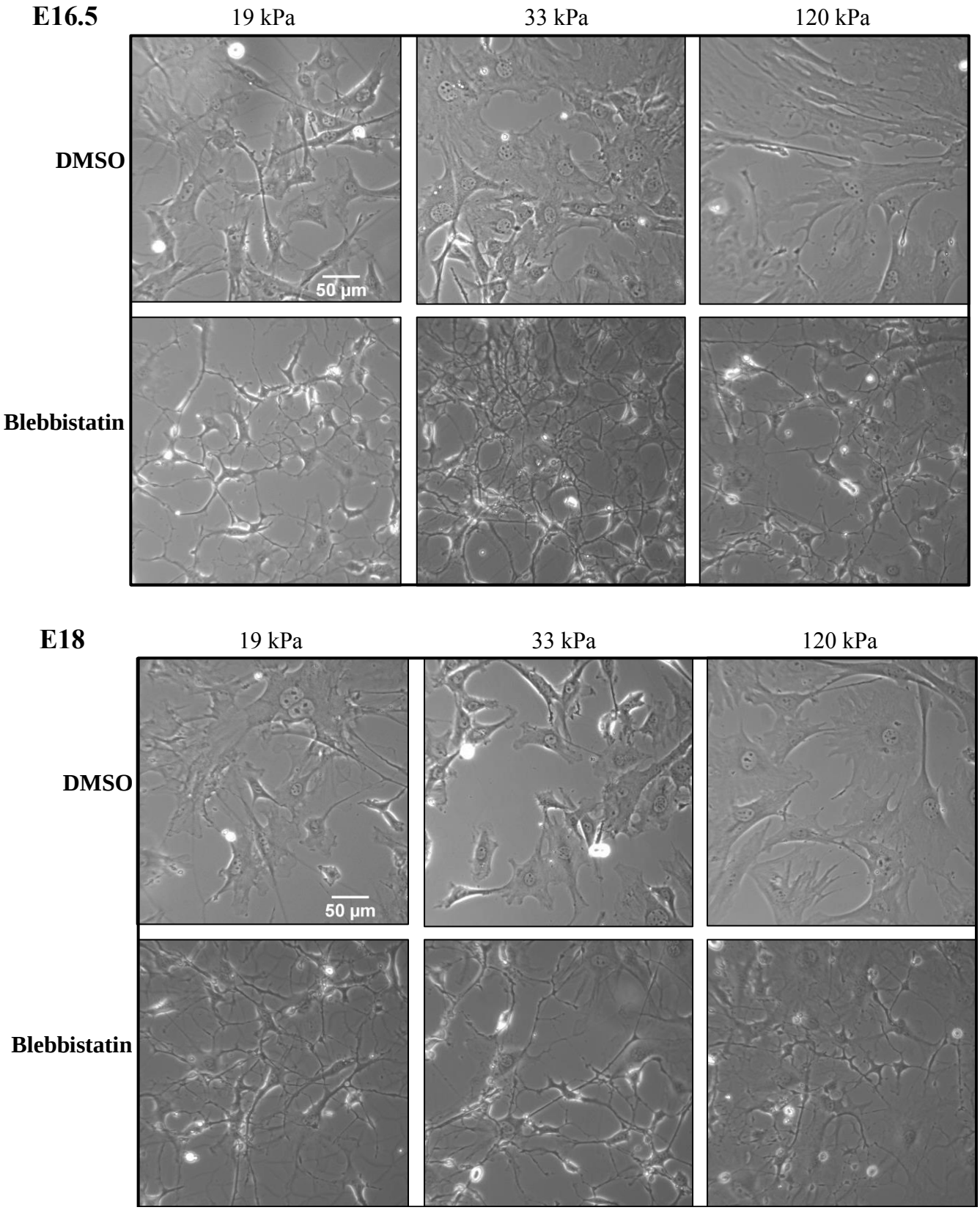
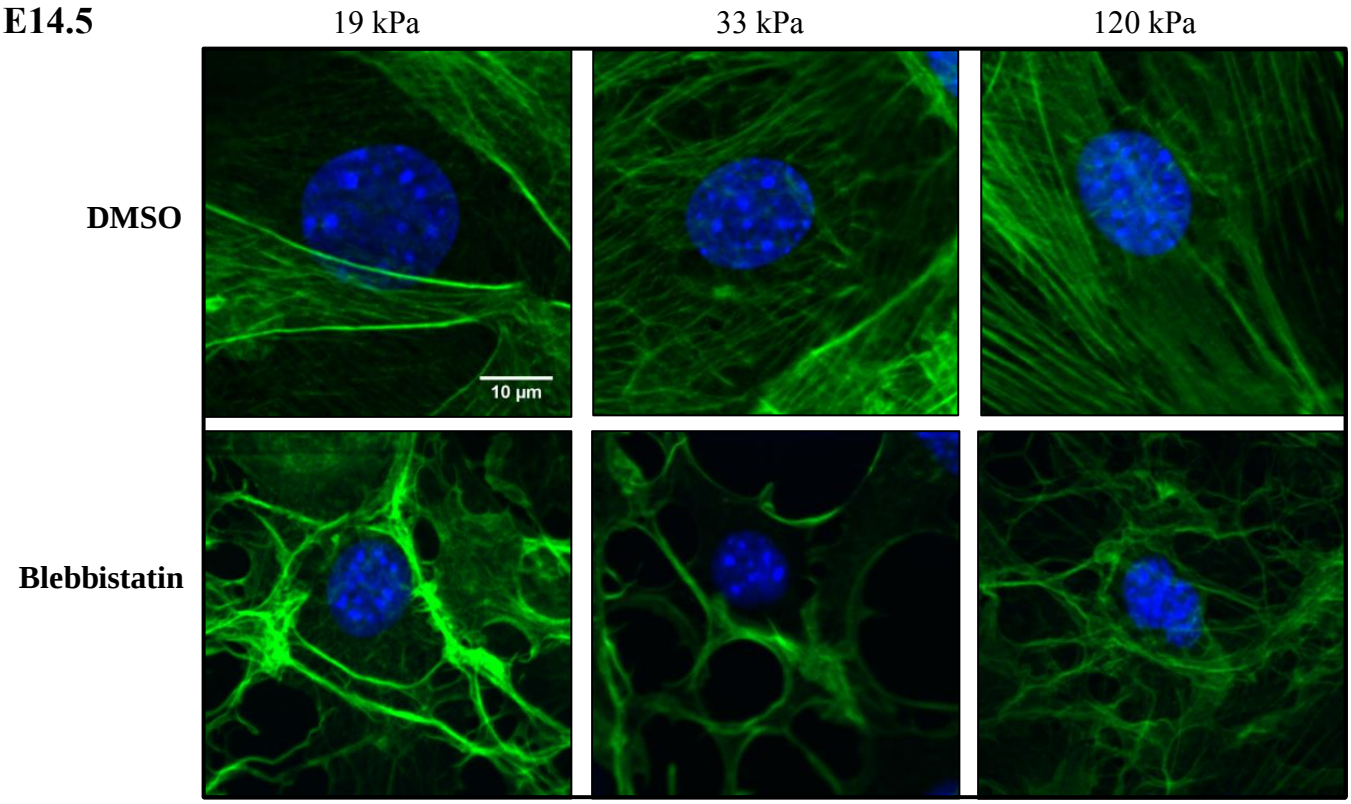


Figure 5-5: Blebbistatin treatment changed the morphology of mouse TPCs. TPCs of several stages (E14.5, 16.5, 18) were seeded on polyacrylamide gels of various elastic moduli (19, 33, 120 kPa) and cultured for 24 hours. Cells were treated with 25µM blebbistatin or 0.5% DMSO (vehicle control) and cultured for another 24 hours. Images were taken after 24 hours of treatment.

5.3.2 Blebbistatin disrupts the actin cytoskeleton

Immunofluorescent staining was used to visualize actin morphology. Mouse TPCs of several developmental stages (E14.5, 16.5, 18, n= 2 biological replicates, 2 experimental replicates) were cultured on polyacrylamide gels of various elastic moduli (19, 33, 120 kPa) for 48 hours. Actin fibers were stained with phalloidin, and imaged by confocal microscopy. In all conditions tested, blebbistatin treatment resulted in dramatic actin disorganization. Compared to the aligned, contiguous actin fibers of the control samples, TPCs treated with blebbistatin showed substantial actin disorganization (Figure 5-6). After blebbistatin treatment, no obvious differences in actin morphologies were observed by TPCs cultured on PA gels of different elastic moduli. There no longer appeared to be regulation of the actin cytoskeleton by substrate elastic modulus.



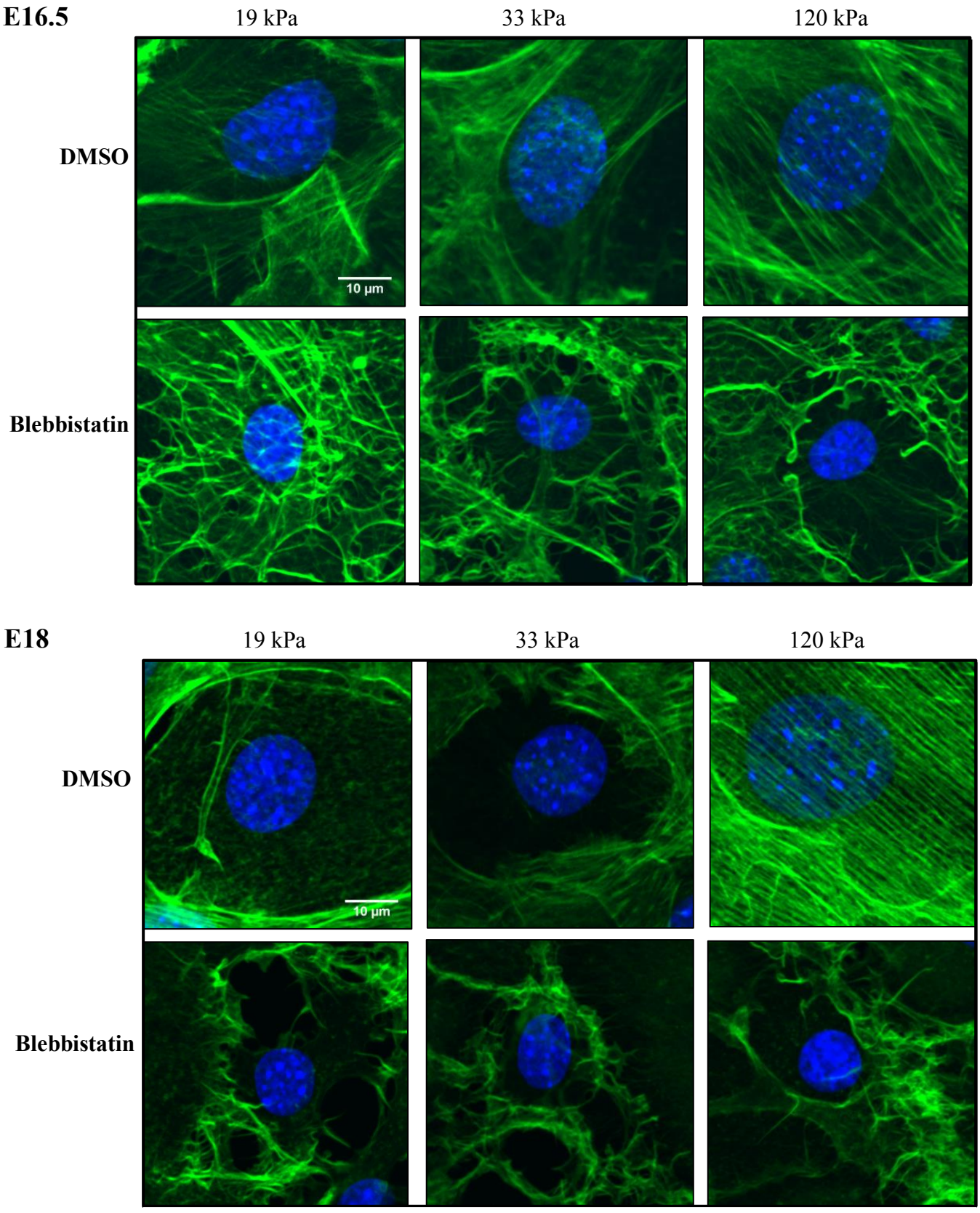
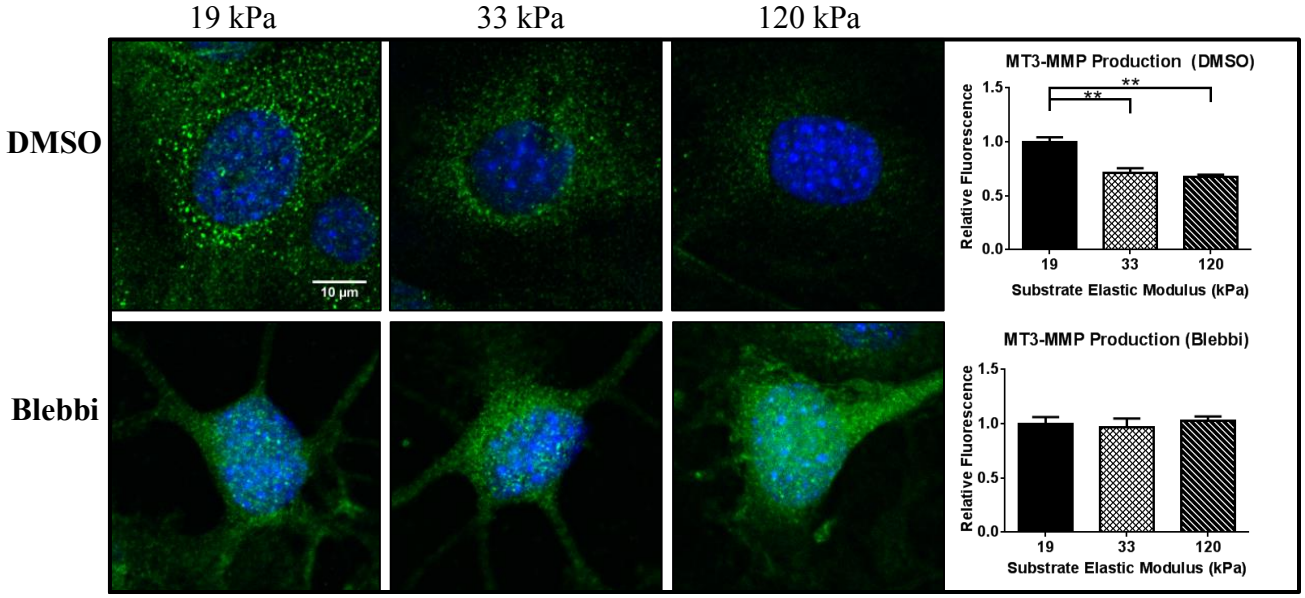


Figure 5-6: Actin stress fibers (green) are disrupted by 25µM blebbistatin in mouse TPCs (E14.5, 16.5, 18). Vehicle control samples (0.5% DMSO) showed aligned actin fibers, with alignment increasing with substrate elastic modulus. TPCs treated with blebbistatin showed disrupted actin filaments and appear to no longer respond to substrate elastic modulus. Cell nuclei were co-stained with DAPI (blue).

5.3.3 Actin cytoskeleton disruption abolishes the influence of elastic modulus on MT3-MMP protein production

After it was shown that blebbistatin altered cell morphology by disrupting the actin cytoskeleton, experiments were conducted to investigate the effect of actin disruption on MT3-MMP protein production levels. Mouse TPCs (E14.5, 16.5, 18, n= 2 biological replicates, 2 experimental replicates) were cultured on polyacrylamide gels of various elastic moduli (19, 33, 120 kPa) for 24 hours in normal growth media, and then treated with 25 μM blebbistatin or 0.5% DMSO for an additional 24 hours. Levels of MT3-MMP protein production were measured by immunofluorescence staining. Results showed that mouse TPCs treated with DMSO showed significant differences in MT3-MMP protein production when cultured on PA gel substrates of different elastic moduli. MT3-MMP production was greater by TPCs cultured on gels of lower elastic modulus (Figure 5-7), following a similar trend to those left untreated (Figure 4-6). Interestingly, mouse TPCs treated with blebbistatin showed no differences in MT3-MMP production resulting from culture on gels of different elastic moduli (Figure 5-7). These results may suggest that the actin cytoskeleton is a necessary component in the regulation of MT3-MMP by elastic modulus.


19 kPa
33 kPa
120 kPa
DMSO
Blebbi
10 µm
MT3-MMP Production (DMSO)
**
**
Relative Fluorescence
1.5
1.0
0.5
0.0
19
33
120
Substrate Elastic Modulus (kPa)
MT3-MMP Production (Blebbi)
Relative Fluorescence
1.5
1.0
0.5
0.0
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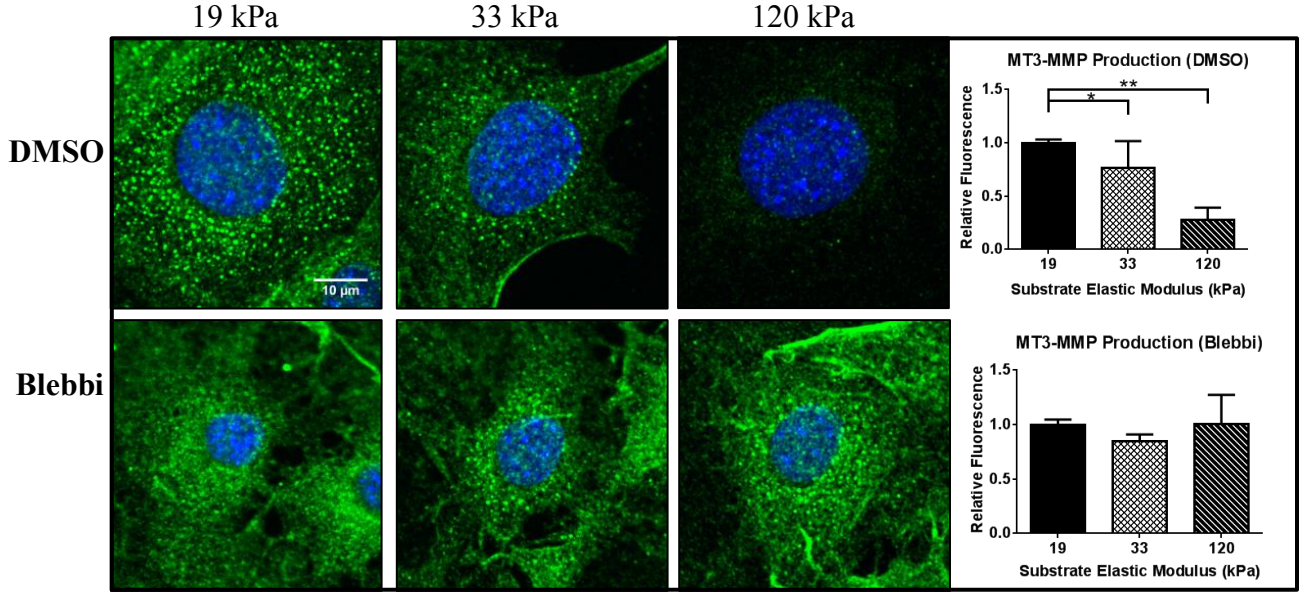
120
Substrate Elastic Modulus (kPa)


19 kPa
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MT3-MMP Production (DMSO)
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Relative Fluorescence
1.5
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Substrate Elastic Modulus (kPa)
MT3-MMP Production (Blebbi)
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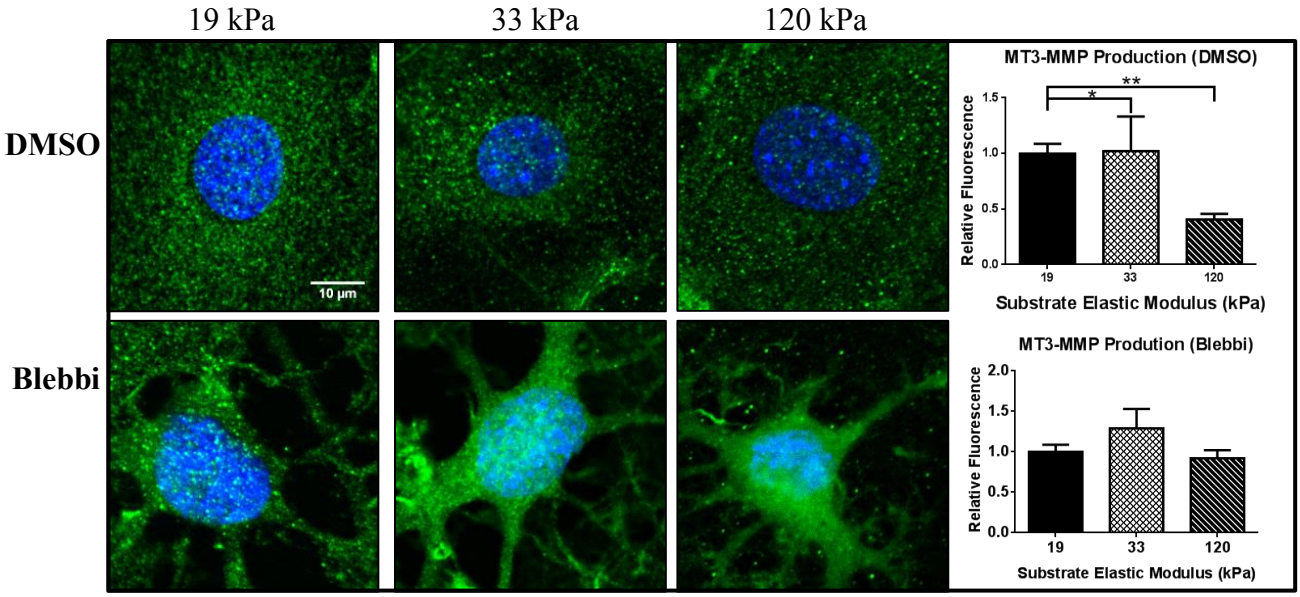

Figure 5-7: Mouse TPCs treated with DMSO vehicle control show significant differences in MT3-MMP production (green) when cultured on PA gel substrates of different elastic moduli. In comparison, TPCs treated with blebbistatin show no regulation of MT3-MMP production resulting from changes in substrate elastic modulus. (* $p<0.05$, ** $p<0.01$). Cell nuclei were co-stained with DAPI (blue).

5.4 Discussion:

In this chapter, the influence of disrupting the actin cytoskeleton with blebbistatin on MT3-MMP protein production was investigated. Embryonic mouse TPCs were seeded on polyacrylamide gels and treated with blebbistatin to disrupt the organization of the actin stress fibers. Changes in MT3-MMP protein levels were measured using immunofluorescence staining and quantified using image analysis. Results showed that 24-hour treatment with 25 µM blebbistatin significantly increased MT3-MMP protein production by all developmental stages tested (E14.5, 16.5, and 18), as compared to vehicle control samples (0.5% DMSO) (Figure 5-7).

5.4.1 Blebbistatin regulates cell morphology

Brightfield images confirmed that the blebbistatin treatment was sufficient to produce a dramatic change in TPC morphology in all developmental stages tested. Blebbistatin treatment effectively caused changes in cell morphology, which was a byproduct of the lack of cytoskeletal organization (Figure 5-5). This illustrated just how important the actin cytoskeleton is to cell behavior. Without the rigid actin structure, cell morphology was greatly disrupted, which then led to changes in protein production.

5.4.2 Blebbistatin disrupts the actin cytoskeleton

To confirm that the blebbistatin-induced change in cell morphology was, in fact, caused by the disruption of the actin cytoskeleton, phalloidin was used to fluorescently label actin fibers. Samples were visualized by confocal microscopy, and changes in cytoskeletal actin organization were observed. Control samples treated with 0.5% DMSO showed normal actin organization, with long, intact, stress fibers. TPCs treated with blebbistatin showed severely disordered actin morphology. IF staining results showed clustered, segmented actin filaments which appeared to have no overall alignment. These results appeared very similar to those published previously of vascular smooth muscle cells treated with blebbistatin [33]. Interestingly, there appeared to be no differences in actin morphology by TPCs cultured on polyacrylamide gels of different elastic moduli. This supports the important role of the actin cytoskeleton in allowing cells to perceive and respond to changes in ECM elastic modulus.

5.4.3 MT3-MMP protein regulation by the actin cytoskeleton requires a functional actin cytoskeleton

In addition to visualizing actin organization, duplicate samples were stained and measured for MT3-MMP protein content. Control samples reinforced the trend observed in the previous chapter, in which MT3-MMP protein production decreased in response to increased substrate elastic modulus. These samples were effectively identical to untreated samples, suggesting that the vehicle control, 0.5% DMSO, did not influence the production of MT3-MMP in response to elastic modulus. Interestingly, TPCs treated with blebbistatin showed no differences in MT3-MMP production when cultured on PA gel substrates of different elastic moduli. These results indicate that the actin cytoskeleton may be a necessary factor in the regulation of MT3-MMP by elastic modulus. While control samples responded to changes in substrate elastic modulus by altering MT3-MMP protein production, blebbistatin-treated samples which lost the integrity of the actin cytoskeleton failed to show regulation of MT3-MMP production by elastic modulus.

Interestingly, the net increase in production was dependent upon the substrate elastic modulus. It appeared that TPCs cultured on the 19 kPa polyacrylamide gels, in which MT3-MMP protein production was already high, experienced a smaller blebbistatin-induced increase in protein production than TPCs cultured on the 120 kPa gel. Previous, we showed that untreated TPCs cultured on 120 kPa gels showed low levels of MT3-MMP protein production.

This suggests that there may be a maximum level of MT3-MMP protein production under these *in vitro* conditions.

Overall, these results showed an important relationship between MT3-MMP and the actin cytoskeleton. Solely through the disruption of the actin cytoskeleton, it appeared that mouse TPCs lost their ability to regulate MT3-MMP protein production.. This suggests that MT3-MMP is regulated, at least in part, through the actin cytoskeleton. This conclusion supports previous work in which it was shown that MT3-MMP overexpression results in actin disorganization [24]. Here, this relationship was approached from the opposite direction; by disrupting the actin cytoskeleton and observing changes in MT3-MMP expression. Shofuda, et. al. showed that the interaction was likely between MT3-MMP and the focal adhesion kinase, but this was not addressed with experiments described here. An interesting future direction would include measuring changes in focal adhesion kinase levels in parallel with levels of MT3-MMP in response to blebbistatin-induced actin disorganization.

Chapter 6: Discussion, Conclusions, and Future Directions

6.1 Discussion and conclusions

6.1.1 Background

Currently there is a limited understanding of the factors that influence embryonic tendon progenitor cells. A better understanding of normal embryonic tendon development at the cellular level may lead to improved tissue engineering strategies and identifying better markers of tendon formation. To approach this goal, we must first elucidate which factors regulate cell behavior, matrix assembly, and mechanical properties during development. The first objective of this thesis was to characterize MT3-MMP gene expression and protein spatiotemporal distribution throughout tendon development, which has so far only been briefly studied in the literature. The second objective was to investigate elastic modulus as a potential regulator of MT3-MMP behavior. Tendon elastic modulus changes throughout development and certain MMPs have been shown to be regulated by elastic modulus. Specifically, MMP-3 and MMP-13 gene expression by rat annulus fibrosus cells has been shown to be upregulated when cultured on polyacrylamide gels of greater modulus [5].

Quantitative PCR and immunofluorescent staining (IF) were utilized to measure MT3-MMP gene and protein production, respectively, in embryonic tendon and by tendon progenitor cells (TPCs). Next, mouse TPCs were cultured

on polyacrylamide gels of different elastic moduli, and changes in MT3-MMP protein production were measured. Finally, blebbistatin was used to disrupt the actin cytoskeleton and changes in MT3-MMP protein production were measured.

6.1.2 MT3-MMP characterization

Immunofluorescent staining of cultured mouse TPCs showed that MT3-MMP protein production is conserved through the processes of cell isolation, FACS sorting, and multiple passages. It was shown that when cultured on substrates of equal modulus, MT3-MMP protein production by TPCs did not change significantly. To better understand how MT3-MMP functions *in vivo*, further characterization experiments were conducted on embryonic mouse and chick tendon.

By IF staining of embryonic mouse and chick tendon sections, it was shown that MT3-MMP was produced throughout embryonic tendon development in both species. Interestingly, MT3-MMP protein production in embryonic chick tendon remained high throughout most of development (HH 35 to HH 40), but then decreased significantly during HH 43. These results support data published by Jung, et. al., which showed by Western blot that MT3-MMP protein production is higher during HH 40 than HH 45 [17]. Furthermore, this trend coincides inversely with embryonic chick tendon elastic modulus, which remains low during HH 30 through HH 40, and then increases sharply during HH 43 [9]. Taken together, these results suggest that increases in substrate elastic modulus are associated with decreases MT3-MMP protein production. One caveat to these

results is the fact that tendon decreases in cell density throughout development [34]. The change in MT3-MMP protein production could be due to a decreased number of cells, but this could not be determined using IF staining. To better approximate the levels of MT3-MMP protein, production as a function of cell membrane surface area would be appropriate, but that was not addressed within these experiments.

6.1.3 MT3-MMP production was regulated by substrate elastic modulus and production was mediated by the actin cytoskeleton

By culturing mouse TPCs on polyacrylamide gels of various elastic moduli, MT3-MMP protein production was found to decrease proportionally in response to increased substrate elastic modulus. Interestingly, qPCR results showed that gene expression was not affected by substrate elastic modulus, suggesting that post-translational interactions were responsible for the change in production. It has been shown in the literature that MT3-MMP overexpression results in actin disorganization, so the actin cytoskeleton was investigated as a potential regulator of MT3-MMP production [24]. Accordingly, the actin cytoskeleton was disrupted with blebbistatin and changes in MT3-MMP expression were measured. Interestingly, MT3-MMP protein production increased with blebbistatin-induced cytoskeleton disorganization. These results support those of a previous study, which showed that MT3-MMP overexpression resulted in actin disorganization [24]. Here, the relationship was approached from the opposite direction, by altering actin organization and measuring changes in MT3-MMP production, and the same trend was observed.

It is important to understand the impact of the morphology change on these results. Blebbistatin clearly had a significant impact on the overall size of the cell bodies by inducing contraction, resulting in cells with a smaller surface area and likely greater height. This may have caused problems in the imaging process because only a 'slice' of the cell is measured. Future Western blot studies could measure MT3-MMP protein production normalized to GAPDH production, to account for any differences in cell number. Immunofluorescent staining paired with Western blot would both show spatiotemporal distribution and give a quantitative measure of MT3-MMP protein production, respectively, and would certainly improve the quality of results described here.

6.1.4 Significance in the field of tendon research

The motivation behind characterizing MT3-MMP was to gain information about embryonic tendon development and factors that influence MMP expression. MMPs have been shown to be influential during tendon development, especially in terms of extracellular matrix (ECM) remodeling. MT3-MMP can digest many of the ECM proteins found in tendon, and also activates MMP-2, which has been shown to regulate tendon fibril growth [17-18]. Our results showed that total MT3-MMP protein production changes throughout embryonic chick tendon development, which lends credence to the influential role of the protein during tendon development. Further studies are required to fully elucidate the role of MT3-MMP in tendon development, but here we provided a detailed description of MT3-MMP production levels and spatiotemporal patterns throughout the later stages of embryonic tendon development.

Furthermore, MT3-MMP protein production was shown to be regulated by elastic modulus. This information may be useful for informing the design of tissue engineering scaffolds and their mechanical properties. Given that MT3-MMP production depended on elastic modulus, the selection of substrate modulus for tissue constructs will be extremely important.

These results may also be relevant in the field of tendon wound healing. Upon injury, the highly aligned collagen structure of tendons is disrupted and results in changes in mechanical properties [35]. For example, significant differences in tendon elastic modulus have been shown between wounded and unwounded sheep tendons of embryonic and adult animals [35]. Specifically, wounded tendons were of lower elastic modulus than unwounded tendons. Taken with the data presented in these studies, MT3-MMP may be upregulated in cases of tendon injury and may play a role in ECM turnover and repair. Future studies investigating MT3-MMP production in tendon before and after injury would help elucidate the potential role of MT3-MMP in tendon wound healing.

6.2 Future Directions

6.2.1 Knockout experiments to better understand MT3-MMP function

A common method of characterizing the function of a particular protein is to reduce or completely knock out its expression by siRNA treatment. By comparing to control samples, side effects resulting from reduced protein production can be identified, and protein functions can be inferred. Previous studies have knocked out MT3-MMP gene expression in mice and shown a

multitude of negative effects on bone length, chondrocyte proliferation, apoptosis, and overall survival rate [20]. Unfortunately, this study did not examine the effects on tendon development. By utilizing a similar MT3-MMP knockout mouse system, much could be learned about the influence of MT3-MMP during tendon development. Specifically, studies exploring the effects on tendon cell actin organization and whole tendon elastic modulus would be very interesting.

6.2.2 Western blot in support of immunofluorescent staining

It should be noted that the combination of immunofluorescent staining and confocal imaging is not a 100% reliable method of measuring protein production. While IF staining can accurately label quantities of the protein of interest, confocal microscopy may lead to bleaching effects. By repeatedly exposing a sample to the powerful confocal lasers, the secondary antibody fluorescence signal weakens over time and may result in an inaccurate measurement of protein levels. Additionally, confocal detectors have an upper limit of detection. Above this limit, all levels of fluorescence cannot be distinguished, resulting in an over-exposed image. In these experiments, however, care was taken to ensure no samples were over-exposed. Western blot can also be used to quantify protein levels by directly measuring total protein content normalized to a constitutively produced protein, such as β-actin or GAPDH. In future studies, Western blot would be very helpful in supporting data presented in this thesis.

6.2.3 Three-dimensional gel system

In designing experiments to explore the effect of substrate elastic modulus on MT3-MMP expression, the two-dimensional polyacrylamide gel system was utilized. Future studies could involve the repeat of experiments described herein, but with the use of a three-dimensional scaffold. In other projects, our lab has used three-dimensional alginate hydrogels and seen good results. By adjusting crosslinking density, elastic modulus can be controlled. By culturing TPCs in alginate gels, we may be able to gain a better understanding of how MT3-MMP behaves in an environment closer to that of natural tendon.

Chapter 7: References

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